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# PROCEEDINGS

## 4th INTERNATIONAL CONFERENCE ON BIOSCIENCES AND BIOTECHNOLOGY

# ADVANCING LIFE SCIENCES FOR HEALTH (ANTI AGING DEVELOPMENT IN PARTICULAR) AND FOOD SECURITY

21st-22nd September 2012
Udayana University Denpasar-Bali, Indonesia

EDITORS:
YENNI CIAWI • NI MADE PHARMAWATI • YAN RAMONA • DEWA MADE SUKRAMA
SHOJI OHGA • JOO YOUNG CHA • I DEWA KETUT HARYA PUTRA
MADE MASTIKA • KETUT SUADA • YAYA RUKAYADI

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# Proceedings

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*4th INTERNATIONAL CONFERENCE ON BIOSCIENCES AND BIOTECHNOLOGY*

*"Advancing life sciences for health (anti aging development in particular) and food security"*

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# PREFACE

This proceedings compiles all papers presented in the 4th International Conference on Biosciences and Biotechnology (ICBB) held at the Udayana University, Bali on 21st - 22nd September 2012.

Seventeen plenary presentations were delivered by keynote and invited speakers with international reputations from Japan, Australia, Malaysia, Philippines, Korea, USA and Indonesia and a total of 126 papers (oral and poster presentations) compiled in this proceedings, which were presented in the conference by 148 contributors from 45 institutions in nine countries (Indonesia, Japan, Australia, Thailand, Korea, USA, Pakistan, Malaysia, Philippines).

We thank those who involve in the organizing committee for their hardworking. While it was a huge task, it was a privilege for us in editing this proceedings and work together with the referees who reviewed the papers.

We realized that this book is still far from perfect. Therefore, valuable criticisms on the content will highly be appreciated for the improvement of our Proceedings in the future.

We hope that the papers contained in this proceeding will prove useful in developing further study in the area of Biosciences and Biotechnology.

Editors

# FOREWORDS - HEAD OF ORGANIZING COMMITTEE

This proceedings is a compilation of papers presented in the 4th International Conference on Biosciences and Biotechnology 2012, organized by the Udayana University and it was held in accordance to the Golden Jubilee of Udayana University. The conference consisted of 17 plenary presentations delivered by International invited speakers from Japan, Korea, USA and Australia, Malaysia, Philippines, and Indonesia, covering general aspects of Biosciences and Biotechnology. Besides this plenary session, there were also four satellite symposia, covering areas of **health**, **agriculture, food science**, **biodiversity and environmental sciences**. Totally 126 contribution papers (oral and poster presentation) were presented in the conference. The efforts of the presenters to prepare their contribution papers for this conference are highly appreciated.

This conference was aimed to gather scientists, government officers and industries in Bioscience related disciplines, so that they can discuss and share their expertise, experience and expand networking. Therefore, in this very occasion, I would also like to extend my sincere appreciation to our distinguished guests and invited speakers, as well as all participants who travelled a long way to Bali to present their work and shared their expertise.

This Conference was financially supported by mainly the Rector of Udayana University and some other contributors. In this occasion, on behalf of the committee, I would like to thank their generous support for the conference. My thanks should also go to all people who were involved in the committee of the conference. Without their hard working and efforts, I am afraid we would not be able to make this event to happen.

Last but not least, I hope this book will be useful and provide the readers with valuable information for their scientific works.

Head of the Organizing Committee
**Dr. dr. Dewa Sukrama, MSi., Sp.MK**

# FOREWORDS - RECTOR OF UDAYANA UNIVERSITY

**Om Swastyastu,**

Bioscience and Biotechnology has developed very rapidly and has affected research direction and its achievements have been remarkable all over the world. Many countries have put forward their program and economic development based on bioscience and biotechnology. Biotechnological applications have contributed for community welfare and for economic development. We have known industries that made insulin, tissue culture for burn injury recovery, production of superior plant cultivars, enzyme production, bioremediation, microbe engineering such as probiotic, fermentation and vaccine production, detection for the cause of disease that become easier with the invention of PCR technique, immunohistochemical, and others. Beside that, biotechnology also develops in medical forensic such as identification of victims of terrorism based on DNA fingerprinting. Therefore, from this conference we hope that we all can learn more about biotechnological latest advance through a number of invited speakers presentation and sharing knowledge among participants.

As it was mentioned by the Chairman of the Organizing Committee that this conference was aimed to gather scientists, government officers and industries in bioscience related disciplines, so that they can discuss and share their expertise, experience and expand networking. Therefore, on behalf of the Udayana University, I would like to appreciate the efforts of all participants, especially all outstanding scientists or foreign invited speakers, to come to Bali and shared their expertise in the conference. I understand that all participants have spent much time for this conference, and therefore I must give high appreciation on all of those efforts and dedication.

To compile all papers presented in the conference, a proceedings is produced by the organizing committee of this conference. I hope this proceedings will be useful for all of us and will serve as part of scientific resources for the development sciences, especially biosciences and biotechnology in the future.

Finally, I wish you all had a fruitful conference and hope that it provided new ideas and strategies for the application of Biosciences and Biotechnology in the industries.

Rector of Udayana University,
**Prof. Dr. dr. I Made Bakta, SpPd. (KHOM).**

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# PLENARY PRESENTATIONS

# GENETIC MODIFICATION FOR IMPROVING IRON CONTENT IN RICE AND VALIDATING TOLERANCE GENE

**Dr. Inez Hortense S. Loedin**
*GRiSP Program Theme Leader for Gene Validation*
*Head of Genetic Transformation Laboratory*
*International Rice Research Institute*
*Indonesian Institute of Sciences*

*(Abstract not available)*

# DEVELOPMENT OF *IN PLANTA* TRANSFORMATION METHOD USING *Agrobacterium tumefaciens* THAT IS SIMPLE AND EFFICIENT AS WELL AS APPLICABLE TO VARIOUS PLANTS

**Mineo Kojima**
*Professor Emeritus, Shinshu University, Japan*
*E-mail address; kojima.m@silk.plala.or.jp*
*Mailing address: 2-46, Gokuraku, Meito-ku, Nagoya, 465-0053, Japan*
*Phone & Fax: +81-(0)52-701-3873*

## ABSTRACT

A simple and efficient *inplanta* transformation method was developed. In the method,meristems of either apical or axillary buds of immature plants or apical buds of embryos in imbibed seeds, depending on plants, were inoculated by *Agrobacterium tumefaciens* after being pricked with a needle. The inoculated plants were grown to maturation in pots under non-sterileconditions. Transformation was demonstrated by several lines of evidence obtained with mostly the progenies of $T_1$ generation; phenotypic inheritance from $T_0$ plants to plants of the following generation, resistance of seed germination to antibiotics, detection of β-glucronidase activity in transformantsof $T_1$ generation, detection of transgene by Southern blot and PCR analyses in $T_1$generation transformants and rescue from T1 generation transformants of the plasmids composed of T-DNA of binary vector and flanking plant genomic DNA. The diverse species of plants such as buckwheat (*Fagopyrum esculentum*), mulberry (*Morus alba* L.), kenaf (*Hibiscus cannabinus*), rice (*Oryza sativa*), wheat (*Triticum aestibvum* L.), maize (*Zea mays*), and soybean (*Glycine max*) were shown to be efficiently transformed by our *in planta* method.

# BIOTECHNOLOGY: IMPORTANCE IN SUSTAINABLE FUTURE

**Fusao Tomita, Ph. D.**
*IUMS Ambassador to Asia Emeritus Professor of Hokkaido Univ.*
*Adjunct Professor of Open U. of Japan*

**ABSTRACT**

Biotechnoogy is highly multidisciplinary; it has its foundations in many areas including biology, microbiology, molecular biology, genetics, chemistry, and chemical and process engineering. It involves the practical application of organisms or their cellular components to make and service industry and environmental management.

In recent years, genome analyses have been progressed rapidly and contributed immensely to compiling of base sequences. By the use of those data, GM crops can be achieved rapidly and will contribute to not only food security but also to bio-energy etc. As to human welfare, iPS has progressed rapidly and we will soon have better ways to combat many problems in the future.

Metagenomics are becoming very important technology for ecological survey of microbes in various places such as soil, water, human gut, etc. and also studies of viable-but-not-culturable microbes.

Biotechnology will be and is very important technology in sustainable future, since we must live on Earth where we now know we have limited resources and we must live in the recyclable society. Biotechnology is the most important one in this respect.

# APPLICATIONS OF MYCORRHIZAL FUNGI IN SUSTAINABLE HORTICULTURAL PRODUCTION SYSTEMS

**Fred Davies**
*Regents Professor & AgriLife Research Faculty Fellow Department of Horticultural Sciences*
*Texas A&M University College Station, Texas 77843-2133*

## ABSTRACT

Mycorrhiza means "fungus roots" which is a symbiotic association between specific fungi and the fine, young roots of higher plants. The majority of agronomic and horticultural plants strictly speaking do not have roots, rather they form mycorrhiza. Mycorrhiza are unique in that their hyphae do not penetrate the vascular stele of the host plant, unlike vascular wilt organisms such as *Verticilium*, *Fusarium*, etc. Mycorrhiza can enhance efficiency of plant roots to absorb water and macro- and microelements from the soil or container media. This helps reduce fertility and irrigation requirements, increases drought resistance and plant resistance to pathogens. Mycorrhiza enhance plant health and vigor and minimize stress. Minimizing stress is particularly important because with less stress there is greater plant resistance to pathogens and pests and a reduction in pesticide usage. There are opportunities for the utilization of mycorrhiza with the challenges facing the horticultural industries and the incorporation of good agricultural practices (GAPs). Water quantity and quality are key limiting environmental factors. GAPs include reducing irrigation water usage and subsequent irrigation run-off. GAPs also include changing cultural practices for more efficient utilization of fertilizers, fungicides and pesticides for sustainable crop production systems. There are commercial opportunities to produce and market more stress-efficient plants that use less water and fertilizer, and have greater resistance to environmental stress, and resistance to biotic stress caused by disease and insects. This presentation will cover practical applications of mycorrhiza for sustainable horticultural production systems.

# ZYMOGRAPHY OF EXTRACELLULAR PROTEASES IN *Bacillus subtilis*

**Takeko Kodama[1], Keiji Endo[1], Katsutoshi Ara[1], Katsuya Ozaki[1] and Junichi Sekiguchi[2 #]**
*[1]Biological Science Laboratories, Kao Corporation, 2606 Akabane, Ichikai, Haga, Tochigi 321-3497, Japan,*
*[2]Department of Bioscience and Textile Technology, Interdisciplinary Graduate School of Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda, Nagano 386-8567, Japan*
[#]Corresponding author: jsekigu@shinshu-u.ac.jp
Phone: +81-(0)268- 21-5344 Fax: +81-(0)268- 21-5345

**ABSTRACT**

In *Bacillus subtilis*, AprE, Bpr, Epr, Mpr, NprB, NprE, Vpr and WprA have been identified as extracellular proteases. We determined protease activities from the culture supernatant of *Bacillus subtilis* using a 1D zymogram gel containing gelatin. The highest protease activities were found at the late stationary phase of a 75-h culture. To distinguish the proteases, the zymogram pattern of the wild type was compared with the protease-deficient mutants (*aprE*, *bpr*, *epr*, *mpr*, *nprB*, *nprE*, *vpr* and *wprA*). Activities of AprE, Bpr, Mpr, and Vpr were estimated by gelatin zymography. In addition, for the *aprE* mutant, the zymogram profile of Bpr was very different from that of the wild type. This suggested that AprE is involved in processing the Bpr protein.

***Keywords***: protease, zymography, gelatin, *Bacillus subtilis*

# METAL BIOTRANSFORMATION BY RHIZOBACTERIA FOR PHYTOREMEDIATION

**Irfan Dwidya Prijambada**
*Graduate School of Biotechnology, Gadjah Mada University, Jl. Teknika Utara, Barek, Yogyakarta 55281, INDONESIA.*
*Dept of Agricultural Microbiology, Fac. of Agriculture, GadjahMada University, Jl. Flora, Bulaksumur, Yogyakarta 55281, INDONESIA.*

**ABSTRACT**

Activities in industry and agriculture have resulted in soil contamination by metals. Metal contamination of soil causes a massive threat to environment. Phytoremediation is the use of a plant to remove, degrade and sequester pollutants from the environment. Phytostabilization and phytoextraction are among subsets of technology that can be classified into phytoremediation to handle the metal polluted environment. In phytoextraction, plants remove metals from the soil and concentrate them in the harvestable parts of plants. Therefore the metals are preferably accumulated in the shoot. On the other hand, in phytostabilisation plants reduce the mobility and bioavailability of pollutants in the environment either by immobilization or by prevention of migration. Accumulation of the metal in the root may satisfy these criteria. Both phytoextraction and phytostabilization take advantage of the ability of plants to absorb metals from contaminated soil environments. Exploitation of metal uptake into plant biomass as a method of soil decontamination is limited by plant productivity and the concentrations of metals achieved. Rhizobacteria have the ability to alter metal uptake and its translocation by plant. Rhizobacteria have been shown to possess several mechanisms capable of altering metal bioavailability for uptake into roots including their ability to catalyze redox reactions leading to changes in metal mobility in soil and propensity for uptake into roots. At the same time, rhizobacteria have also been shown to possess mechanisms capable of altering metal translocation from roots into shoot including their ability to produce organic acids which act as chelator.

***Keywords***: Metal, Biotransformation, Rhizobacteria, Phytoremediation

# STRATEGY OF THE RICE BLAST FUNGUS TO DEAL WITH BLAST RESISTANT CULTIVARS OF RICE

**Teruo Sone**
*Research Faculty of Agriculture, Hokkaido University, Sapporo Japan*

## ABSTRACT

*Magnaportheoryzae*is the causal agent of rice blast, the most devastating disease of rice. In order to control the disease, fungicides or resistant cultivars have been developed, but their longevity was prevented by frequent mutation of the pathogen. The breakdown of the resistant cultivar has been recorded many times, typically several years after the release of the new cultivar.*Magnaporthe* genes involved in the resistant cultivar breakdown are avirulence (AVR) genes, which participate with corresponding resistance (R) genes in Gene-for-Gene theory. A pathogen that carries an AVR gene cannot invade the host plant with the corresponding R gene.

*AVR-Pia* in*Magnaportheoryzae*is an AVR gene that exhibits avirulence toward the rice *Pia* resistance gene, which had been found in Japanese cultivar Aichiasahi. We have cloned the gene from Japanese isolate Ina168. *AVR-Pia* encodes a protein consists of 85 amino acids which contains a predicted 19-amino acid signal peptide. Expression profile of *AVR-Pia*was investigated *in planta*using qRT-PCRof inoculated rice leaf and fluorescent microscopy of rice leaf sheath inoculated by *M. oryzae*expressing GFP-tagged AVR-Pia protein. Strain Ina168was inoculated onto either compatible or incompatible rice. The expression was first detected at 24 hoursafter inoculation (hai). After that, on compatible rice, the expression was increased until 60 hai, whereas it was reduced after 30 hai on incompatible rice.The expression of AVR-Pia::EGFP was successfully detected during infection of intact leaf sheathof compatible rice cultivar. EGFP was accumulated at BIC (biotrophic interfacial complex, Khang et al., 2010)-like structure of invasive hyphae. In addition, AVR-Pia protein was also successfully detected from blast-inoculated rice leaf sheath by Western blotting. Molecular weight of the detected AVR-Pia showed that the signal peptide was removed. Theseresults suggest the function of AVR-Pia as invasive effectors.

*AVR-Pia* gene was conserved in Japanese field isolates in all-or-nothing manner. In Ina168, three copies of *AVR-Pia* were conserved in the same chromosome. The only one*avr-Pia* mutant, Ina168m95-1, was revealed to lack the *AVR-Pia* gene. Sequencing analysis of *AVR-Pia* flanking region revealed that three *AVR-Pia* genes were deleted by homologous recombination between two copies of DNA-type transposon *Occan* located adjacent to *AVR-Pia* genes.

Further elucidation of the mechanism of interaction with host R gene or mutationwill lead us to establish a novel strategy of the disease control.

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**Prof. K. Kawakita**

*(Abstract not available)*

# AGE REVERSAL TO STAY YOUNG AND HEALTHY

**Wimpie Pangkahila**
*Center for Study of Anti-Aging Medicine Department of Andrology and Sexology Medical Faculty Udayana University*
*Indonesian Center for Anti-Aging Medicine (INCAAM)*
*wimpie.pangkahila@gmail.com*

**ABSTRACT**

There is still a common belief that old people are those who develop many diseases and other unhealthy condition without good looking. But it is known two kinds of age, chronological age and physiological age. Physiological age is based on how someone looks or functions.

There are many factors causing aging process that are divided into two groups, internal factor and external factors. If all causative factors of aging are inhibited or overcome, aging process can be delayed, even reversed. The reversal of physiological age makes people look healthier and younger with better even optimal function of the body system. During aging process, many changes occur in body system. All changes caused by or

related to aging process are termed as biomarkers of aging. Biomarkers of aging may be one or more parameters of physical, physiological, biochemistry, or molecular changes related to aging process. Biomarkers can be found during the 3 phases of aging process (subclinical, transition, clinical).

Based on the biomarkers, intervention can be performed to delay even reverse aging process. The fact that aging process occurs gradually is an advantage concerning the intervention because there is a chance to delay even reverse the process.
The interventions are as follows 1. Practice healthy life including all aspects in daily life those are enough sleep, activities and relaxation balance, healthy diet, regular exercise, avoid toxic substance, avoid or manage stress, avoid consume any substance (mixture, medicine, supplement) without recommendation, and healthy sexual life, 2. Perform regular medical checkup, 3. Get treatment based on the biomarkers. There are many basic studies and clinical data show that physiological age can be reversed to younger age with optimal function.

The conclusion is aging process can be intervened and treated, and physiological age can be reversed to younger age with optimal function. In this condition people can stay young and healthy.

***Keywords***: chronological age- physiological age- aging process-biomarker-age reversal

# THE REPRODUCTIVE PHYSIOLOGY OF A MARSUPIAL, THE TASMANIAN BETTONG (*Bettongiagaimardi*)

**R. W. Rose**
*School of Zoology, University of Tasmania, Hobart, AUSTRALIA 7001*
*Email: randy.rose@utas.edu.au*

## ABSTRACT

The reproductive physiology of the Tasmanian Bettong (*Bettongiagaimardi*) conforms in many respects to that found in the related kangaroos. Gestation and the oestrous cycle are of similar duration (21-22 days), and a postpartum oestrus/matingcan result in a blastocyst that remains quiescent (=embryonic diapause) throughout most of pouch life. Reactivation of the blastocyst occurs when the young in the pouch nears independence or is removed (RPY). The furred young always vacates the pouch when a new young is born due to physiological changes within the mother associated with the imminent birth. Pouch life is relatively short (106 days).

During the oestrous cycle, there was a significant trough on day 2 in basal body temperature and an increase on day 3 and the temperature remained raised until day 10, during which time plasma progesterone concentrations are also high; the temperature then fell 2 days before oestrus. This fall corresponds to a decrease in concentration of plasma progesterone and in the numbers of leucocytes in vaginal smears.

There was a transient peak of progesterone (0.7 ng/ml) early in gestation at Day 4 RPY. After Day 6 RPY, progesterone levels remained elevated (1.2-1.5 ng/ml) until they dropped sharply to basal levels on the day of birth.Injections of prostaglandin ($PGF_{2}$□) caused contractions of the pouch and pouch vacation by the young; oxytocin had a similar effect but with a longer latency. In addition both hormones induced birth behaviors in adult and immature females *and* males!

# DNA MIICROARRAY BASED EXPRESSIONAL PROFILING OF *hrpXo* DEPENDENT UP-REGULONS IN *Xanthomonas oryzae* pv. *oryzae*

**Byoung Ho So[1], Jongun Kim[1], and Hee Wan Kang[1,2*]**
*[1]Graduate School of Biotechnology and Information Technology, Hankyong National University, Ansung 456-749, Korea*
*[2]Institute of Genetic Engineering, Hankyong National University, Ansung 456-749, Korea*
**Corresponding author: Hee-Wan Kang*
*Graduate School of Biotechnology and Information Technology, Hankyong National University, Ansung 456-749, Korea*
*Tel: +82-31-670-5420; Fax: +82-31-676-2602; E-mail: kanghw2@hknu.ac.kr*

**ABSTRACT**

Thirty-mer oligonucleotides for microarray analysis were designed from the annotated open reading frames (ORFs) in the whole genome sequence of *X. oryzae* pv. *oryzae* KACC10331. Mutant *hrpXo::TN* and wild-type strain KACC10859 were cultured in *hrp* inducing medium (XOM2), and cDNAs, which were synthesized from the total RNA samples from both strains, were hybridized on a DNA microarray. The microarray data showed that 210 genes were down-regulated more than 2-fold in *hrpXo*::TN, while 115 genes were up-regulated more than 2-fold. The HrpXo regulons differently included 54 hypothetical genes: type III secretory genes (11 *hrp* genes); genes encoding type III secretory effectors (*xopP* genes and *avr/pthA*); genes encoding type II secretory effectors (6 proteases, a lipase, a polygalacturonase, and 4 cellulases); 7 iron-uptake genes; 6 *pil* genes encoding fimbrial assembly membrane proteins; and 14 transposon genes. Significant plant-inducible promoter (PIP) sequences were newly identified on HrpXo regulons. The validity of the expressional profiles was further confirmed by reverse transcription (RT)-PCR.

***Keywords***: *X. oryzae* pv. *oryzae*, DNA Microarray, HrpX regulons

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**Prof. K. Maeda**

*(Abstract not available)*

# A PERSPECTIVE ON GLOBAL FOOD SECURITY- WHERE FROM HERE?

**Acram Taji[1] and Tanya Tapingkae[2]**
*[1]Queensland University of Technology, AUSTRALIA*
*[2]Chiang Mai Rajabhat University, THAILAND*

**ABSTRACT**

As the world population continues to increase, it is projected that the demand for food will require the doubling of agricultural production over the next forty years. Producing such a large amount of food sustainably in the face of many constraints is one of the major challenges faced by humanity in this century. Constraints such as input resources, availability of arable land, water, energy cost, and uncertainties of climate will put further pressures on food production. There is an urgent need to pay attention to food production whilst maintaining the quality of the resource base from which it is produced. Urgent actions are required to overcome an emerging food crisis. These actions must be coupled with adaptation and mitigation measures in relation to climate change, sustainable management of water, land and other natural resources, including the protection of biodiversity.

# POTENTIAL APPLICATIONS OF FINGERROOT [*Boesenbergia rotunda* (L.) MANSF. A] FOR INDUSTRIAL NATURAL COSMETIC PRODUCTS

**Yaya Rukayadi**
*Department of Food Science, Faculty of Food Science and Technology, and Laboratory of Natural Products, Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM),*
*43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
*yrukayadi@food.upm.edu.my*

**ABSTRACT**

Fingerroot [*Boesenbergia rotunda* (L.) Mansf. A], synonyms: *Kaempferia pandurata, Gastrochilus pandurata, Boesenbergia pandurata,* is a perennial herb of the Zingiberaceae. Vernacular names of fingerroot are Chinese keys (English), petits doigts (France), *kracai* (Thailand) and *temu kunci* (Indonesia and Malaysia). The distribution of fingerroot is native to Java and Sumatra, and cultivated in India, Sri Lanka, Malaysia, Thailand and Southern China. It has been used as a spice in the Indonesian and Thai cuisine. In folk medicine in Suriname and Southeast Asia, the rhizome of fingerroot is used for swelling, wounds and diarrhea. The fresh rhizome of fingerroot has been used as a folk medicine for the treatment of colic, dry cough, rheumatism, muscle pain, and as an aphrodisiac. Several studies reported various activities of fingerroot, including antiinflammatory, antitumor, antidiarrhea, antidysentery, antiflatulence, and antiepidermophytid effects. Panduratin A, isopanduratin A and 4-hydroxypanduratin A are the typical chalcone compounds found in fingerroot. Chalcones belong to a group of phenolic compounds in the flavonoid and widely occur in nature as pigments. Chalcones has a broad bioactive spectrum such as anticancer, antibacterial and antifungal activities. In previous studies, chalcone compounds in fingerroot showed anticancer, antioxidative and antimutagenic. We recently reported that the compound has activity to prevent and treatment of skin aging by UV, treatment hyperpigmentation**,** prevent periodontal inflammation, antistaphylococcal and antienterococcal. Clinical test analysis showed that compound was active *in vivo* and was not toxic to the host cells. Thus, there are some challenges and opportunities in applying fingerroot for industrial natural cosmetic products.

***Keywords***: *Boesenbergia rotunda*, fingerroot, *temu kunci*, natural cosmetic

# USE OF MUTATED *ALS* GENES AS SELECTABLE MARKER FOR GENETIC TRANSFORMATION OF PLANTS, AND NON-GM HERBICIDE RESISTANT RICE HAVING A MUTATION IN THE *ALS* GENE

**Tsutomu Shimizu[1], Koichiro Kaku[1], Tomonori Fujioka[1], Kiyoshi Kawai[1], Kinya Toriyama[2] and Hiroaki Ichikawa[3]**
[1] Life Science Research Institute, Kumiai Chemical Industry, Co., Ltd., Kikugawa, Shizuoka 439-0031, Japan
[2] Laboratory of Environmental Plant Biotechnology, Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 981-8555, Japan
[3] National Institute of Agrobiological Sciences, Kan-non-dai, Tsukuba, Ibaragi 305–0856, Japan
Email: t-shimizu@kumiai-chem.co.jp

**ABSTRACT**

Widely used selectable markers for genetic transformation of plants are bacterial resistance genes for antibiotics such as kanamycin and hygromycin. Herbicide-resistant genes from bacterial sources such as 5-enolpyruvylskimate-3-phosphate synthase gene (*EPSPS*) and phosphinothricine acetyltransferase gene (*Pat*) are also used as selectable markers. There are, however, concerns and objection for use of the antibiotic and herbicide resistance genes derived from bacteria as selectable markers, though these genes have been proven to be innocuous to human health. Meanwhile, herbicide-resistant genes from plants can be used both for the generation of herbicide-resistant crops and as selectable markers. Mutated genes of acetolactate synthase (*ALS*), which confer resistance to ALS-inhibiting herbicides, have been shown to be useful not only for the generation of herbicide resistant crops but also for introducing foreign traits into crops as selectable markers. Application of the mutated *ALS* genes as a selectable marker appears to contribute to minimize public concern regarding genetically modified (GM) crops. Mutated *ALS* genes from crops are also helpful for introducing useful crop genes into crops by self-cloning as host-derived selectable marker genes. And also, herbicide resistant non-GM crops with a mutated *ALS* gene, which are generated through mutagenesis and selection, are useful to facilitate weed control and reduce labour in farming. Herbicide resistant non-GM rice with a mutated *ALS* gene gives the best opportunity to farmers for controlling troublesome weedy rice.

***Keywords***: acetolactate synthase, ALS, AHAS, pyrimidinylcarboxylates, bispyribac-sodium

# CLONING OF FLOWERING GENES (*WjFLC* AND *WjFT*) IN WASABI (Japanese horseradish) AND MONITORING OF FLOWERING RESPONSEWITHTHEIR EXPRESSION

**Masayuki Nozue[1], , Hiroyoshi Kubo[2] and Kiyoshi Yoshida[3]**
[1]*Division of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, Tokida 3-15-1, Ueda 386-8567, Japan*
[2] *Department of Biology, Faculty of Science, Shinshu University, Asahi 3-1-1, Matsumoto 390-8621, Japan*
[3]*Marui Co. Ltd., Toyoshina 4932, Azumino 399-8205, Japan*

## ABSTRACT

Wasabi (*Wasabia japonica*) is a commercially important crop in Japan. We isolated a *FLC* ortholog (*WjFLC*) and *FT* ortholog (*WjFT*) from wasabi.Predicted amino acid sequence encoded by *WjFLC* and *WjFT* showed 89% and 85% identities with *FLC* and *FT* of Arabidopsis, respectively. The expression of *WjFLC* was high in October and reduced in November when flower buds are formed in wasabi. On the other hand,expression of *WjFT* was not detected in October and was slightly detected in November. Thereafter,*WjFT* was highly expressed later in February.We examined the best condition for initiation of flower bud formation under various artificial environments by monitoring the flowering response of wasabi using *WjFLC* and *WjFT*.

# UTILIZATION OF ELECTRIC PULSED POWER ON FRUITING OF EDIBLE MUSHROOMS

**Shoji Ohga**
*Division of Forest Environmental Sciences, Department of Agro-environmental Sciences,*
*Kyushu University, Fukuoka 811-2415, Japan*
*ohga@forest.kyushu-u.ac.jp*

## ABSTRACT

Effect of pulsed power was investigated on fruit body formation of 10 edible mushrooms, *Lentinula edodes*, *Glifola frondosa, Pholiota nameko, Flammulina velutipes, Hypsizygus marmoreus, Pleurotus ostreatus, Pleurotus eryngii, Pleurotus abalones, Agrocybe cylindracea* and *Sparassis crispa*. Pulsed power of 100-170 kV was directly charged to the substrate just before fructification. The effect of the pulsed power resulted to promote for 10 edible mushrooms fructification. The treatment especially stimulated the fructification on *Pleurotus* species.

# ORAL PRESENTATIONS: FOOD SCIENCE AND TECHNOLOGY

# HUMAN FACTORS CONSIDERATION IN FOOD INDUSTRY TO MINIMIZE HEALTH PROBLEMS AS IMPACT OF HUMAN-MACHINE INTERACTION

**Triarti Saraswati[1] and Timothy Ekaputra[2]**
*[1,2] Industrial Engineering Department, Faculty of Engineering, Swiss German University*
*EduTown, BSD City, Tangerang, Indonesia*
*triarti.saraswati@sgu.ac.id[1]*
*timothy.ekaputra@hotmail.com[2]*

**ABSTRACT**

Modern food industry invests a large number of automated machinery. But, most of these industries do not realized that the interaction between machine and human is a complex system and pose impact to the human. Human plays an important role in this relationship as the only flexible component but still negligible by the company. Human operators work under unnatural posture with high repetitive movement over a prolonged period of time. For example in packaging department, workers have to sit for a long time and only use hands to arrange products into its package using certain movements. Or they have to bend their body to move raw materials from one place to another. This will lead to the increase of discomfort in the musculoskeletal system, in this case low back pain. The productivity would hence be affected by this condition. The purpose of this study is to increase awareness of human factors in food industry in order to optimize productivity. This study tried to analyze factors that will trigger low back pain and other health problems. Motion study, psychological problems must be evaluated to find the best solution to this problem. A proposed design consideration will be presented to minimize discomfort in the musculoskeletal system.

***Keyword:*** Human-machine interaction, musculoskeletal disorder, human factors

## INTRODUCTION

Food industry is a highly competitive business. This food industry may vary from home-made industry to fully automated industry. The fully automated industry will provide higher productivity than traditional food industry done manually. This fully automated industry play an important role in improving the quality and hygiene. The food industry lag behind other industries in adopting this new technology, but this industry has been successful in putting many automatic processes into place (Gunasekaran, 2009). The automation system might be integrated for the whole system, from receiving raw materials to shipping finished products, or only applied in particular process of food manufacturing.

Automation provides lots of benefits to industry, such as improved productivity, improved product quality and at the end will improve profitability to company and customer satisfaction. But, automation also has hidden impact to human workers that management never realized.

Food industry is highly labor intensive. Most of the manual work in food manufacturing requires rapid, repetitive, and monotonous movement and consequently, workers get bored very easily. These are the problems with job simplification. Job simplification fragmented task so that each worker produced only a small part of the finished product (Schultz, 2006). The goal was to reduce every manufacturing process to the simplest elements that could be mastered by low-skilled worker. The consequences of job simplification are boredom and monotony result from repetitive movement. This repetitive movements may lead to fatigue or even injury in wrists, hands, shoulders, neck, and back (Schultz, 2006).

Automation itself gives problem to human operator, as machine is considered to be a constant factor in human-machine interaction, incapable of being changed to meet human needs (Schultz, 2006). It is the human operator that would have to adapt in order to finish the job, no matter how uncomfortable, tiring, or unsafe the equipment was. The human has to bend, twist or doing awkward movement in order to adjust with the machine. Unconsciously, health problem may arise, such as low back pain or musculoskeletal disorder.

In food industry, the interaction between human and machine may occur in every stage of production, for example selecting raw materials and materials handling, to quality control. Heat, vibration and noise from the machine also give health impact to workers, such as hearing loss and headache (Timothy, 2012).

Human factors engineering approach is needed to address the problem arise in human-machine interaction in food manufacturing industry. Human factors engineering has an objective to minimize the potential of human error and accidents and encouraging human performance of assigned activities as efficiently and effectively as possible (McSweeney et al, 2009).

## MATERIALS AND METHOD

Factors which have impact on the workers in human-machine interaction need to be analyzed. This factors can be found through questionnaire that distributed among the workers. Questionnaire is a structured technique for data collecting that consist of a series of questions, in written or verbal, that need answer from respondents (Malhotra, 2007). Cross-sectional design, a type of research design that involve the collection of information from sample population only once, was used.

The data collected then analyzed statistically in order to find the correlation between the nature of the job and health problems facing by the workers. This correlation is important to determine factors that contribute to health problem in order to find solution that will be able to minimize health problems.

In more advance research, motion study then will be conducted to evaluate the movements that caused health impact of the workers. Biomechanics of the workers need to be evaluated to assess unnecessary movements and reduce movement that will cause injury to human muscle.

## RESULT AND DISCUSSION

Study of the impact of low back pain in poultry industry found (Ekaputra, 2012) that noise cause loss of hearing, vibration make headache to workers and low back pain are common problem in packaging department workers. This low-back pain suffered by most of the workers, even the young one with couple of years working experience in this manufacturing.

When the workers were given five minutes stretching, the result showed positive effect. They feel refreshed, less fatigue, less pain on their back, and more focused for their body and mind. This finding showed that workers engage in heavy physical activity need regular rest break because muscle in continuous use will get tire and become less effective (Schultz, 2006). Regular rest break also reduce repetitive motion injuries to hands and wrists.

This study also tried to find the relation between shift work and health problem, but all shifts showed the same complaint. But, shift one, two, and three showed different result in productivity. Shift one is the morning shift with eight working hours showed the lowest level of productivity (as shown in table 1). Shift two is afternoon shift with 7 hours while shift three with duration of 6 hours working.

**Table 1.** Productivity level for every shift

| Month of April | | | |
|---|---|---|---|
| Shift | Average | Max average | Productivity |
| 1 | 277.2 | 389.53 | 71% |
| 2 | 286.57 | 363.58 | 79% |
| 3 | 287.08 | 323.20 | 89% |

Table 1 showed that the shortest working hours yield the highest level of productivity, means that the longer they have to sit or stand to do their job, the more fatigue they suffer and they will become less productive. This means that the nature of their job, in their interaction with machine, has to be improved. The motion study will be helpful in redesign the work to minimize fatigue. The analysis of every movement is important in order to eliminate unnecessary movement to prevent injury.

Human factor engineering play an important role in manufacturing where human-machine interaction exist. Human factor is an important factor to consider to increase productivity and in the end will increase

profit to a company. But, human operator or labor worker, most of the time, were ignored by management since they are low level workers with low skills and intelligence.

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# THE STUDY OF STABILITY OF ANTIOXIDANT ACTIVITY IN BUTTERFLY PEA (*Clitoria ternatea*) EXTRACT SYRUP

**Nani Pasaribu, Muzi Marpaung, and Fanny Indriyani**
*Food Technology Department, Life Sciences Faculty, Swiss German University, Indonesia*
*Corresponding author: nani.pasaribu@sgu.ac.id*

**ABSTRACT**

The effects of ascorbic acid, sugar and sodium citrate addition to the stability of butterfly pea extract syrup were studied by Response Surface Methodology by Box-Behnken design. The stability of the syrup was observed for 14 days by measuring total phenolic content, antioxidant activity, monomeric and polymeric anthocyanin content. The interaction between sugar and sodium citrate improved the stability of monomeric anthocyanin, while stability of phenolic content was affected by sugar. Ascorbic acid and sodium citrate helped to inhibit the degradation of phenolic compound. Polymeric anthocyanin was proven to be more stable than monomeric anthocyanin. Six syrup formulations were obtained to be the most stable and sensory analysis was held to determine which formulation was the most acceptable for the panelists. The most stable and accepted formulation was syrup that contained 37.55 g sugar, 0.3 g sodium citrate, 3.2 g citric acid and 60 mg ascorbic acid per 40 ml of extract.

***Keywords:*** Butterfly Pea (*Clitoria ternatea L.*), Antioxidant, Sugar, Sodium Citrate, Ascorbic Acid, Anthocyanin, Phenolic

## INTRODUCTION

In recent years, functional drinks have become very popular. Convenience and health have been the most important factors why consumers choose functional drinks. Functional drink can be defined as a product that includes herbs, vegetables, fruits juice or additional vitamins and minerals, which provide specific health benefits. Sports drinks, energy drinks and enhanced juices are some products that marketed as functional drinks. One of the health benefits of functional drink that is in trend now is as antioxidant and it protects the drinkers from free radicals.

Free radicals are actually a natural by-product of body metabolism. Our body has mechanisms to deal with these free radicals, but bad lifestyle can produce excess free radicals more than our body can handle. Smoking, excessive exercise, stress, radiation, polluted environment and consuming too much deep-fried food can cause excess of free radicals. They become problem because they attack our body and damage key cellular molecules such as DNA. Damaged DNAs are prone to develop cancer, cause premature aging, heart disease, arthritis, cataract, chronic fatigue syndrome, etc.

Antioxidant compounds are needed to deactivate these excess free radicals. They protect cells against the damaging effects of reactive oxygen species which is among the major causative factors of many chronic and degenerative diseases. Antioxidant can neutralize free radicals before they can cause harm and help undo damage caused to specific cells.

One good example of the source of antioxidant compound is *Clitoria ternatea L*. which is also commonly known as butterfly pea is a climber herb plant that originated from tropical Asia. The flowers are dark blue and contain high anthocyanin (Terahara *et al*., 1996). Anthocyanin is one of the most abundant natural pigments and has been long proven to have antioxidant properties. The anthocyanins of butterfly pea petals are mainly ternatins which are derived from anthocyanidin named delphinidin (Wongs-Aree *et al.*, 2006). According to Kahkonen & Heinonen (2003), delphinidin has higher activity in preventing oxidation than cyanidin, peonidin, pelargonidin, malvidin and petunidin.

Butterfly pea is one of the Ayurveda medicines, a system of traditional medicine native to India. It also has been used widely in Thailand, Malaysia, Burma, the Philippines and even Indonesia. Besides having function as food colorants (Gupta *et al.,* 2010) and antioxidant, ternatins also give other health benefits, for instance enhance vision (Ghosh & Konishi, 2007), control type II diabetes, reduce risk of coronary heart disease and prevent cancer (Bagchi *et al.,* 2004; Fossen *et al.,* 1998; Gulcin *et al.,* 2004). Ternatins also can act as antimicrobial agent (Uma et al., 2009), blood platelet aggregation inhibitor (Gupta et al., 2010) and immunomodulator (Daisy & Rajathi, 2009).

In Indonesia, butterfly pea can be found growing wild in the yard. Moreover, butterfly pea is a non-seasonal plant, easy to cultivate and very suitable to Indonesia tropical climate. It needs no special treatment, can adapt to various types of soil and also a drought tolerant plant. Regarding all of the good properties and functions of butterfly pea, its utilization should be improved.

Some people may have recognized the health effect of butterfly pea. But, as fresh butterfly pea petals are inconvenient to be directly consumed and not preferable because of the color and aroma, further process is needed to improve its palatability. One of the examples is by processing the flowers into functional drink. Butterfly pea flower itself has been commonly consumed in Thailand as herbal tea or cold drink with addition of lime and sugar.

The formulation of the functional drink becomes very important because anthocyanins are very unstable. The antioxidant activity and color stability of the butterfly pea extract are easily degraded. It is correlated with its structural features and is also affected by some factors such as heat, pH, light, oxygen, sugar, enzymes and metal ions (Rein, 2005). In the previous thesis research by Ria Amelia (2011), the effect of pH, temperature during storage, presence of light and type of packaging had been studied. But, further research about how the functional drink is formulated and treated in order to preserve the health function is needed considering that there are many factors affecting its stability.

Lowering water activity by adding sugar and processing the extract into thicker solution or syrup can be protective against anthocyanins degradation to improve the quality of the product and minimize the degradation of antioxidant activity (De Ancos et al., 1999b). Ascorbic acid also is added to improve the antioxidant activity to add the health benefit of the drink, while sodium citrate is added to act as acidity regulator and sequestrant to improve the stability of the product. This research will focus on how those ingredients will give effect to the anthocyanin content and antioxidant activity stability of the product. The experiment is designed by using Response Surface Methodology with Box-Behnken design. Conventional optimization process cannot give an indication of the interactive effects between any two factors in a multi-variable system. Response surface methodology can avoid the limitations of conventional methods.

**Fundamental Theory**

In Southeast Asia, the blue flowers have been used as food colorant. A syrupy blue drink made from the blue petals of butterfly pea named as nam dok anchan is also very famous in Thailand.

In ancient days, the flower also has been used to treat hair loss and grey hair (Kamkaen & Wilkinson, 2009). The blue petals contain several flavonoids such as quercetin, isoquercitin, robinin, ternatin, delphinidin, pethunidin and malvidin. The anthocyanin can act as antioxidant and anti-inflammatory agent. Quercetin for is effective in reducing blood pressure in hypertensive people (Edward *et al.,* 2007). According to Rao *et al.* (2003), ternatins shows to serve as an anti-inflammatory agent in animal studies. Study has showed that the ethanol extract of the flowers has weaker anti-oxidant activity than the aqueous extract. (Kamkaen & Wilkinson, 2009). Isoquercitrin found in blue butterfly pea flowers has also been proven to improve symptoms in patients with chronic venous insufficiency when used as a constituent with other flavonols (Schaefer *et al.,* 2003). Butterfly pea flowers also can act as anti-diabetic compound (Terahara *et al.,* 1998; Daisy *et al.,* 2009).

Butterfly pea flower petals have been reported to contain mainly ternatins. There are around fifteen of them, and most of them are malonylated delphinidin 3,3'5'-triglucosides having 3'5'-side chains with alternative D-glucose and p-coumaric acid units and delphinidin 3-O-(2"-O-α-rhamnosyl-6'-O-malonyl-β-glucoside (Terahara *et al.,* 1996; Kazuma *et al.,* 2003). According to a research by Fukumoto & Mazza (2008), delphinidin has the highest antioxidant activity, followed by cyanidin, peonidin, pelargonidin, malvidin and petunidin.

The phytoconstituent found in the young butterfly pea flowers are the minor delphinidin glycosides, eight anthocyanins (ternatins C1, C2, C3, C4, C5, D3 and preternatins A3 and C4). Besides those anthocyanins, there are also other anthocyanins that were isolated in recent study, it were ternatins A1, A2, A3, B1, B2, B3, B4, D1 and D2 (Terahara *et al.,* 1996; Kazuma *et al.,* 2003).

## MATERIALS AND METHODS

### Materials and Chemicals

The main materials used in this research are butterfly pea flowers *(Clitoria ternatea L.*). The fresh flowers were collected from the private plantation in Pamulang-Tangerang, Indonesia. The flowers were carried to the laboratory and extracted. 2,2-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteau reagent and gallic acid were obtained from Merck (Germany).

### Preparation of *Clitoria ternatea L.* Extract

The flower petal of *Clitoria ternatea L.* were harvested and separated between the white and blue parts. The blue parts of the flowers were cut into small pieces and put into plastic film. It was blanched for 6 minutes. Then the blanched petals were extracted with water in a waterbath shaker at 60°C for 30 minutes. 40 ml of water was added for each 10 g of blue petals. The suspension was filtered.

### Analysis of Stability

The syrup samples were stored in refrigerator for 2 weeks. The stability analysis including total phenolic content assay, antioxidant activity assay and monomeric anthocyanin content assay were performed.

### Total Phenolic Content Assay (Waterhouse, 2002)

Total phenolic content was determined by using Folin Ciocalteau method according to the method described by the International Organization for Standardization (ISO) 14502-1 (122). Briefly, 0.2 ml of sample was mixed with 1 ml of Folin-Ciocalteau reagent (previously diluted 10-fold with distilled water). 0.8 ml of 7.5% w/v sodium carbonate solution was added to the mixture. After 60 minutes incubatedat room temperature, absorbance was read at 765 nm using spectrophotometer. Results were expressed as mg gallic acid equivalents in 1 l of syrup sample (mg/l GAE).

### Antioxidant Activity Assay (Prakash, 2001)

The antioxidant activity was measured using the DPPH radical scavenging activity method. This method also used spectrophotometer with DPPH as standard. The sample (0.1 ml) was added with 0.75 ml of 96% ethanol and 0.15 ml od DPPH solution. Absorbance at 515 nm was read after 30 minutes incubation at dark place. The percent of antioxidant activity was calculated as [(a-b)/a]x100 (a=absorbance of control; b= absorbance of sample).

### Monomeric Anthocyanin Content Assay (Giusti & Wrolstad, 2001; AOAC, 2005)

Anthocyanin content was measured using pH-differential method. Two set of the same sample were prepared in 0.025 M potassium chloride solution and in 0.4 M sodium acetate solution adjusted respectively to pH 1.0 and 4.5 with HCl. The absorbance of each mixture was measured at 546 nm and 700 nm against a distilled water blank using UV-visible spectrophotometer. The absorbance of the sample was calculated according to the equation below:

$$\mathrm{Abs(A)} = \left[\left(A_{546} - A_{700}\right)_{\mathrm{pH1}} - \left(A_{546} - A_{700}\right)_{\mathrm{pH4.5}}\right]$$

Anthocyanin was calculated as delphinidin-3-glucosides according to the following equation:

$$\text{Anthocyani n content } (\mathrm{mg/l}) = \frac{\left(\mathrm{Abs\ x\ Mr\ x\ DF\ x\ CF}\right)}{\left(\varepsilon\ \mathrm{x}\ 1\right)}$$

**Experimental Design**

This research was studied by using Response Surface Methodology with three-factorial Box-Behnken experimental design as established using Design Expert software (8.0 trial version). The variable input parameters were ascorbic acid concentration of 0-60 mg/serving, sugar concentration in the range 30-40 g/serving and percentage of sodium citrate to total of sodium citrate and citric acid in the range 0-20%, the factor levels being coded as -1 (low), 0 (medium) and 1 (high), respectively. The three independent variables were designated as A (ascorbic acid concentration, B (sugar concentration and C (percentage of sodium citrate) respectively, for statistical computations. The range and levels used in the experiments are listed in Table 1.

Table 1. Independence Factors and their Coded Levels used for Optimization

| Variable | Real Values of Coded Levels | | |
|---|---|---|---|
| | -1 | 0 | +1 |
| Ascorbic acid concentration, A | 0 | 30 | 60 |
| Sugar concentration, B | 30 | 35 | 40 |
| Sodium citrate percentage, C | 0 | 10 | 20 |

A total of 17 runs were performed to find the optimum formulation, according to the design proposed by Box-Behnken design that consists of 17 runs, in which 5 of them were the replication of center point of each factors. The samples were monitored every two day for two weeks.

**Sensory Analysis**

The sensory analysis was done to obtain information about the panelists' acceptance level on the product. Three optimum formulation that have high initial anthocyanin content, antioxidant activity and total phenolic content with minimum degradation of antioxidant activity and anthocyanin content were chosen based on the solutions provided by Design Expert. The sensory test was done using 9 hedonic scales, in which 9 had the highest acceptance level and 1 had the lowest acceptance level. The samples were served to 45 untrained panelists. The panelists were asked to evaluate those three samples based on four attributes, which were color, aroma, taste and overall performance of the samples.

## EXPERIMENTAL RESULTS

**Monomeric Anthocyanin Content**

The 2-Factorial Interaction (2FI) equation for predicting the optimal initial monomeric anthocyanin content was achieved according to Box-Behnken experimental design and input variables, the empirical relationship between the response and the independent variable in the coded units based in the experimental results was given by:

$$Monomeric\ anthocyanin = 27.04 - (0.15 \times A) - (1.47 \times B) + (1.09 \times C) + (3.88 \times AB) - (1.24 \times AC) - (3.37 \times BC)$$

The results from the ANOVA for the equation are presented in Table 2. The ANOVA suggested that the equation and the actual relationship between the response and the significant variables represented by the equation were adequate. The p-value was lower than 0.05, indicating that the model may be considered to be statistically significant. AB and BC were the only significant parameters. The other factors whose p-values are listed as being greater than 0.05 in Table 2 were not significant factors.

Table 2. P-value for Response Surface 2FI Model for Initial Monomeric Anthocyanin Content

| Source | P-value | Significance |
|---|---|---|
| Model | 0.0172 | Significant |
| A-Ascorbic Acid | 0.8547 | not significant |
| B-Sugar | 0.0938 | not significant |
| C-Sodium citrate | 0.2011 | not significant |
| AB | 0.0062 | Significant |
| AC | 0.2968 | not significant |
| BC | 0.0134 | Significant |
| Lack of Fit | 0.8646 | not significant |

There was interactive correlation between ascorbic acid and sugar in the syrup. As can be seen in the equation, ascorbic acid and sugar had an interactive correlation, which means the monomeric anthocyanin was higher when the concentration of those both ingredients were at the highest point. Sugar and sodium citrate interaction also gave significantly different effect to the monomeric anthocyanin content, but they worked in an opposite way. The monomeric was higher when either the sugar was high and sodium citrate was low or the other way around. The equation indicated that sugar played dominant role in interaction with the other factors in determining the initial amount of monomeric anthocyanin.

The correlation among the factors can be seen clearer in contour plot. When the sodium citrate concentration was 0%, the monomeric anthocyanin was the highest in syrup that contained high sugar and high ascorbic acid concentration (Figure 1). On the other hand, when the sodium citrate was 20%, the anthocyanin was highest if the sugar and ascorbic acid concentration was the lowest (Figure 2). The sodium citrate concentration was related to citric acid concentration. If the concentration of sodium citrate was 20%, then the 80% was citric acid. After observing the effects correlated to each other, the monomeric anthocyanin was higher when the sugar content was high accompanied with lower pH. According to Tsai *et al.* (2005), sucrose can decrease monomeric anthocyanins especially during heating. In the presence of acid, the sucrose was hydrolyzed to become glucose and fructose which preserved the monomeric anthocyanin better than sucrose. That is why the monomeric anthocyanin was higher when the sugar and acid concentration was high.

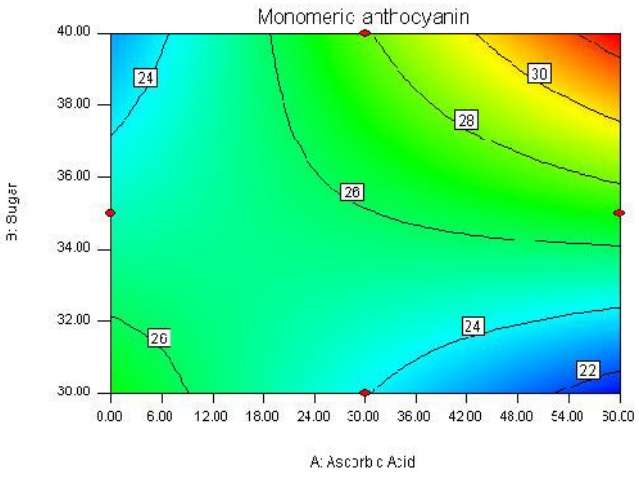


Figure 1. Effect of Sugar and Ascorbic Acid on Initial Monomeric Anthocyanin Content (0% Sodium Citrate)

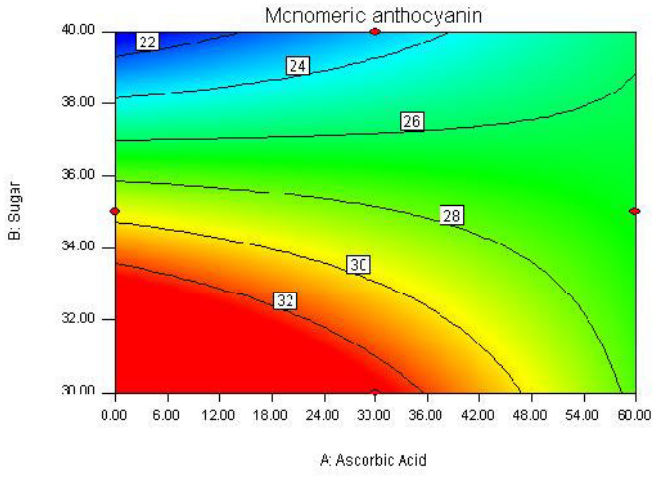


Figure 2. Effect of Ascorbic Acid and Sugar on Initial Monomeric Anthocyanin Content (20% Sodium Citrate)

Formulation with low concentration of citric acid and sugar had higher monomeric anthocyanin than the sample with high concentration of citric acid and sugar because sample that was too acidic can cause negative error to the result of the monomeric anthocyanin content assay because the principal used was the pH differential method. According to Giusti & Wrolstad (2005), the method was based on the change of pH, in which oxonium red-coloured form of monomeric anthocyanin predominates at pH 1 and hemiketal colorless form of the monomeric anthocyanin predominates at pH 4.5. This was why the absorbance was measured at both pH. Sample with very low pH can influenced the pH of the solution after the buffer had been added. The buffer's capacity might be exceeded because the sample was too acidic and caused the pH was not 4.5 when measured by spectrophotometer. It caused the result of monomeric anthocyanin result to be lower than it really was.

The high acidity of the sample brought two effects. First, it was accompanied by high sugar, it prevent anthocyanin from decreasing. Second, when the sample contained low sugar, the effect of acid became negative because it influenced the reading of the pH differential method to measure the monomeric anthocyanins.

After the syrup was stored for two weeks, the monomeric anthocyanin content was measured again. According to Box-Behnken experimental design, 2FI equation was achieved. The relationship between the response and the variables in coded units based on the experimental results was given by:

*Monomeric Anthocyanin = 101.41 – (0.52 x A) + (8.12 x B) + (2.89 x C) – (11.25 x AB) – (4.11 x AC) + 18.82 x BC)*

The results from the ANOVA for the equation are presented in Table 3. The model p-value was lower than 0.05. It indicated that the model can be considered as statistically significant. The only significant parameter was BC. The other factors whose p-values are listed as being greater than 0.05 in Table 3 were not significant factors.

As can be seen from the equation, ascorbic acid and sodium citrate gave no significant effect to the stability of monomeric anthocyanin while sugar and the interaction of ascorbic acid and sugar gave considerable effect despite it is not significant. However, sugar and sodium citrate have interactive relation. The formulation that had high stability of anthocyanin was sample with either both high concentration of sugar and sodium citrate. The relation between those two factors also can be seen in Figure 3. The most stable monomeric anthocyanin was showed by the red area in the contour plot.

Table 3. P-value for Response Surface 2FI Model for Monomeric Anthocyanin Stability after 14 Days

| Source | P-value | Significance |
|---|---|---|
| Model | 0.0384 | Significant |
| A-Ascorbic Acid | 0.8966 | not significant |
| B-Sugar | 0.0645 | not significant |
| C-Sodium citrate | 0.4771 | not significant |
| AB | 0.0691 | not significant |
| AC | 0.4744 | not significant |
| BC | 0.0067 | Significant |
| Lack of Fit | 0.2686 | not significant |

When sodium citrate was diluted into the product, it was ionized to sodium ions. Sodium ions, which are metal ions, are able to enhance the color of anthocyanins. It was due to the ability of the anthocyanin and metal ions to form metal chelation (Osawa, 1982). Moreover, butterfly pea petals contained mostly delphinidin based anthocyanin. According to Osawa (1982), delphinidin based anthocyanin is more capable of metal chelation because they have more than one free hydroxyl group in the B-ring. A research done by Li *et al.* (2009) indicated that metals can improve the stability of monomeric anthocyanin.

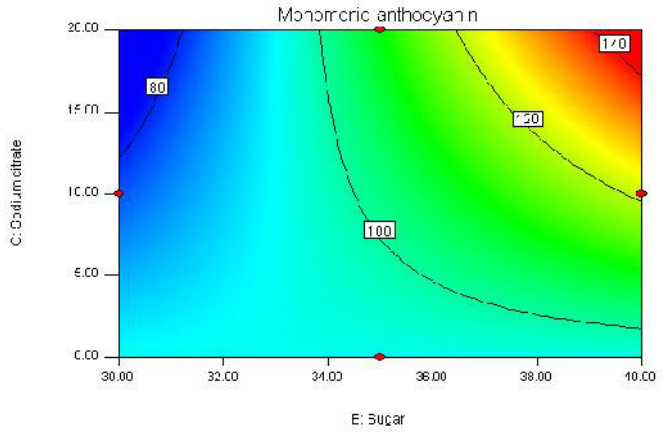


Figure 3. Effect of Sodium Citrate and Sugar on Stability of Monomeric Anthocyanin (0 mg of Ascorbic Acid)

Sodium ion that originated from the sodium citrate added to the syrup also can stabilize the monomeric anthocyanin through metal chelation. Both sugar and sodium citrate gave synergic effect to the monomeric anthocyanin content in long term, which was different from the initial value. In term of the stability, the sugar contained in the sample improved the stability of the monomeric anthocyanin because it inhibits the condensation reaction and reduce water activity, thus prevent hydrolysis reaction **(**Wrolstad *et al.* 1990).

**Antioxidant Activity**

The antioxidant activity of the syrup after formulation was analyzed and compared by calculating the amount of DPPH consumed per liter syrup. High amount of DPPH consumed means that the sample has high antioxidant activity. According to Box-Behnken, the relationship between the response and the variables followed 2FI model. It was represented by this equation in coded units:

*Antioxidant Activity = 3799.98 + (2629.94 x A) – (594.72 x B) + (135.56 x C) – (829.74 x AB) – (131.02 x AC) - (78.21 x BC)*

The results from the ANOVA for the equation are presented in Table 4. The p-value suggested that the equation and the relationship between response and variable were statistically significant. A was a highly significant factor, while B and AB were also significant. The other factors whose p-value greater than 0.05 were not significant factors.

The antioxidant activity based on design expert analysis was affected by two factors, which were ascorbic acid concentration and sugar concentration. Ascorbic acid itself is a reducing agent that also can act as antioxidant (Frei *et al.*, 1989). The addition of ascorbic acid certainly increased the antioxidant activity of the syrup and more DPPH was consumed by the sample compared to sample without ascorbic acid. The higher the concentration of ascorbic acid, the higher the antioxidant activity is.

Table 4. P-value for Response Surface 2FI Model for Total DPPH Consumed on Day 0

| **Source** | **P-value** | **Significance** |
|---|---|---|
| Model | < 0.0001 | Significant |
| A-Ascorbic Acid | < 0.0001 | Significant |
| B-Sugar | 0.0060 | Significant |
| C-Sodium citrate | 0.4472 | not significant |
| AB | 0.0065 | Significant |
| AC | 0.6006 | not significant |
| BC | 0.7536 | not significant |
| Lack of Fit | 0.0848 | not significant |

Sugar unexpectedly gave the opposite effect to the antioxidant activity. The antioxidant activity of syrup that contained high sugar was lower than in the sample with lower sugar concentration. The effect of sugar to antioxidant activity was predicted to relate with sugar hydrolysis by acid. Sugar that was used in this experiment was sucrose. Sucrose is easily hydrolyzed, even by weak acid, through the addition of water. The hydrolysis converts sucrose into glucose and fructose (Lowe, 2010). Ingredients used in the formulation of the syrup certainly very possible in causing the hydrolysis of sucrose. It can be catalyzed by citric acid and ascorbic acid. According to Tikekar (2011), fructose in apple juice is able to significantly increased ascorbic acid degradation while glucose and sucrose did not.

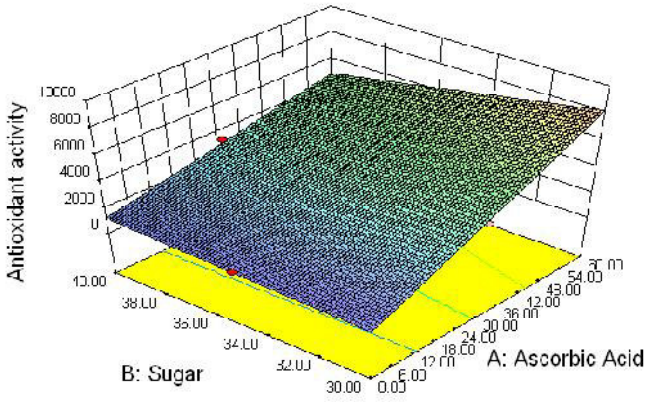


Figure 4. Effects of Sugar and Ascorbic Acid on Initial Antioxidant Activity

Based on the experiment data, the DPPH consumed in sample containing high sugar and high ascorbic acid was lower than the sample containing low sugar and high concentration of ascorbic acid (Figure 4). This may because of the fructose resulted from the sucrose hydrolysis caused the degradation

of ascorbic acid. Less ascorbic acid means decrease in antioxidant activity.

The syrup product is supposed to have high antioxidant activity. Even though on day 0, the sample contained high antioxidant, it cannot be assumed that the antioxidant properties would remain the same during storage after 2 weeks period. The stability of antioxidant activity was analyzed by using ANOVA. The linear equation that showed the relation between the response and the independent variables in the coded units based on the experiment result was given by:

*Antioxidant Activity = 75.66 + (6.16 x A) + (14.89 x B) + (3.74 x C)*

The results from the ANOVA for the equation are presented in Table 5.The p-value of the model was lower than 0.05, indicating that the model may be considered to be statistically significant. B was the only significant parameters, while the other factors were insignificant because the p-values were greater than 0.05.

Table 5. P-value for Response Surface Linear Model for Total DPPH Consumed after 14 Days

| Source | P-value | Significance |
|---|---|---|
| Model | 0.0298 | Significant |
| A-Ascorbic Acid | 0.2140 | not significant |
| B-Sugar | 0.0076 | Significant |
| C-Sodium citrate | 0.4426 | not significant |
| Lack of Fit | 0.0730 | not significant |

Based on the equation, ascorbic acid and sodium citrate did not give any significant difference to the stability of antioxidant activity. There was only one factor that gave significant effect to the stability of antioxidant which was sugar concentration. It can be concluded that higher concentration of sugar can minimize the degradation of antioxidant activity (Figure 5).

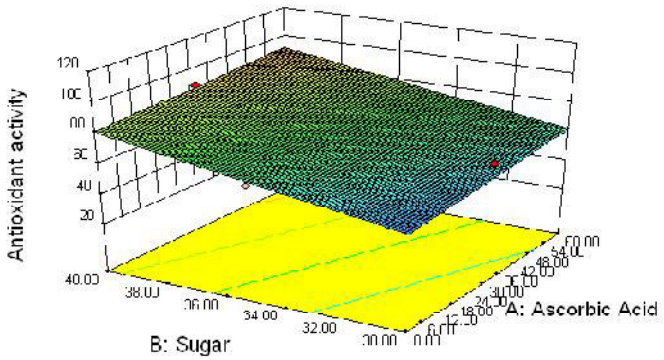


Figure 5. Effect of Sugar to Antioxidant Activity after 14 Days of Storage

Sugar can inhibit condensation reaction between anthocyanin molecules in the butterfly pea extract (De Ancos *et al.* 1999). Besides that, high sugar concentration decreased water activity. Anthocyanin degraded more slowly with decreasing water activity (Erlandson *et al.*, 1972). Water availability also affected the hydrolytic reaction, which can be the cause of antioxidant activity in the product (Jimenez *et al.*, 2011). As the anthocyanin can be preserved better, the antioxidant activity also went through very small degradation when the sugar concentration was high.

**Total Phenolic Content**

Total phenolic content in each sample was also measured because butterfly pea extract contains not only anthocyanins but also other phenolic compounds that also have health functions. The Reduced Lineal Model was used to predict the optimal total phenolic content. The relationship between response and variables as coded units was given by:

*Total phenolic content = 700.12 + (54.72 x A) – (43.81 x C)*

The results from the ANOVA for the equation are presented in Table 6. The ANOVA suggested that the equation and the relationship between the response and variables were statistically significant. A was the only significant factors, while the others whose p-values greater than 0.05 in Table 6 were insignificant factors.

Table 6. P-value for Response Surface Reduced Linear Model for Total Phenolic Content on Day 0

| Source | P-value | Significance |
|---|---|---|
| Model | 0.031 | Significant |
| A-Ascorbic Acid | 0.0345 | Significant |
| C-Sodium citrate | 0.0819 | not significant |
| Lack of Fit | 0.1963 | not significant |

Based on the ANOVA, the samples showed significant different among each other. Sugar and sodium citrate concentration gave no significant different to total phenolic content among the sample. On the other hand, ascorbic acid apparently gave significant impact to the total phenolic content in the samples (Figure 6).

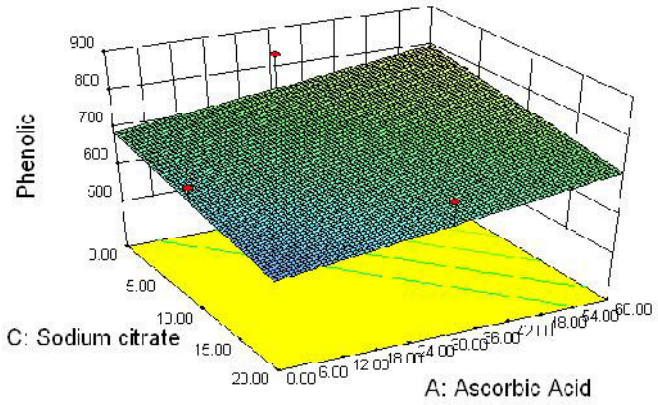


Figure 6. Effect of Ascorbic acid and Sodium Citrate on Initial Phenolic Content

Based on Altunkaya *et al.*, (2008), the decrease of phenolic content in fruits and vegetables can be prevented by using of ascorbic acid as a reducing agent. Furthermore, ascorbic acid had synergic effect with total phenolic content of fruits (Kähkönen *et al.* 2001).

After storage period of 2 weeks, the total phenolic content went through degradation. The quadratic model showed the relation between response and variables. Here is the equation in coded units.

*Phenolic Content = 86.40 – (1.19 x A) + (3.88 x B) + (5.94 x C) – (2.71 x AB) + (12.95 x $A^2$) + (4.93 x $B^2$) – (6.38 x $C^2$)*

The significance of each factor that gave influence to the stability of phenolic content can be seen in Table 7 based on the p-value. The ANOVA suggested that the equation and the actual relationship between the response and the significant variables were statistically significant. C and $A^2$ were both significant factors.

Table 7. P-value for Response Surface Reduced Quadratic Model for Total Phenolic Content Stability after 14 Days

| Source | P-value | Significance |
|---|---|---|
| Model | 0.0121 | Significant |
| A-Ascorbic Acid | 0.5972 | not significant |
| B-Sugar | 0.1080 | not significant |
| C-Sodium citrate | 0.0233 | Significant |
| AB | 0.4023 | not significant |
| A^2 | 0.0019 | Significant |
| B^2 | 0.1348 | not significant |
| C^2 | 0.0625 | not significant |
| Lack of Fit | 0.7561 | not significant |

It can be seen that sodium citrate and ascorbic acid gave significant effect to the stability of phenolic content, while the other compounds gave no significant effect. Phenols compound can react with active metals like sodium to yield phenoxides and hydrogen (Clark, 2004). The interaction with metal resulted in more stable form. That is why when the sodium citrate is high, phenol degradation was minimized (Figure 7).

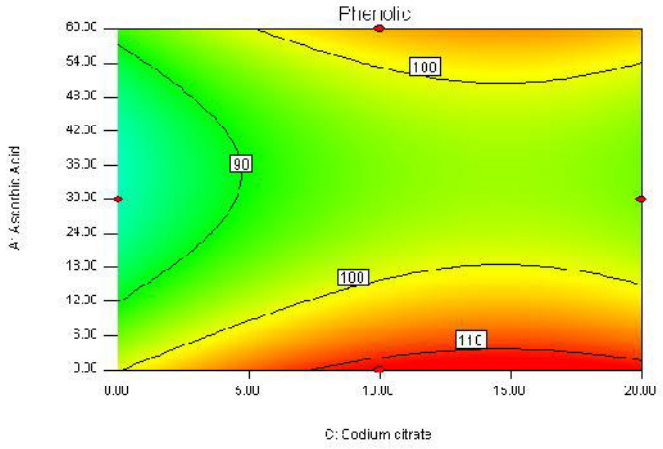


Figure 7. Effect of Sodium Citrate and Ascorbic Acid on Total Phenolic Content on Day 14

The effect of ascorbic acid was more dominant than the effect of sodium citrate. Based on Lattanzio (1989), ascorbic acid can protect phenol compounds from degradation, in the presence of oxygen and other compounds that can cause degradation. The ascorbic acid effect followed the quadratic model, which means that the phenolic compound reached maximum when ascorbic acid concentration was the lowest or the highest (Figure 8). When the ascorbic acid was in medium level, the phenolic compound was not the maximum. The total phenolic compound in the syrup was contributed also by anthocyanin. According to Sarma (1997), anthocyanin can protect ascorbic acid from degradation. Anthocyanin was also belong to phenol groups, will be degraded. This will cause the total phenolic compound also decreased.

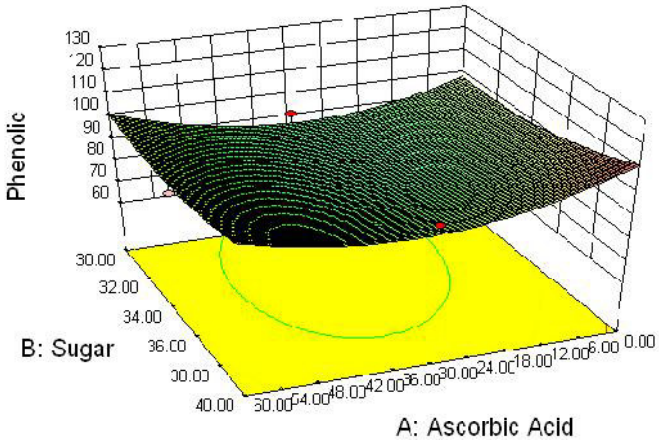


Figure 8. Effect of Ascorbic Acid to the Total Phenolic Content on Day 14

**Sensory Analysis**

Three samples (Table 8) were picked based on some criteria. They have the highest initial monomeric anthocyanin content, highest antioxidant activity and high phenolic compounds content. The usage of citrate was also minimized because it did not give significant effect to the stability of syrup. Besides those criteria, the formulas also have the lowest level of degradation of monomeric anthocyanin and antioxidant activity.

Table 8. Three Optimum Formulas

| Ascorbic Acid (mg/500 ml) | Sugar (g/500ml) | Sodium Citrate (g/500ml) | Citric Acid (g/500ml) |
|---|---|---|---|
| 60 | 34.78 | 0 | 3.5 |
| 60 | 40 | 0.4242 | 3.0758 |
| 60 | 37.55 | 0.30275 | 3.19725 |

The result of the sensory analysis turned out that the panelists preferred the second and third samples in overall equally. The first was less accepted because it tasted too sour.

## CONCLUSIONS

From this research, it can be concluded that higher concentration of sugar resulted in the decrease in monomeric anthocyanin, polymeric anthocyanin and antioxidant activity degradation. However, the sugar concentration gave no significant effect to the initial total phenolic content. Sucrose can protect anthocyanin from degradation. It reduced water activity and inhibited condensation reaction of anthocyanin in the system.

Sodium citrate also improved the stability of the syrup. Higher concentration of sodium citrate correlated with high concentration of sugar reduced the anthocyanin degradation. Phenolic compound was also stabilized by the addition of sodium citrate. Sodium citrate was ionized in the solution, resulted in sodium ions that formed metal chelation with anthocyanin or phenolic compound and stabilized them.

Ascorbic acid did not give significant effect to the stability of anthocyanin content and antioxidant activity, although the presence of ascorbic acid can increase the antioxidant activity and health benefit of the product. This research also proved that ascorbic acid improves the stability of phenolic content.

The best formulation was characterized by having the maximum initial anthocyanin content, antioxidant activity and total phenolic content with the minimum degradation after 14 days of storage

period. The result of best formulation was syrup that contains 60 mg of ascorbic acid, 37.55 g of sugar, 0.3 g of sodium citrate, 3.2 g of citric acid and 40 ml of extract. All of them were diluted with water to total volume of 500 ml.

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# EFFECT OF INITIAL CONCENTRATION OF $CO_2$ AND STORAGE TEMPERATURES ON THE CHARACTERISTICS OF PACKED BROCCOLI

**Ida Bagus Putu Gunadnya[1], John Janes[2] and Cokorda Istri P. Mudati[1]**
[1]Department of Agricultural Engineering, Faculty of Agricultural Technology,
Udayana University, Denpasar, email: gunadnya@ftp.unud.ac.id
[2]Curtin University, Perth, Western Australia, email: j.janes@curtin.edu.au

## ABSTRACT

Modified atmosphere packaging (MAP) has been used widely to pack fresh produces. Some efficacies of MAP when combined with cold storage are to lengthen shelf life and to retain quality of fresh fruits and vegetables. Broccoli as one of important and valuable vegetables has a very short shelf life and rapid rate of wilting. Besides, its crop is easy to bloom and grow turning its green color to yellowish green. To lengthen the shelf life of broccoli and to alleviate the rate of change on its characteristic, it is useful to conduct experiment utilizing MAP technology. Changing the initial gas concentration inside the broccoli packages by flushing with $CO_2$ gas is expected to hasten MAP effect on the broccoli during storage. The experiment was carried out for 6 days with 2 days interval of observation using a two factors completely randomized design. The first main factor was 4 levels of initial $CO_2$ concentrations, namely 0%, 4%, 8%, and 12% $CO_2$ (v/v). The second main factor was cold storing with two storage temperatures at 20 and 5°C. Weight loss, crop growth, ascorbic acid and chlorophyll contents were observed during storage. The results of experiments showed that all parameter observed were significantly affected by the two main factors. Combination of these two main factors affected all parameters, except the changing in weight loss and also ascorbic acid content of broccoli after 2 and 4 days of storage. Adding 4% and 8% $CO_2$, and storing at 5°C gave significant difference on all parameter.

***Keywords:*** vegetables, broccoli, MAP, cold storage, quality.

# FEEDING YACON TUBER-CONTAINING OLIGOFRUCTOSE INCREASES INTESTINAL FERMENTATION AND ALTERS MICROBIAL COMMUNITY STRUCTURE IN RATS

**Ni Wayan Arya Utami*, Michiko Tanaka, Teruo Sone, Cindy Nakatsu, and Kozo Asano**
[1]*Graduate School of Agriculture, Hokkaido University, Sapporo, Japan*
[2]*School of Public Health, Udayana University, Bali, Indonesia*
[3]*Department of Agronomy, Purdue University, Indiana, USA*
**Corresponding author: utami@chem.agr.hokudai.ac.jp*

## ABSTRACT

Tubers of the *Smallanthus sonchifolius* (yacon) contain mainly fructo-oligosaccharides, thus yacon tuber potentially has prebiotics properties. Aims of this study were to compare the effect of yacon and fructo-oligosaccharides on cecal weight, short-chain fatty acid (SCFA) concentration, pH, and microbial community structure. Eighteen male Sprague-Dawley rats were randomly divided into one of three groups (n=6/group), yacon group (Basal Diet (BD)+10% yacon powder), FOS group (positive control) (BD+5% FOS), and negative control (BD+8% cellulose) for 28 d. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes was performed to characterize differences of fecal and cecal microbial communities. Generally, total cecal SCFA were higher on the yacon diet than on FOS; both were two times higher than the negative control, decreased cecum pH similar to results of the purified FOS diet. Cecal content weight was higher on FOS diet. Cluster analysis of PCR-DGGE fingerprint patterns showed that cecal and fecal bacterial communities in rats fed yacon were similar to rats fed fructo-oligosaccharides, and were separate from the control. Temporal analysis of microbial community in rat fecal samples indicated that changes on the yacon diet were seen earlier than on the fructo-oligosaccharides diet. Preliminary sequencing of common bands to rats fed yacon and fructo-oligosaccharides represent *Lactobacillus* species. Dietary incorporation of yacon tuber, similar to FOS, by providing SCFA, lowering pH and increasing lactobacilli, may be beneficial in improving gastrointestinal health.

***Keywords:*** yacon, DGGE, fructo-oligosaccharides, Lactobacilli, rats

# CHARACTERISTICS OF MICROORGANISMS IN BLACK TEA BROTH (KOMBUCHA) IN BALI

**Pande Putu Ayu Sukmawati[1], Ni Putu Eka Leliqia[1], and Yan Ramona[2]**
*[1]Pharmacy Department, Faculty of Mathematics and Natural Sciences, Udayana University, Kampus Bukit Jimbaran, Bali.*
*[2]Biology Department, Faculty of Mathematics and Natural Sciences, Udayana University, Kampus Bukit Jimbaran, Bali.*
*leliqia@gmail.com*

**ABSTRACT**

Kombucha tea is a mixture of tea and sugar fermented with a symbiotic association of acetic acid bacteria and *yeasts* and is known to have antioxidant activity. It has been reported that microorganisms in Kombucha has a varied composition and this composition will affect its activity. This present study aimed to determine characteristics of microorganisms present in Kombucha cultured from local resources (Bali). The results showed that Acetobacter and Schizosaccarhomyces, Saccarhomyces, Torulaspora, and Zygosaccharomyces were found to be dominant microorganisms involved in the process of fermentation.

***Keywords:*** Bacteria, Bali, Black tea, Kombucha, Yeast.

# ORAL PRESENTATIONS: BIODIVERSITY AND ENVIRONMENT

# PRELIMINARY STUDY ON THE PRODUCTION OF BIOETHANOL USING *Codium geppiorum* AS THE CARBON SOURCE AND NUSA PENIDA LIMESTONE AS THE ADSORBENT

**I Wayan Karta[1], Ni Made Puspawati[2], Wiwik Susanah Rita[2], NGAM Dwi Adhi Suastuti[2], and Yenni Ciawi[3*]**
*[1]Department of Chemistry, Universitas Pendidikan Ganesha, Singaraja Bali, Indonesia*
*[2]Department of Chemistry, Udayana University, Denpasar, Bali, Inodnesia*
*[3]Engineering Faculty, Udayana University, Denpasar, Bali, Indonesia*
*[*]Corresponding Author: yenniciawi@yahoo.com*

**ABSTRACT**

The aims of this research are to investigate (1) the effect of inoculum concentration and length of fermentation on the concentration of ethanol produced by consortium of yeast using *Codium geppiorum* as the carbon source and (2) the effect of burning temperature and mass of limestone used as adsorbent on the concentration of ethanol produced. This research was a true experimental with completely randomized design 3 x 4 factorial using 2 variables *i.e.* amount of inoculum and length of fermentation. The ethanol concentration obtained was measured using Gas Chromatography techniques and data was analysed using two ways Anava software SPSS 17.0. The results showed that $F_{count} > F_{table}$ (38.212 > 2.51) with the probability of 0.000 indicating that there was interaction between yeast concentration and length of fermentation. The optimum result for the fermentation process was found by applying 20% inoculum on 25 g of algae for 7 days fermentation which gave an average of 3.03% ethanol.. It was also found that $F_{count} > F_{table}$ (3.082 > 2.51) with probability of 0.022 for the process of ethanol dehydration suggesting that there was interaction between temperature of activation and mass of limestone used in ethanol dehydration process. The optimum result was obtained by using 50 g of limestone activated at 800°C producing ethanol with the concentration of 99.15%. Therefore, it can increase the concentration of bioethanol from 28.92% to 83.78%. In conclusion, variations in the concentration of yeast and length of fermentation using *Codium geppiorum* significantly influence the levels of ethanol produced, and variations of activation temperature and mass of limestone used as adsorbent significantly affect the increase of the ethanol concentration.

***Keywords:*** bioethanol, *Codium geppiorum*, dehydration, limestone, yeast

## INTRODUCTION

Fuel grade bioethanol has ethanol content of more than 99.5%. It can be used as alternative energy because of its high oxygen content, higher octane number than gasoline, biodegradable, and renewable. High oxygen content will improve combustion efficiency and reduce pollution caused by vehicle exhaust such as hydrocarbons, carbon monoxide, and particulate, or greenhouse gases. Higher octane number will reduce the occurrence of knocking and can replace the function of additives in petrol (methyl tertiary butyl ether/MTBE or tetra ethyl lead). In addition, ethanol also has higher flame rate and higher steam calor than gasoline. This will give a higher compression ratio and shorter burn time (Balat et al., 2007).

Until present, many types of raw material have been utilized to produce fuel grade bioethanol, e.g. cane sugar, sorghum, palm sap, sweet potato. These raw materials are foodcrop and require large terrestrial plantation area. Therefore, alternatives for other raw materials is urgently needed. Seeweeds is one of that which has many advantages e.g. it can grow extensively, less land-competition with agricultural crop, absorbing $CO_2$, as well as renewable energy sources (Nahak et al., 2011). This research used *Codium geppiorum*,which can be found in the Nusa Lembongan, with a carbohydrate content of 69.10% (Puspaningrat et al., 2011).

The process of bioethanol production consists of pretreatment, hydrolysis, fermentation, and purification. Hydrolysis is carried out by using acid or by enzymatic reaction (Hamelinck et al., 2005). Fermentation is carried out by yeast or bacteria. In this research, the inocula was commercially available starter and is commonly used for traditionally fermented cassava in Indonesia, which consist of a consortium of microorganisms; *Amylomyces rouxii, Mucor sp., Rhizopus sp.; Saccharomycopsis fibuligera, Saccharomycopsis malanga, Burtonii pichia, Saccharomyces cerevisiae and Candida utilis*, *Pediococcus* sp. and *Bacillus* sp. Some of the microbes are non-ethanol producers but they produce useful enzymes for ethanol production (Kusnadi et al., 2009).

Fermentation yields affected by the type and amount of inoculum, sugar concentration and enzyme concentration, duration of fermentation, acidity (pH), temperature, air (oxygen), and other nutrients. The number of microbes will affect the concentration of enzymes in the fermentation which in turn will affect ethanol production. Optimum length of fermentation is important to ensure efficient ethanol production (Kusuma, 2010; Jumari et al., 2009; Bamforth, 2005).

Fermentation usually produced less than 15% ethanol as the producers can not tolerate high ethanol concentration. Therefore, further step is needed to increase the ethanol concentration. Distillation usually produces ethanol less than 95% due to the azeotropic properties. Further purification is usually done by dehydration to separate the mixture of ethanol-water by eliminating the water. This can be done by adsorption or absorption.

This study employ Nusa Penida's limestone which were activated to dehydrate ethanol to produce higher concentration of bioethanol. The chemical composition of the limestone is 87.35% CaO, 1.12% $Al_2O_3$. 10:34% $SiO_2$. 0.85% $Fe_2O_3$. 0.07% $TiO_2$ and 0.20% BaO (Arimbawa, 2010). Nusa Penida limestone contains high content of aluminum silicate which cause higher mineral structure density in the rock (Kusumoyudo, 1986).

This study aimed to determine (1) the influence of inoculum concentration and length fermentation on ethanol production by the consortium using *Codium geppiorum* as the carbon source, and (2) the influence of activation temperature and amount of the limestone on the concentration of ethanol in the dehydration process.

## MATERIALS AND METHODS

*Codium geppiorum* used in this research was collected from Nusa Lembongan, limestone was collected from Nusa Penida, yeast NKL was bought from local market, sulphuric acid, distilled water, sodium hydroxide, and ethanol (92.51%, 99.8% and 30%) are pro analysis grade. Instruments used in this study was a gas cromatographVarian 3300.

The method of this research was a true experimental with completely randomised design 3 x 4 factorial with two variables. The data analysis was assisted with SPSS 17.0 software.

### *Pretreatment of Codium geppiorum*

Fresh seeweeds were collected from the coastal área of Nusa Lembongan and sun-dried. It was then soaked in water for 24 hours, dried, and ground it into powder.

### *Hydrolysis*

Each sample (25 g) was put into erlenmeyer and 3.5% sulphuric acid was added with a ratio 1:8 (sample to acisd). Then, the sample was heated at a temperature of 110$^o$C and stirred at scale 8 for 1 hour. After cooling, pH was adjustedd to 4-5 by adding 4 M NaOH. The completion of hydrolysis recation was tested with Benedict's reagent.

### *Activation of yeast (starter preparation)*

Yeast consortium was inoculated with 25 mL of 1% glucose solution in a 50 mL erlenmeyer and sealed. It was then incubated at 30$^0$C for 24 hours.

### *Fermentation*

25 g of hydrolysate algae was inoculated with yeast consortium with variation of concentration: 1.25 g, 2.5 g, and 5 g. It was then put into containers with sealed and incubated at room temperature (27$^0$C-30 $^0$C) for 3, 5, and 7 days. It was done in triplicates.

***Fractional Distillation***

Bioethanol in the fermentation broth was separated by fractional distillation at $70^0$C-$90^0$C. Distillate was collected in Erlenmeyer, the volume was measured and ethanol concentration were determined using gas chromatography. The samples with highest level of etanol was then dehydrated using Nusa Penida limestone.

***Activation of Nusa Penida limestone***

Prior to use as adsorbent aaaa9dring agent), limestone of Nusa Penida was activated with variation of temperatures. 5 kg of crushed limestone was heated at a temperature of 100 $^0$C, 800 $^0$C, 900 $^0$C, and 1000 $^0$C respectively for 2 hours in a furnace Nabertherm.

***Testing of Optimum Dehydration***

100 mL of 92.51% ethanol was dehydrated by adding activated limestone as dehydrating agent with variation of mass (50g, 75g, 100g) to obtain the optimum amount of limestone used which give highest concentartion of ethanol. The mixtures were soaked for 24 hours. The resulting solutions were distilled at 70 $^0$C-80 $^0$C for 1 hour. The volume of destilate was measured and the ethanol concentration were determined by gas chromatography. It was done in triplicates. The best dehydration condition (temperature activation and mass of limestone) will be applied to dehydrate (purify) crude bioethanol.

***Determination of ethanol concentration by gas chromatography***

The concentration of etannol was determined using Gas Chromatograph Varian 3300. Etanol 30 % was used as standard to determine concentration of bioethanol produce during fermentation while etanol 99.8 % was used as standard to determine the biethanol concentration after dehydrating with activated limestone.. The ethanol content of each sample was calculated by the following equation.

$$\text{Ethanol concentration (\%)} = \frac{\text{Sample area}}{\text{Standard Area}} \text{ x Standard concentration (\%)}$$

## RESULTS AND DISCUSSION

The level of bioethanol produced from *Codium geppiorum* by varying concentration of inoculum and length of fermentation is depicted in figure 1. While the statistical analysis with SPSS is summarized in Table1.

Table 1. Anova Test Results on Two Line SPSS 17.0 Levels of Ethanol produced by *Codium geppiorum* with different concentration yeast and length of fermentation.

| Variables | Sum of square | Degree of freedom | Average of sum of square | $F_{count}$ | $F_{table}$ 5% | Sig. |
|---|---|---|---|---|---|---|
| Treatment | 138.750[a] | 11 | 12.614 | 119.876 | 2.22 | 0.000 |
| Intercept | 202.393 | 1 | 202.493 | 1924.431 | 4.26 | 0.000 |
| Inocula concentration | 96.120 | 3 | 32.040 | 304.498 | 3.01 | 0.000 |
| Length of fermentation | 28.866 | 2 | 14.433 | 137.166 | 3.40 | 0.000 |
| Interaction of inocula concentration and length of fermentation | 13.764 | 6 | 2.294 | 21.802 | 2.51 | 0.000 |
| Error | 2.525 | 24 | 0.105 | | | |
| Total | 343.768 | 36 | | | | |
| Total treatment | 141.275 | 35 | | | | |

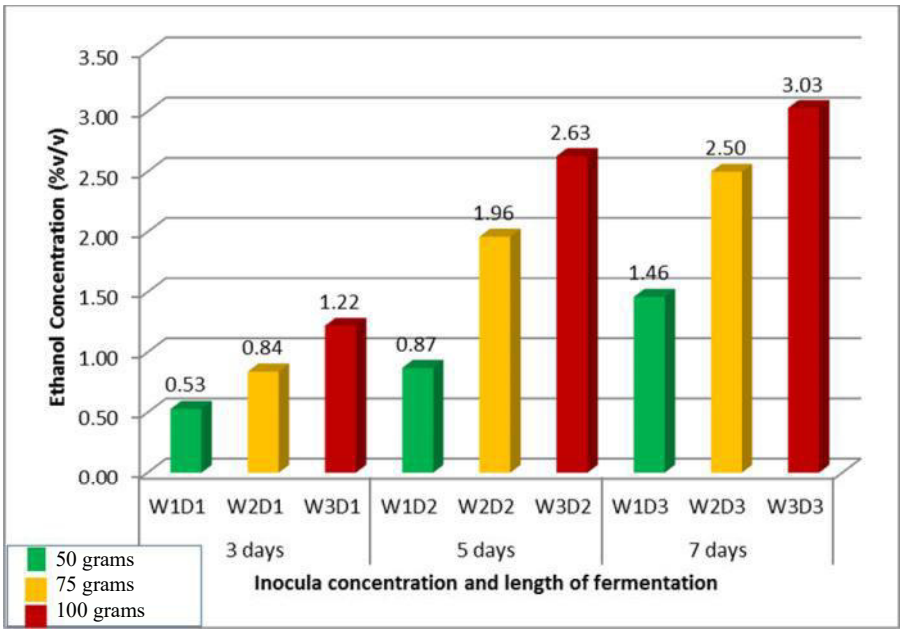


Figure 1. The average levels of Ethanol Production using *Codium geppiorum* as the carbon sourse at varied Yeast concentration and Fermentation Time

Table 2. The Results of Two Line SPSS 17.0 Anava Test on Concentration of Ethanol after Dehydration at varied activation Temperature and amount of Nusa Penida Limestone

| Variables | Sum of square | Degree of freedom | Average of sum of square | Fcount | Ftable 5% | Sig. |
|---|---|---|---|---|---|---|
| Treatment | 412.673[a] | 11 | 37.516 | 4.203 | 2.22 | 0.002 |
| Intercept | 311129.684 | 1 | 311129.684 | 34853.320 | 4.26 | 0.000 |
| Activation temperature | 115.145 | 3 | 38.382 | 4.300 | 3.01 | 0.015 |
| Amount of limestone | 132.446 | 2 | 66.223 | 7.418 | 3.40 | 0.003 |
| Interaction of temperature and amount of limestone | 165.082 | 6 | 27.514 | 3.082 | 2.51 | 0.022 |
| Error | 214.244 | 24 | 8.927 | | | |
| Total | 311756.601 | 36 | | | | |
| Total treatment | 626.917 | 35 | | | | |

The results of fermentation showed that the values of $F_{count} > F_{table}$ (38.212> 2.51) with a probability of 0.000, which means that there is a probability of interaction between yeast concentration and the lenght of fermentation. The optimum result of average ethanol content 3.03% was obtained at W3D3, when 20% inocula was applied for 7 days fermentation on 25 grams of algae samples (Figure 1.)

Longer fermentation time produces more biomass which in turn produce more alcohol. It was also found that higher amount of yeast added to convert sugar into alcohol resulted in higher concentration of alcohol production.

The monosaccharide concentration resulted from hydrolysis of polysaccharide in the algae depends upon the amount of samples used, the concentration of sulphuric acid, length of contact with acid, and agitation (Razif, 2011). The sample will tend to produce a lot of high sugar substrate, this will have an impact on the increase in the concentration of sugar will result in a higher ethanol productivity. This is due to the increasing number of substrates available for yeast to produce more ethanol. However, excess sugar can affect the production of ethanol due to mechanism called substrate inhibition (Roukas, 1996).

The results of dehydration of ethanol (Table 2.) shows that Fcount> Ftable (3.082> 2.51) with a probability of 0.022 which means that there is interaction between activation temperature and amount of limestone. The optimum results was achieved by M1T1 (50 grams limestone and activated at 800 $^0$C) with an average ethanol content of 99.15%. The ethanol content in the fermentation broth (28.91%) has been increased to 83.78% by dehydration with the activated limestone.

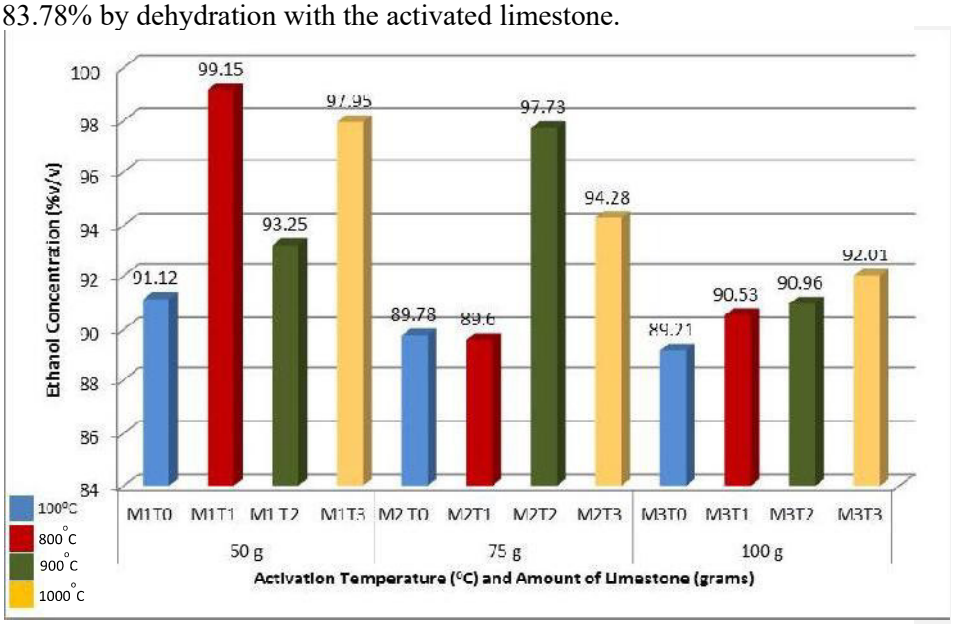


Figure 2. Ethanol Concentration after Dehydration Process at varied mass and activated temperature of Nusa Penida Limestone

The un-activated limestone decreased the levels of the ethanol (lower than 92.51% ethanol in the feeds). This is may be due to the absorption of ethanol in addition to the absorption of water. At 800$^0$C, higher amount of limestone did not produced higher concentration of ethanol. This is may be due to less ethanol was absorbed by 50 grams limestone than the other amount (75 grams and 100 grams). It was also showed that the lowest concentration of ethanol produced by using 100 grams of limestone which may be due to that much of the ethanol was trapped in limestone structures (Killic, 2006).

It can be concluded that (1) the addition of yeast and length of fermentation significantly affect the concentration of ethanol produced using Codium geppiorum as the carbon source, and (2) activation temperature and amount of Nusa Penida limestone has a significant effect on the ethanol content.

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# SOCIO-ECOLOGICAL LANDSCAPE CHANGE AS A PREAMBLE TO URBAN WATERSHED REJUVENATION, KANSHI OF THE JHELUM RIVER BASIN, POTOHAR PLATEAU, PAKISTAN

**Khalida Khan[1], Sachiko Takahi[2] Rabia Faridi[3] and Razi Iqbal[4]**
*[1]Centre for Integrated Mountain Research (CIMR) University of the Punjab, Lahore-54590, Pakistan; cimrpu@yahoo.com*
*[2]Faculty of Education and Human Studies, Akita University, 1-1 Akita City Akita, Japan*
*[3] Centre of Excellence in Microbiology, University of the Punjab, Lahore-54590, Pakistan;*
*[4]Graduate School of Engineering & Resource Science, Akita University, Jap*an

## ABSTRACT

Changing Geo-envir**o**nmental factors and process are entirely different from those of urban anthropogenic systems. At the same time these systems are self-motivated, diverse and prone to procedural shifts, and are associated with change. Resultantly, corrections between Geo-environmental and human factors distress sustainability differently. Ill-planned urbanization is one of the major causes to disturb natural ecosystem sustainability. This paper will highlight socio-cultural landscape changes along the Kanshi sub watershed (1,111 km²) of Jhelum River basin located in the Potohar Plateau and its intimate involvement for indigenous urban sustainability. Kanshi River flows in southward and then joins Jhelum River in an eastward direction. The study area (Kanshi) location is in latitude 33°14'54.59"N and longitude 73°36' E, tehsil Gujar Khan and tehsil Kahuta of district Rawalpindi. Tran-disciplinary approach is incorporated in this research to appraise social-ecological landscape systems dynamics and urban morphology. By exercising Environmental impact assessment techniques and using on-site morphological and biological parameters, urban watershed quality will be assessed. It is pertinent to considerate momentary performance of social-ecological landscape systems and their response to interruption. So that, reliable management strategies could be initiated that may rejuvenate, enhance capabilities and ensure sustainable development in fastly deteriorated urban watersheds.

***Keywords:*** Urban landscape morphology; dynamicity; stream water quality; socio-ecological landscape systems

# REDUCING WATER HARDNESS USING EXTRACT OF *KETAPANG* (tropical betel nut) LEAVES

**Iryanti Eka Suprihatin**
*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Udayana University, Bali, Indonesia*
*iryantis@hotmail.com*

**ABSTRACT**

Water hardness have been notoriously raising diverse problems from as simple as reducing the function of soap to damaging machinery such as boiler. Various ways of minimizing water hardness involving addition of chemicals and heating are known. This presentation offers a natural material locally available to treat water hardness. *Ketapang* (tropical betel nut) leaves contain humic acid which is known as a ligand capable of binding metal atoms in a complex (coordinative) chemical bond. This capability is used to bind calcium and magnesium known as the causes of water hardness. The investigation in using the extract of tropical betel nut leaves to decrease water hardness is discussed in this presentation.

# ORAL PRESENTATIONS: HEALTH

# FERTILITY ENHANCING EFFECTS OF *Gynura procumbens* METHANOLIC EXTRACT ON DIABETIC INDUCED MALE RATS

**Nani Rahayu Mohd Radzuan* and Mahanem Mat Noor**
*School of Bioscience and Biotechnology*
*Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Malaysia*
**Correspondence author: naniradzuan17@yahoo.com*

**ABSTRACT**

*Gynura procumbens* or Sambung Nyawa is a type of herb used to treat numerous types of diseases such as rheumatoid, hypertension and diabetes. Diabetes mellitus is a type of metabolic disease and one of its complications is infertility among male patients. This study aims at evaluating the potential of traditional herbs in enhancing fertility among diabetic patients. In this study, the ethyl acetate fraction of *Gynura procumbens* methanolic extract was used. Some 60 rats were divided into 5 groups. Diabetic were induced to four of these groups and another group acted as normal control. Three of the diabetic groups were treated with 50 mg/kg *Gynura procumbens* extract (n=12), 300 mg/kg *Gynura procumbens* extract (n=12) and 300 mg/kg metformin (n=12) respectively. The fourth diabetic group acted as negative control. Sexual behavior tests were done to each group during treatment period. Six rats from each group were then sacrificed after one week while the rest were sacrificed after two weeks of treatment. Results showed that two weeks treatment with low dose of *Gynura procumbens* (50 mg/kg) produced the highest number of sperm count as well as high grade a sperm (most progressive) in comparison with diabetic control group. The mounting latency of *Gynura procumbens* treated diabetic rats also decreases while the mounting frequency increases significantly ($p<0.05$) in comparison with the diabetic group suggesting that the extract possesses aphrodisiac effect. This study showed that *Gynura procumbens* extract enhances the fertility and libido of diabetic rats efficiently in low dose and in short period of treatment intervals.

***Keywords*:** *Gynura procumbens*; diabetes; sperm quality; sexual behavior

## INTRODUCTION

Diabetes mellitus (DM) is one of the most chronic diseases in the world. A diabetes patient will usually experience hyperglycemia, polyurea, polydypsia as well as recurrent and severe infections (WHO 2011). DM may also affect male reproductive function at multiple levels. More than 90 % of DM male patients experience sexual dysfunction which includes impotency, decrease in libido as well as infertility (Amaral *et al.* 2006). Increase in production of ROS (reactive oxygen species) among DM patients is also said to be the reason for impairment in sperm production and fertility (Wright *et al.* 2006)

Treatment of DM as well as other diseases using synthetic drugs such as metformin had often caused unwanted side effects. A study done on rabbits had shown that treatment with metformin had caused the sperm quality of the rabbits to decrease (Naglaa *et al.* 2010). This has led patients to opt for alternative treatment which often uses medicinal or herbal plants such as *Gynura procumbens.*

*Gynura procumbens* (GP) also known as "Sambung Nyawa" is a type of herbacious plant used in treatments of many ailments such as rashes, inflammation, kidney diseases, fever, migraine, constipation and cancer (Perry 1980). Studies on its extract revealed that GP possess antioxidant property which may contribute to its anti-hyperglycemic (Akowuah *et al.* 2002), anti-cancer (Jenie and Meiyanto 2008) and anti-inflammatory (Iskander *et al.* 2002) activities. An antioxidant study had been done to analyze the antioxidant content of various GP extracts. The results showed that the ethyl acetate fractions, which contain the highest amount of phenolic compounds, exhibited the greatest antioxidant activity (Rosidah *et al.* 2008). Hence, current study was done using ethyl acetate fractions of GP methanolic extract. Diabetic male rats will be treated with this extract in order to discover the potential of this GP extract in enhancing the fertility of diabetic male rats.

## MATERIALS AND MEHODS

### Extraction and fraction of *Gynura procumbens* leaves

The leaves were obtained from the Plant House, Faculty of Science and Technology, University of Kebangsaan Malaysia. Extraction was done according to method of Rosidah *et al.* (2008). The leaves

of GP were dried in an oven at 45°C and ground into powder. The dried powdered leaves were further extracted with methanol in a Soxhlet apparatus (Soxtec System-Texator, Sweden). The methanol extract was concentrated by a rotary evaporator (Büchi Rotavapor® R-200/205) and then resuspended in water and fractionated to ethyl acetate fraction using a separating funnel.

**Animals**

Male Sprague Dawley rats of either sex aged two months were obtained from Animal House, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. All rats were fed standard diet and water.

**Experimental design**

In this study, 60 male rats were divided into five groups. Diabetic were induced to four of these groups and another group acted as normal control. Three of the diabetic groups were treated with 50 mg/kg GP extract (n=12), 300 mg/kg GP extract (n=12) and 300 mg/kg metformin (n=12) respectively. The fourth diabetic group acted as negative control. Sexual behavior tests were done to each group during treatment period. Six rats from each group were then sacrificed after one week while the rest were sacrificed after two weeks of treatment.

**Induction of diabetes**

Diabetes was induced after rats were fasted overnight by a single intraperitoneal injection of 55 mg/kg STZ, dissolved in sodium citrate buffer pH 4.5. Fasting blood glucose levels of the rats were determined prior to STZ injection using Glucometer Accucheck Active® Roche Diagnostic (Canada) on the seventh day after the induction. Rats with blood glucose concentrations above 300 mg/dl were considered diabetic and were used in the experimental group.

**Sexual behavior test**

Sexual behavior test were done once in two days throughout the treatment period. Sexually receptive females were prepared according to method of Agmo (1997). A male rat was acclimatized for five minutes before introducing a receptive female rat in the test arena. Parameters observed were mount frequency and mount latency. Mount latency depicts the time from introduction of the female until the first mount activity. Observations were made within 30 minutes.

**Sperm sample preparation**

After animals were sacrificed, caudal epididymis were removed to obtain the sperm samples and were put into 15 ml prewarmed rat sperm isolation medium known as Biggers, Whitten and Whittingham (B.W.W.) medium (Biggers *et al.* 1971). Sperm samples were then incubated at 37°C with 5% CO2 for 30 minutes. The sperm sample was then assessed based on two parameters; sperm count and motility.

## RESULTS AND DISCUSSION

The present investigation showed that induction of diabetes impaired the male rats' sexual activity. However, tratement with GP extract had proven to assist in normalizing the blood glucose level of diabetic male rats (data not shown) as well as the rats' libido. Table 1 shows that mounting frequency was significantly increased in treated groups in comparison with the diabetic control group. Similarly, a positive result was also achieved for mount latency. Diabetic rats treated with 50 mg/kg GP extract (low dose) took less time to mount the female rats towards the end of treatment period (Table 2). This suggests that GP extract possesses aphrodisiac effect and continuous administration of this extract at the dosage of 50 mg/kg helped in enhancing the sexual activity of diabetic male rats.

**Table 1:** Mount frequency

| Group | Mean time ± SE |
|---|---|
| | Mount frequency |
| Normal control | 9.00 ± 3.07 |
| Diabetic control | 0.10 ± 0.10 |
| Metformin | 1.00 ± 0.76 |
| Low dose (50 mg/kg) | 1.70 ± 0.88 |
| High dose (300 mg/kg) | 2.30 ± 1.24 |

After one week of treatment, six rats from each group were sacrificed and its sperm count and motility were assessed. A week later, another six rats were sacrificed and the same procedure for sperm quality assessment was done.

Induction of diabetes towards the male rats had significantly decreased the sperm count and motility. However, treatment with GP extract and metformin resulted in increment of both sperm count and sperm motility of diabetic male rats. Figure 1 shows the effects of GP extract and metformin on sperm count of diabetic rats. Treatment with 50 mg/kg GP extract for two weeks had significantly ($p<0.05$) increased the sperm concentration of diabetic male rats.

**Table 2:** Mount latency (NA = no activity recorded)

| Group | Mean time ± SE (minute) | | | | | |
|---|---|---|---|---|---|---|
| | Day | | | | | |
| | 1 | 4 | 7 | 10 | 12 | 14 |
| Normal control | 3.75 ± 0.96 | 4.00 ± 2.64 | 4.00 ± 2.65 | 3.33 ± 2.31 | NA | 4.00 ± 2.82 |
| Diabetic control | NA | NA | NA | NA | NA | NA |
| Metformin | 15.5 ± 10.54 | 7.50 ± 3.53 | NA | 10 ± 7.11 | NA | 16.8 ± 10.33 |
| Low dose | NA | 2.00 ± 1.41 | 6.00 ± 5.15 | 4.25 ± 2.22 | 4.25± 3.40 | 2.00 ± 1.41 |
| High dose | 18.40 ± 11.17 | 24.40 ± 12.52 | 18.80 ± 15.34 | 25.20 ± 9.65 | 15.60 ± 13.83 | 17.80 ± 12.60 |

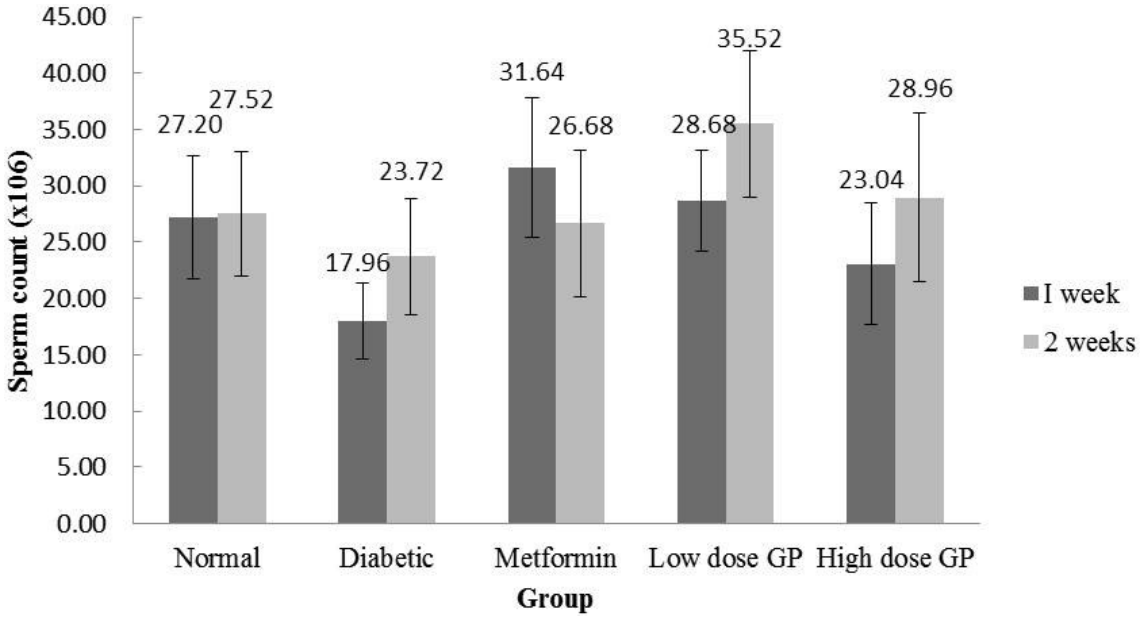


**Figure 1:** Sperm count assessment

Table 3 displays the percentage of sperm motility based on motility grades for each experimental group. There are four sperm motility grades, where grade a (>25 μm/s) depicts the most progressive sperm movement, grade b (5-24 μm/s) and c (<5 μm/s) represents the category of intermediate movement of sperms meanwhile immotile or non-moving sperm are placed in grade d (0 μm/s). In reference to Table 3, treatment with GP extract has succeeded in increasing the percentage of grade a sperm in diabetic rats while the metformin treatment did not. Progressive sperms are important in fertilization process especially during the sperm's movement towards the egg because the sperms has a very short life span which is 6-12 hours (Turner 2003).

The ability of GP extract in enhancing the fertility of diabetic male rats may have been contributed by its' antioxidant content. Rosidah *et al.* (2008) had discovered two important flavonoids in GP extracts which are kaempferol and astragalin. Another study had even discovered quercertin (Akowuah *et al.* 2002). Flavonoids are a class of secondary plant phenolics with significant antioxidant and chelating properties. Their cardioprotective effects stem from the ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving reactive oxygen species (Heim *et al.* 2002).

This study concerns the effects of ethyl acetate fractions of GP methanolic extract on fertility of diabetic male rats. Taken together, these results lead us to conclude that DM had adverse effects on sperm quality as well as sexual behavior. Treatment with 50 mg/kg of GP extract for two weeks had proven to enhance the sexual activity as well as the sperm quality of diabetic male rats. Meanwhile, treatment with metformin did not demonstrate similar effects.

**Table 3:** Sperm motility assessment

| Group | Percentage of sperm motility based on Motility Grades (%) | | | |
|---|---|---|---|---|
| | a | b | c | d |
| 1 week treatment | | | | |
| Normal | 28.10 | 32.85 | 8.17 | 30.88 |
| Diabetic | 12.28 | 17.38 | 15.63 | 54.70 |
| Metformin | 17.04 | 21.65 | 16.00 | 45.30 |
| Low dose | 37.57 | 21.18 | 11.60 | 29.65 |
| High dose | 39.28 | 16.19 | 10.85 | 33.68 |
| 2 weeks treatment | | | | |
| Normal | 28.54 | 33.97 | 9.51 | 27.98 |
| Diabetic | 12.79 | 18.85 | 6.89 | 61.48 |
| Metformin | 16.79 | 16.79 | 10.25 | 56.16 |
| Low dose | 38.41 | 11.69 | 11.96 | 37.93 |
| High dose | 23.86 | 14.31 | 31.05 | 30.78 |

## ACKNOWLEDGEMENT

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# ANTI-NEUROINFLAMMATORY EFFECTS OF CARDAMONIN, A CHALCONE DERIVATIVE ISOLATED FROM *Alpinia rafflesiana*

**Syahida Ahmad[1,2]*, Yuh-lit Chow[1], Sharmili Vidyadaran[3]and Nordin Haji Lajis[1,4]**
[1] *Institute of Bioscience,* [2] *Faculty of Biotechnology and Biomolecular Sciences,* [3] *Faculty of Medicine and Health Sciences,* [4] *Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor DE, Malaysia.*
**Correspondence author: syahida@biotech.upm.edu.my*

## ABSTRACT

The increasing prevalence of neurodegenerative diseases has prompted investigation into innovative therapeutics over the last two decades. Non-steroidal anti-inflammatory drugs (NSAIDs) are among the therapeutic choices to control and suppress the symptoms of neurodegenerative diseases. However, NSAIDs-associated gastropathy has hampered their long term usage despite their clinical advancement. On the natural end of the treatment spectrum, our group has shown that cardamonin (2′,4′-dihydroxy-6′-methoxychalcone) isolated from *Alpinia rafflesiana* exerts potential anti-inflammatory activity in activated macrophages. Therefore, we further explored the anti-inflammatory property of cardamonin as well as its underlying mechanism of action in IFN-γ/LPS-stimulated microglial cells. In this investigation, cardamonin shows promising anti-inflammatory activity in murine microglial cell line (BV2) by inhibiting the secretion of pro-inflammatory mediators including nitric oxide (NO), prostaglandin $E_2$ ($PGE_2$), tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). The inhibition of NO and $PGE_2$ by cardamonin are resulted from the reduced expression of inducible nitric oxide synthase (iNOS) and cycloxygenase-2 (COX-2), respectively. Meanwhile the suppressive effects of cardamonin on TNF-α, IL-1β and IL-6 were demonstrated at both protein and mRNA levels, thus indicating the interference of upstream signal transduction pathway. Our results also validate that cardamonin interrupts nuclear factor-kappa B (NF-κB) signalling pathway via attenuation NF-κB DNA binding activity. Interestingly, cardamonin also showed a consistent suppressive effect on the cell surface expression of CD14. Taken together, our experimental data provide mechanistic insights for the anti-inflammatory actions of cardamonin in BV2 and thus suggest a possible therapeutic application of cardamonin for targeting neuroinflammatory disorders.

***Keywords:*** *Alpinia rafflesiana;* anti-inflammatory; cardamonin; microglia; NF-κB

## INTRODUCTION

Neurodegenerative diseases for instance, Alzheimer's disease, Parkinson's disease, multiple sclerosis, HIV-associated dementia and Creutzfeldt-Jacob disease are associated wit neuroinflammation. Increasing evidence had revealed that microglia play a dual role in the central nervous system (CNS) by being either protective or detrimental to the neuronal cells depending on their state of activation (Block *et al*., 2005). Prolong activation of microglia has been linked to the pathogenesis of neuroinflammation as they excessively release neurotoxic molecules including nitric oxide (NO), prostaglandin $E_2$ ($PGE_2$) and a series of pro-inflammatory cytokines (Tambuyzer *et al*., 2009; Zielasek and Hartung, 1996). These harmful pro-inflammatory mediators eventually caused neuronal cell loss in the affected brain region (Block *et al*., 2005). Studies had shown that the expression of these mediators are transcriptionally regulated by nuclear factor kappaB (NF-κB) in microglia. Being the key mediator in this pathway, activated transcription factor NF-κB dimer (p50/p65) will bind to the specific promoter regions upon microglial stimulation and subsequently allow the transcription of these downstream genes (Nakamura, 2002). Therefore, pharmacological agents that regulate NF-κB signaling and pro-inflammatory mediators production thus insight a potential therapeutic approach in anti-neuroinflammatory drugs development. Previous studies have shown that cardamonin (2′,4′-dihydroxy-6′-methoxychalcone) exerted promising anti-inflammatory activity in activated macrophage system (Israf *et al*., 2007; Syahida *et al*., 2006; Hatziieremia, 2006). However, no study have been reported on modulatory effect of cardamonin on the functional activation of microglial cell. Hence, in this study, we investigated the pharmacological effects of cardamonin isolated from *Alpinia rafflesiana* towards pro-inflammatory mediators expression and NF-κB signaling pathway activation in IFN-γ/LPS-stimulated microglial cell line (BV-2).

## MATERIALS AND METHODS

### Sample preparation

Cardamonin or 2',4'-dihydroxy-6'-methoxychalcone ($C_{16}H_{14}O_4$, MW270.28) was isolated from fruits of *Alpinia rafflesiana* as previously described (Mohamad *et al*., 2004).

### Cell culture and stimulation

Murine microglial cell line (BV-2) (kindly provided by Dr. Sharmili Vidyadaran from UPM) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1% penicillin and streptomycin, 0.5% fungizone, 0.1% gentamycin, 0.3% insulin, 1% non-essential amino acid, and were maintained in a humidified incubator with 5% $CO_2$ at 37 ºC. BV2 cells were induced with 100 U/ml of recombinant mouse interferon-gamma (IFN-γ) (eBioscience) and 1 μg/ml of lipopolysaccharide (LPS) (*Escherichia coli*, strain 055:B5) (Sigma), followed by treatment with cardamonin at different concentrations for different time points accroding to the assays.

### Anti-inflammatory bioassays, gene & protein expression, and cell signaling pathway evaluations

Following the treatment, different assays were performed to evaluate the production of NO using Griess assay, $PGE_2$ and pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) using enzyme-linked immunosorbent assay (ELISA), the gene expression of the pro-inflammatory cytokines using reverse transcription-polymerase chain reaction (RT-PCR) and cytotoxicity effect of cardamonin towards BV2 cells through MTT assay. Besides that, iNOS, COX-1 and COX-2 protein expression were determined using western blot whereas NF-κB DNA binding was evaluated by electrophoretic mobility shift assay (EMSA). Lastly, flow cytometry method was carried out to determine the cell surface expression of CD14.

### Statistical analysis

Statistical analysis applied was one-way analysis of variance (ANOVA) followed by Dunnett test using GraphPad Prism 5.0. All results were of three independent experiments and presented as mean ± SEM unless otherwise stated. Statistical significance of difference between groups was accepted at $P<0.05$.

## RESULTS & DISCUSSION

Several clinical studies have shown the abundantly accumulation of NO and $PGE_2$ in the cerebrospinal fluid of patients who suffered from neuroinflammatory diseases (Combrinck *et al*., 2006; Rejdak *et al*., 2004; Montine *et al*., 1999). Therefore in this study, the effects of cardamonin on NO and $PGE_2$ secretion were evaluated in microglia, the well-characterized crucial cellular system involved in neuroinflammation. In order to mimic the pathological condition of neuroinflammation, microglial cell line (BV-2) were stimulated with IFN-γ/LPS and resulted in the augmentation of NO and $PGE_2$ secretion. As shown in Figure 1, treatment of cardamonin has showed a dose-dependent inhibition of NO and $PGE_2$ production wih $IC_{50}$ value of 27.45 ± 0.46 μM and 2.52 ± 0.12 μM, respectively. The inhibitory activity was not due to the cytotoxicity effect (data not shown).

In addition, western blotting results indicated that the inhibitory action of cardamo on NO and $PGE_2$ secretion were due to the down-regulation of iNOS and COX-2 expression (Figure 2). Interestingly, the inhibitory effect of cardamonin on $PGE_2$ was mediated by the suppression of COX-2 but not COX-1 expression, thus indicating the selectivity of cardamonin towards COX-2 expression. These novel selective inhibitory effects could eventually minimize

the gastrointestinal complications by maintaining the COX-1 expression with physiological role of gastroprotection.

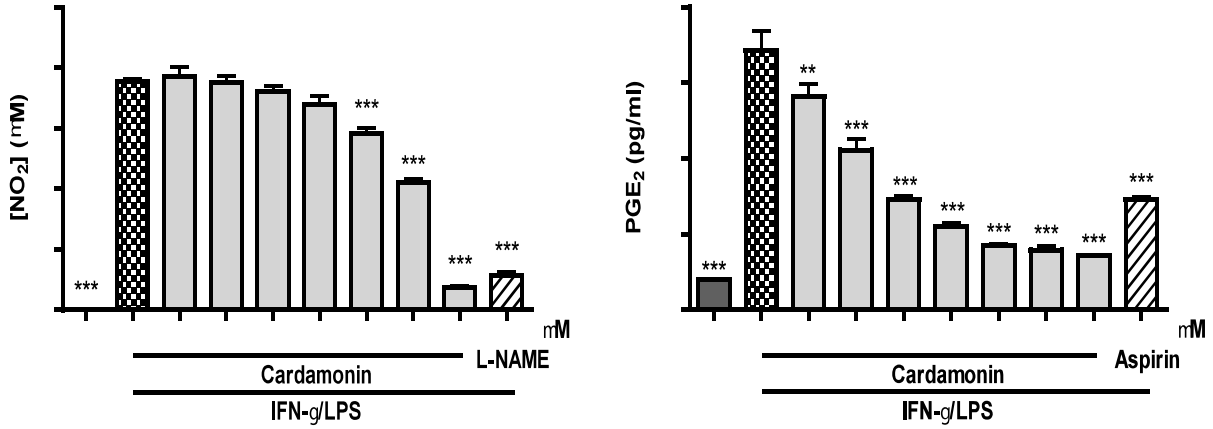


**Figure 1. Effects of cardamonin on a) NO and b) $PGE_2$ production in IFN-γ/LPS-induced BV2 microglial cells.** Cells were stimulated with 100 U/ml recombinant murine IFN-γ and 1 µg/ml LPS and treated with increasing concentration of cardamonin for 24 h. Nitrite and $PGE_2$ level in culture supernatant were determined by Griess reaction and $PGE_2$ EIA kit (Cayman, USA). The $IC_{50}$ was calculated at 27.45 ± 0.46 µM. and 2.52 ± 0.12 µM, respectively. L-NAME (250 µM) and aspirin (50 µM) were used as positive drug control for inhibiting NO and $PGE_2$ production, respectively. C; Basal level of nitrite/$PGE_2$ production without IFN-γ/LPS treatment. All values are the mean ± S.E.M. of three independent experiments. ***P*<0.01, ****P*<0.001, significantly different from IFN-γ/LPS-treated control group.

In chronic neuroinflammation, elevated levels of pro-inflammatory cytokines exacerbated the progression of neurodegenerative diseases (Heneka *et al*., 2012). Furthermore, *in vivo* studies show that TNF inhibitor (XENP345) has attenuated the loss of dopaminergic neurons in animal models of Parkinson's disease (McCoy *et al*., 2006). These observations have prompted us to further investigate the effect of cardamonin on the expression of the pro-inflammatory cytokines in microglial cell. As demonstrated in Figure 3, cardamonin significantly inhibited the production of TNF-α, IL-1β and IL-6 due to suppression at their respective genes level.

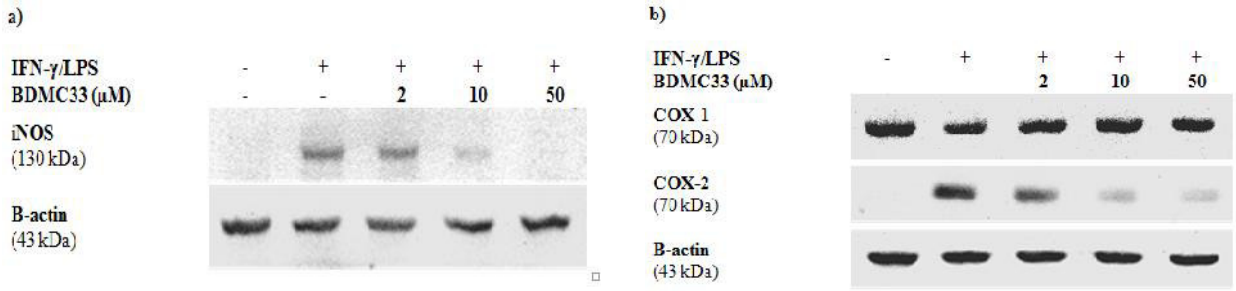


**Figure 2. Effects of cardamonin on a) iNOS and b) COX expression in IFN-γ/LPS-induced BV-2 microglial cells.** Cells were stimulated with 100 U/ml recombinant murine IFN-γ and 1 µg/ml LPS and treated with increasing concentration of cardamonin for 6 h. Whole cells lysate were assayed for protein expression by using Western blotting. Immunoblotting of β-actin expression was used as loading control. C; Basal level of iNOS/COX expression without IFN-γ/LPS treatment.

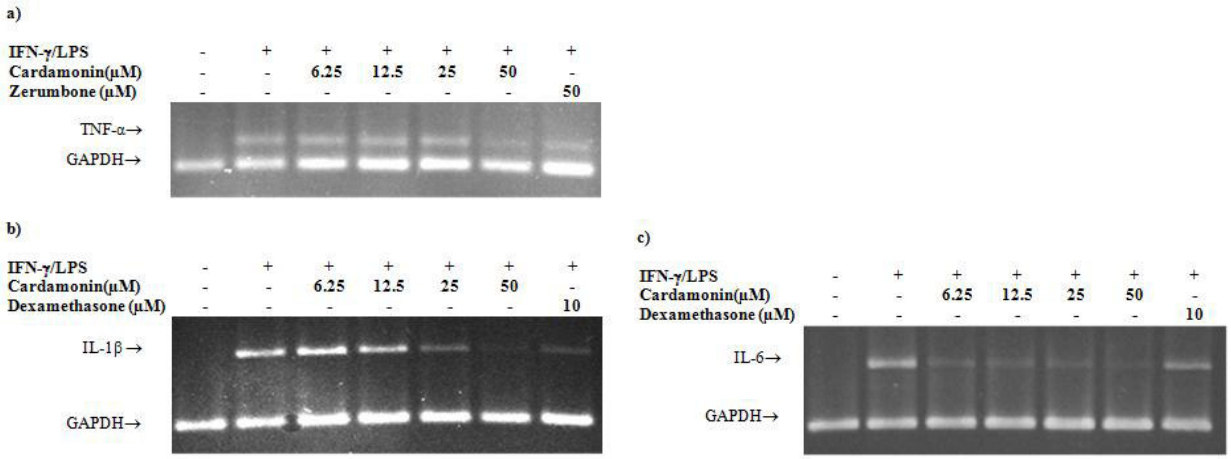


**Figure 3. Effects of cardamonin on a) TNF-α, b) IL-1β and c) IL-6 gene expression in IFN-γ/LPS-induced BV-2 microglial cells.** Cells were stimulated with 100 U/ml recombinant murine IFN-γ and 1 μg/ml LPS and treated with increasing concentration of cardamonin for 6 h. The total RNA of the cells was extracted using RNasey Mini extraction kit (Qiagen, USA) and reverse transcription polymerase chain reaction (RT-PCR) was perfomred using One–step RT-PCR kit (Qiagen, USA). Zerumbone (50 μM) was used as positive control for TNF-α gene expression while dexamethasone (10 μM) was used for IL-1β and IL-6. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as loading control. C; Basal level of TNF-α, IL-1β and IL-6 gene expression without IFN-γ/LPS treatment.

A recent report by Kim *et al.* (2010) had shown promising anti-inflammatory activities of dimethyl cardamonin in inhibiting the gene expression of these cytokines in macrophages. Both dimethyl cardamonin and cardamonin are under the same class of chalcone which share the basic molecular skeleton. Hence, we believed that this similarity of both compounds had contributed to the parallel inhibitory effects observed. Since cardamonin inhibited the cytokine expression at both the protein and gene levels, we speculated that the anti-inflammatory effects of cardamonin was mediated by its up-stream signaling pathways.

In order to validate our speculation, the effect of cardamonin on the NF-κB signaling pathway was investigated. NF-κB is a prominent signaling pathway involved in the induction of a myriad of inflammatory genes. As demonstrated in Figure 4A), cardamonin inhibited NF-κB DNA binding dose dependently in IFN-γ/LPS-stimulated BV-2 cells. Our results were in accordance to previous studies which showed the inhibitory effects of cardamonin and dimethyl cardamonin on NF-κB DNA binding activity in LPS-challenged RAW 264.7 (Hatziieremia *et al*., 2006; Kim *et al*., 2010). The involvement of cardamonin on NF-κB signal transduction was further supported by a number of studies which demonstrated that cardamonin targeted the NF-κB activation by interfering the NF-κB nuclear translocation as well as the phosphorylation and rapid degradation of I-κB kinase (IKK) (Israf *et al*., 2007; Hatziieremia *et al*., 2006; Kim *et al*., 2010).

Accumulating studies have revealed the pivotal role of CD14 as a co-receptor that is involved in LPS recognition and activation of intracellular signaling cascade (Heumann and Roger, 2002). In addition, Reed-Geaghan *et al.* (2009) demonstrated that β-amyloid is incapable to activate the microglial cells isolated from $CD14^{-/-}$ mice, thus suggesting the engagement of co-receptor CD14 is required for microglial activation (Reed-Geaghan *et al*., 2009). As demonstrated in Figure 4B), the protective effects of cardamonin in neuroinflammation can be observed by its consistent suppression on the cell surface receptorexpression of CD14 in IFN-γ/LPS-stimulated BV-2 cells regardless of the concentrations used. Hence, we suggest that cardamonin can abolish

the activation of microglial cell with significant role to suppress the CD14 cell surface expression, which in turn affect the activation of intracellular signaling cascade .

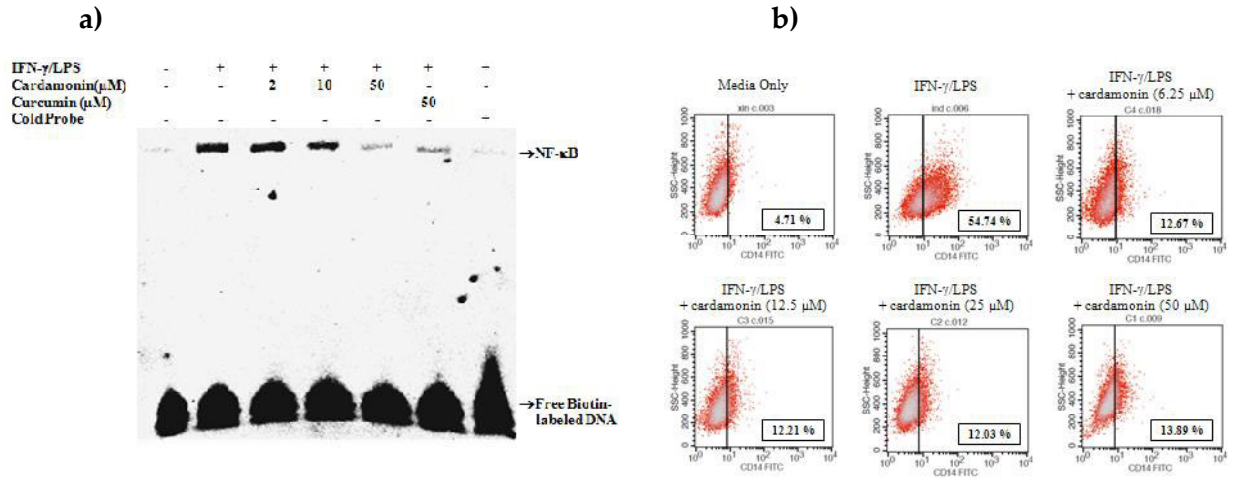


**Figure 4. a) Effects of cardamonin on NF-κB DNA binding activity in IFN-γ/LPS-induced BV2 microglial cells.** Cells were stimulated with 100 U/ml recombinant murine IFN-γ and 1 μg/ml LPS and treated with increasing concentration of cardamonin for 1 h. The nuclear extracts were prepared by using Nuc-Buster protein extraction kit (Novagen, USA) and EMSA was performed using a light shift chemiluminescent EMSA Kit (Pierce, USA). Curcumin (50 μM) was used as the postitive control for NF-κB DNA binding inhibition. C; Basal level of NF-κB DNA binding activity without IFN-γ/LPS treatment. **b) Effects of cardamonin on CD14 cell surface expression in IFN-γ/LPS-induced BV-2 microglial cells.** Cells were stimulated with 100 U/ml recombinant murine IFN-γ and 1 μg/ml LPS and treated with cardamonin for 24 h. Harvested cells were analysed forCD14 cell surface expressionby using flow cytometer. Results shown was one representative of three independent experiments.

In summary, cardamonin isolated from *Alpinia rafflesiana* exhibited inhibitory effects on the inflammatory responses in IFN-γ/LPS-stimulated microglial cell line (BV-2), through the inhibition of activated NF-κB transcription factor binding to the DNA. Results also showed that down-regulation of the responses was contributed partially by the suppression of CD14 cell surface receptor expression. Further *in vitro* investigations are required to find out the molecular target(s) of cardamonin in the NF-κB pathway and to validate its inhibitory effects *in vivo.*

## ACKNOWLEDGEMENT

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# OCCULT HEPATITIS B DETECTED IN HUMAN IMMUNODEFICIENCY VIRUS PATIENTS IN MOEWARDI GENERAL HOSPITAL SURAKARTA

**Afiono Agung Prasetyo[1,2,3]*, Yulia Sari[2,3,4], Paramasari Dirgahayu[2,3,4], Hudiyono[1,3], Dhani Redhono[5], and Seiji Kageyama[6]**

*[1]Department of Microbiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[2]Biomedical Laboratory, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[3]Center of Study of Biotechnology and Biodiversity Research and Development, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[4]Department of Parasitology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[5]Department of Internal Medicine, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[6]Division of Virology, Faculty of Medicine, Tottori University, 86 Nishi cho, Japan*
**Correspondence author: afieagp@yahoo.com; afie.agp.la@gmail.com*

**ABSTRACT**

Human immunodeficiency virus (HIV)-infected patients are at risk of acquiring viral hepatitis. As the introduction of highly active antiretroviral therapy (HAART) reduced the frequency of opportunistic infections and improved survival, viral hepatitis emerged as an important cause of morbidity and mortality in HIV-infected cases. Occult hepatitis B virus infection (OBI) is an important risk factor to induce cirrhosis, hepatocellular carcinoma (HCC) and reactivation of the hepatitis B virus (HBV). However, there is no report about the presentation of OBI in Indonesian HIV patients have ever been published, for the best of our knowledge. In an ongoing molecular epidemiology study of blood borne virus, 195 HIV patients in Moewardi General Hospital Surakarta were enrolled in the study in November 2011-February 2012. The blood samples were aliquoted, subjected for CD4 assay, and fractionated. Plasma was addressed for serological assays, including that of the HBsAg assay. The nucleic acid was extracted from all plasma samples, and subjected for the molecular assays, including that of for the HBV detection. The nested PCR was performed to detect the HBV genome. Only plasma samples with repeatedly detectable HBV DNA were considered positive. Hematological and liver function test were performed in all patients involved in the study. OBI was found in one HIV patient in Moewardi General Hospital Surakarta. The epidemiological and clinical data are going to be presented and discussed. Results indicate the need for adequate management of HIV patients, especially in the presentation of OBI.

***Keywords:*** occult hepatitis B infection, HIV patients, Indonesia

## INTRODUCTION

Human immunodeficiency virus (HIV) and Hepatitis B virus (HBV) infections are real public health problems. In the world, there are more than 30 million people living with HIV/AIDS and about 350 to 400 million chronic HBV carriers (Goldstein *et al*. 2005; Marcellin 2009). HBV infection was reported to increase mortality and morbidity in HIV patients. Indeed, HIV infection increases chronic HBV infection risk and promotes faster progression to cirrhosis and its complications, especially when HBV replication is important (Bonacini *et al*. 2004; Salmon-Ceron *et al*. 2005; Thio 2009).

Occult HBV infection (OBI) is defined as low level HBV replication in the absence of detectable circulating HBV surface antigen. OBI has been implicated in HBV reactivation, advanced liver fibrosis and cirrhosis, reduced interferon response rates, elevated liver enzyme levels, and the development of hepatocellular carcinoma (Blackard *et al*. 2012). However, the prevalence of OBI has not been clearly established in many developing countries, such as Indonesia. Until September 2011, 15.589 HIV patients were diagnosed in Indonesia and 877 are living in Central of Java (Ministry of Health of Indonesia, 2011). The rate of HIV/HBV co-infection in Indonesia is 3.2% (Anggorowati *et al*. 2012), however, the prevalence of OBI has not been clearly established, for the best of our knowledge.

## MATERIALS AND METHODS

### Sample study

In an ongoing molecular epidemiology study of blood borne virus, 195 HIV patients in Moewardi General Hospital Surakarta were enrolled in the study in November 2011-February 2012. All subjects were informed that the study was voluntary. Approval was obtained from the institutional ethical committee

review boards of the Faculty of Medicine of Sebelas Maret University and Dr. Moewardi General Hospital, Surakarta, Indonesia. Written informed consent was obtained from all individuals participating in the study. Blood samples collected from the HIV patients were aliquoted, subjected for blood assays and CD4 assay, and fractionated. All the procedures were conducted according to the principles of the Declaration of Helsinki.

**Blood assays**

Hematological and liver function test were performed in all patients involved in the study. Briefly, Hb (Hemoglobin), Hct (Hematocrit), RBC (Red blood cells), MCV (Mean Corpuscular Volume), MCH (Mean Corpuscular Hemoglobin), MCHC (Mean Corpuscular Hemoglobin Concentration), RDW (Red blood cell Distribution Width), HDW (Hemoglobin Distribution Width), Plt (Platelet), MPV (Mean Platelet Volume), PDW (Platelet Distribution Width), WBC (White Blood Count), Eosinophil, Basophil, Neutrophil, Lymphocyte, Monocyte, LUC (Large unstained cells), SGOT (Serum Glutamic Oxaloacetic Transaminase), and SGPT (Serum Glutamic Pyruvic Transaminase) data were obtained. T Helper CD4+ was measured using TRITEST CD3/CD4/CD45 W/TRUCOUNT (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

**Serological assays**

Subject plasma was separated from whole blood with EDTA and subjected to the following tests. A SERATEC Hepatitis B Quick Test (GesellschaftfürBiotechnologie GmbH, Göttingen, Germany) and anti-HBcAb Mini Vidas® (Biomerieux, Marcy l'Etoile, France) were used for the detection of HBsAg and anti-HBc antibody, respectively. Also we searched for anti-HBs Ab in all HBsAg negative and anti-HBcAb positive patients. All blood samples were checked for the presentation of anti-HCV, anti-HDV, IgM-Toxo, and IgG Toxo. All assays were performed according to the manufacturer's instructions.

**Molecular assays**

Nucleic acid (DNA and RNA) was extracted from 200 µl of plasma by using PureLink Viral RNA/DNA Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A portion of the HBsAg gene was amplified using the primers HBS1F/HBS1R in the first round and HBS2F/HBS2R in the second round, as described previously (Tonetto *et al*. 2009). Only plasma samples with repeatedly detectable HBV DNA were considered positive. To confirm the results, the positive samples also subjected for amplification of full HBV genome as described previously (Chen *et al*. 2010). All samples also screened for HIV RNA, HCV RNA, HDV RNA and TTV DNA.

## RESULTS AND DISCUSSION

**The epidemiological and hematological profiles of the occult Hepatitis B virus patient**

During our molecular epidemiology study of blood borne virus in Moewardi General Hospital Surakarta Indonesia, we detected occult Hepatitis B virus infection in one patient. The patient was male, 31 years old, homosexual, with junior high school educational background. The patient was confirmed infected with HIV one month prior the study, and already received antiretroviral NFV (nelfinavir) + D4T(stavudine) + 3TC (lamivudine). The hematological profiles were described in Table 1. The patient had normocytic/normochromic which may caused by the chronic infections process (anemia of inflammatory response), as seen by an elevated counts of monocytes. In response to inflammatory cytokines, the liver produces increased amounts of hepcidin which increasing internalization of ferroportin molecules on cell membranes. Inflammatory cytokines also appear to affect other important elements of iron metabolism, and probably directly blunting erythropoiesis by decreasing the ability of the bone marrow to respond to erythropoietin (Nemeth *et al.* 2004; Nemeth and Ganz 2006; Zarychanski and Houston 2008). However, the high RDW with normal MCV may indicate that a recent hemorrhage or impaired production in bone

marrow (according to the abnormally low MPV) may occur. Intriguingly, the patient had eosinophilia, indicated that the patient may infected with parasite. However, only antibody IgG for Toxoplasma was found positive in the patient. No elevations of transaminases level were found in the patient. The T Helper CD4+ was very low consistent with previous findings. Occult HBV was associated with low CD4 counts since it may caused by opportunistic reactivation of HBV that resolves as a consequence of HAART induced immune reconstitution and/or the effect of lamivudine (Stuart *et al*., 2009).

**Table 1.** The hematological profiles of the occult Hepatitis B virus patient.

| Name | Results |
|---|---|
| Hb (Hemoglobin) | 6.2 g/dl |
| Hct (Hematocrit) | 18 % |
| RBC (Red blood cells) | 2.01 x 10^6/ul |
| MCV (Mean Corpuscular Volume) | 87.3/um |
| MCH (Mean Corpuscular Hemoglobin) | 30.8 pg |
| MCHC (Mean Corpuscular Hemoglobin Concentration) | 35.3 g/dl |
| RDW (Red blood cell Distribution Width) | 27.7 % |
| HDW (Hemoglobin Distribution Width) | 3.7 g/dl |
| Plt (Platelet) | 356 x 10^3/ul |
| PDW (Platelet Distribution Width) | 46 % |
| MPV (Mean Platelet Volume) | 5.5 fl |
| WBC (White Blood Count) | 3.2 x 10^3/ul |
| Basophil | 0.6 % |
| Neutrophil | 53.7 % |
| Lymphocyte | 16.1 % |
| Monocyte | 13.4 % |
| Eosinophil | 16.2% |
| LUC (Large unstained cells) | 2.9 % |
| T Helper CD4+ | 19 cells/ |
| T Helper CD4+ % of Lymphs | 3% |
| SGOT (Serum Glutamic Oxaloacetic Transaminase) | 13 u/l |
| SGPT (Serum Glutamic Pyruvic Transaminase) | 15 u/l |

**The serological and molecular results of blood borne pathogens of the occult Hepatitis B virus patient**

As our routine procedures, all blood samples in our molecular epidemiology study were screened for the presentation of HIV, HBV, HCV, HDV, HTLV-1/2, TTV, Toxoplasma, fungal, and mycobacterial by serological and or molecular assays. In point of the occult Hepatitis B virus patient, the HBsAg, anti-HBc, anti-HBs were found negative, but the HBV DNA was detected by two different nested PCR. The first nested PCR system was done by detection of part of S gene. The S, X, Core gene were successfully amplified by the second nested PCR system, respectively. No mutation in S gene (in "a" region") was found by sequencing (data not shown). No HIV RNA, TTV DNA, Anti HCV, HCV RNA, anti-HDV, HDV RNA, anti-HTLV-1/2, HTLV-1/2 RNA, TTV DNA, fungal genome, mycobacterial genome, and IgM Toxoplasma could be detected in the occult Hepatitis B virus sample, however IgG Toxoplasma was found positive.

OBI was defined by detection of HBV DNA in blood and or in liver, without the presentation of HBsAg , with or without serological markers of previous HBV infection (anti-HBs and or anti-HBc) (Carreno *et al*., 2008). Other experts define OBI by detection of HBV DNA in the liver, with or without detectable HBV DNA in blood, in patient negative for HBsAg (Raimondo *et al*., 2008). However, most

experts prefer the first definition. Our OBI patient was found negative for HBsAg, anti-HBc, and anti-HBs, respectively, however, positive for HBV DNA. It has been suggested that HIV interferes with the natural history of HBV infection by enhancing HBV replication and decreased hepatitis B e-antigen (HBeAg) seroconversion leading to more severe liver disease, more rapid progression of liver fibrosis and a higher rate of cirrhosis decompensation, end-stage liver disease and hepatocellular carcinoma (Azadmanesh *et al*., 2008; Puoti *et al*., 2006). Even though OBI is most frequently seen in patients with anti-HBc antibody as the only HBV serological marker, in some individuals no anti-HBc or anti-HBs antibody could be detected (Aghakhani *et al*., 2010; Torbenson and Thomas, 2002). The sequencing results of the HBV S gene, showed no mutation on S gene, especially in the region important for induction of immune reaction (the "a" region), indicated that the negative result of HBsAg assay was not caused by the escape mutant.

Although reactivation of HBV infection in HIV-positive/HBsAg negative is rare, it would be possible after withdrawal of antivirals against HBV, such as lamivudine or tenofovir (Bottecchia *et al*. 2011). Bloquel *et al.* (2010) described reactivation for two HIV patients with anti-HBc after withdrawal of HAART with anti-HBV activity. Taken all data together, we concluded that it would be very important to know the HBV virological status of HIV patients before starting antiretroviral therapies in order to avoid reactivations if treatment is stopped.

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# A PILOT PROJECT IN MONITORING SERUM CONCENTRATION FOLLOWING ADMINISTRATION AMIKACIN ONCE DAILY DOSING IN ELECTRICAL BURN PATIENTS AT SUTOMO HOSPITAL

**Rasmaya Niruri[1], Budi Suprapti[2], and Iswinarno D.S[3]**
[1]Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Udayana University, Kampus Bukit Jimbaran, Bali. Indonesia
[2]Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia
[3]Department of Plastic Surgeon, Sutomo Hospital, Surabaya – Medical Faculty, Airlangga University, Surabaya, Indonesia
Correspondence author: rasmaya@yahoo.com

**ABSTRACT**

At Sutomo Hospital, electrical burn patients received once daily dosing (1x750mg i.v. bolus) of amikacin, which is for gram negative bacteria. On several studies observed that 15 mg/kg once daily amikacin often had inadequate therapeutic concentration and inadequate doses may promote antibiotic resistance. To evaluate therapeutic concentration [peak ($C_{max}$) and through ($C_{min}$)] and extended-interval dosing, and to determine factors affecting therapeutic concentration in once daily regimen of 750 mg amikacin. This eight-months study covered all twelve electrical burn patients (age of 15-70). Pregnancy, drug allergy, shock, and receiving medicines which could influence the measurement with *Homogeneous Particle-Enhanced Turbidimetric Immunoassay*" (PETIA) method, and alter amikacin pharmacokinetic were excluded. Three serum concentrations were drawn in one hour, in time range of 1-8 hours, and around 24 hours after amikacin injection, and then measured using PETIA method. One patient reached therapeutic concentration of 32 µg/ml ($C_{max}$/MIC ≥8 (MIC 4 µg/ml); 6 patients achieved $C_{max}$/MIC ≥5 (MIC=4 µg/ml); the rest 5 patients didn't reach it; all patients had $C_{min}$ below 2 µg/ml; and 24 hours interval dosing was inadequate. Based on Pearson's /Spearman's correlation test and multivariate analysis, there were insignificant correlation of covariates (clearance creatinine , albumin, burn size, Unit Burn Standard, Baux Index, Tobiasen Index) with the alter of amikacin pharmacokinetic parameter that could be used to predict the changes in dosage required to attain therapeutic concentration. Thus, it is suggested that 12 hours interval dosing is applied, and monitoring serum concentration in each patient to determine individual amikacin dose.

***Keywords:*** Electrical Burn, Amikacin, Once daily.

## INTRODUCTION

Infection has caused 75% of death in burn victims. Thus controlling infection is important. Amikacin is indicated for gram negative bacteria. In thermal burn, the initial dose can be predicted by burn surface area (Conil, 2006). However in electrical burn, area of destruction is unpredictable because it can be on the vessel due to the passage of electrical current (Moenadjat, 2000). At Sutomo Hospital, electrical burn patients received once daily dosing (1x750mg i.v. bolus) of amikacin. On several studies observed that 15 mg/kg once daily amikacin had often inadequate therapeutic concentration and inadequate dose may promote antibiotic resistance. The aims of this study were to evaluate therapeutic concentration [peak ($C_{max}$) and through ($C_{min}$)] and extended-interval dosing, and to determine factors affecting therapeutic concentration in once daily regimen of 750 mg amikacin.

## MATERIALS AND METHODS

Hospitalized patients in Sutomo Hospital with electrical burn (age of 15-70), who get amikacin (1x750 mg iv bolus) during the secondary phase of burn (after 48 hours) (Conil, 2006), were involved in this study. Pregnancy, drug allergy, shock, suffering from pathological condition which was able to alter amikacin pharmacokinetic (such as ascites), and receiving other medicines which was able to influence measurement of amikacin concentration with *Homogeneous Particle-Enhanced Turbidimetric Immunoassay*" (PETIA) method, and/or to alter amikacin pharmacokinetic were excluded from this research. (Abbot, 2006; McEvoy, 2003). This study was approved by the Sutomo Hospital ethics committee.

Three serum concentrations were drawn in one hour, in time range 1-8 hours, and around 24 hours after amikacin injection, and then determined using Architect ci 8000 Integrated System – Abbot Laboratories USA (based on PETIA) with Reagent Multigent Amikacin. The targeted concentration was

amikacin maximum concentration ($C_{max)}$ between 8-10 times minimum inhibitory concentration (MIC). Burton (2006) mentioned that MIC for most gram negative pathogen bacteria was 4-8 µg/ml. (McEvoy, 2003; Zelenitsky, 2003; Shargel, 2005; Burton, 2006; Chambers, 2006; Scaglion and Paraboni, 2008; Suprapti, 2009). If the $C_{max}$ target was not achieved, the estimated dose ($Do_{new}$ ) was calculated using individual pharmacokinetic parameter [half life ($t_{1/2)}$; elimination rate (k); amikacin clearance ($Cl_{amk}$); volume of distribution($V_D$)] with Sawchuck-Zaske method (Shargel et al., 2005; Burton, 2006; Bauer, 2008).

Dose interval in burn patients based on individual pharmacokinetic parameter was evaluated. It was considered sufficient, if there was no opportunity for bacteria to grow during this time range of interval. It was expected that the dose interval was similar or less than the sum of time for amikacin serum concentration to decrease up to 4-8 µg/ml (determined from pharmacokinetic calculation ) and post antibiotic effect (PAE - based on reference) (Burton, 2006; Suprapti, 2009).

Biological parameters, including albumin and serum creatinine, were measured. Creatinine clearance was estimated using Cockcroft–Gault formula. Albumin, creatinine clearance, and burn severity index (the percentage of burned surface area (SB) with Rules of Nines, the 'Unit Burn Standard' (UBS) the Baux index and the Tobiasen Index) will be used to evaluate its influence on the alteration of amikacin pharmacokinetic parameter on burn patients. Base deficit (BD) and urine output were also documented to evaluate in adequation of resuscitation in order to determine the patients were not in shock condition (Conil, 2006 and Josh, 2005).

Mean and median were calculated for each parameter. To examine relationships between pharmacokinetic parameter and burn severity index / creatinine clearance / albumin were used Pearson's correlation test, if the data had normal distribution, or Spearman's correlation test if the data was not normal distributed. Normality-distribution of the data was determined using Shapiro-Wilk test due to the small sample size. The result was considered significant if P values < 0.05. Finally, multivariate analysis was performed to determine the correlation between independent variables with a dependent variable (pharmacokinetic parameter). Variables, which had P values < 0.25 in bivariate analysis (Pearson or Spearman test), were included in multivariate analysis. All analyses were performed using SPPS (Dahlan, 2011).

## RESULTS

Twelve electrical male burn patients were involved in this study and their characteristics are presented in Table 1.

**Table 1.** Patients Characteristic (n= 12)

| | Min | Max | Mean | SD |
|---|---|---|---|---|
| Age | 17 | 55 | 32.8 | 10.9 |
| Weight (kg) | 42.0 | 74.0 | 56.8 | 10.2 |
| Burn Surface Area (Rules of Nines) | 3.0 | 67.0 | 22.2 | 23.5 |
| Tobiasen Index | 4.0 | 8.0 | 5.6 | 1.7 |
| UBS | 6.0 | 72.0 | 26.2 | 26.9 |
| Baux Index | 27.0 | 85.0 | 55.0 | 20.8 |
| Creatinine clearence (ml/menit) | 81.5 | 179.9 | 133.7 | 34.6 |
| Albumin (g/dl) | 2.05 | 4.6 | 3.1 | 0.7 |

Based on Shapiro Wilk distribution test; the values of age, weight, creatinine clearance, and abumin have normal distribution .

**$C_{max}$, $C_{min}$, and Pharmacokinetic Parameter of Amikacin**

There was a big variability of dose (mg/kg weight) in fixed dosage of amikacin (1x750 mg) that influeced $C_{max}$ value (Fig.1). Based on amikacin serum monitoring, it showed that targeted therapeutic concentrations 64 µg/ml ($C_{max}$/MIC ≥8 (MIC 8µg/ml)) was not achieved by all twelve burn patients, therapeutic concentration 32 ($C_{max}$/MIC ≥8 (MIC 4 µg/ml) was reached by 1 patient ; 5 pasien achieved $C_{max}$/MIC ≥5; and the rest 6 burn patient did not reached $C_{max}$/MIC≥5 (MIC ). All patients have $C_{min}$ values < 2 µg/ml.

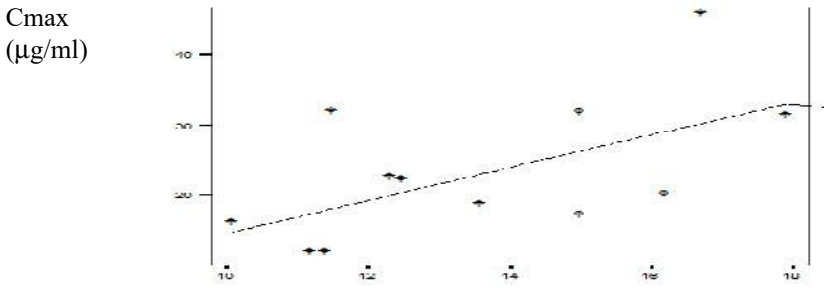


**Fig. 1** Relationship between dose regimen and $C_{max}$ amikacin

Pharmacokinetic parameter were determined using two amikacin concentration (Table 2)

**Table 2.** Pharmacokinetic Parameter Amikacin (750 mg once daily)

| No | Parameter | Mean | Median | SD | Minimum | Maximum |
|---|---|---|---|---|---|---|
| 1 | K ($hr^{-1}$) | 0.5 | 0.4 | 0.1 | 0.2 | 0.7 |
| 2 | t½ (hr) | 1.6 | 1.6 | 0.5 | 0.9 | 2.5 |
| 3 | $V_D$(L/kg) | 0.5 | 0.5 | 0.2 | 0.2 | 0.7 |
| 4 | $Cl_{amk}$(L/hr) | 10.6 | 9.6 | 4.2 | 5.8 | 18.2 |
| 5 | $C_{max}$(µg/ml) | 23.0 | 20.7 | 10.0 | 11.4 | 45.3 |

**Evaluation of Dose Interval**

In all patients showed that 24 hours dose interval of 750 mg once daily in burn patients was not sufficient – 24 hours was more than the sum of time for amikacin serum concentration to decline up to 4-8 µg/ml and PAE (PAE = 8 hours). Based on estimation using pharmacokinetic individual parameter, it showed that 24 hours dosing interval was inadequate in various doses of amikacin (500 to 1500 mg, and 10-30 mg/kg weight).

**Corelations between Pharmacokinetic Parameter and Clinical Characteristic of Patients**

a. $C_{max}$ of Amikacin
Spearman's correlation test showed insignificant correlations between $C_{max}$ amikacin and burn area – Rules of Nines (r= -0.098 and p = 0.761 [p>0.05]); UBS (r= -0.147 and p = 0.648 [p>0.05]); and Tobiasen Index (r=-0.106 and p=0.746 [p>0.05]). Whereas to determine correlation between $C_{max}$ amikacin and Baux Index (r=-0.019 and p= 0.828 [p>0.05]) used Pearson's correlation test, with insignificant as a result.

b. Amikacin Clearence ($Cl_{amk}$)
Pearson's correlation test showed insignificant correlations between $Cl_{amk}$ and creatinine clearance, which was represented renal function (r=0.510 and p = 0.091 [p>0.05]).

c. Half life ($t_{½}$)
Spearman's correlation test showed significant correlations between $t_{½}$ and creatinine clearance, which was represented renal function (r=0.657 and p=0.020 [p<0.05]).

d. Volume of Distribution Amikacin ($V_D$)
Spearman's correlation test showed insignificant correlations between $V_D$ and Burn Area – Rules of Nines (r=0.148 and p=0.646 [p>0.05]); UBS ( r=0.221 and p = 0.489 [p>0.05]); and Tobiasen Index (r= 0.178 and p = 0.579 (p>0.05). On the other hand, Pearson's correlation test was applied to determine the correlation between $V_D$ and Baux Index due to the normal distribution of its data, which has insignificant correlation (r=0.316 and p = 0.317 [p>0.05]). Pearson's correlation test showed significant correlations between $V_D$ and Albumin (p = -0.044 [p<0.05] and r= -0.589).

**Multivariate analysis between Pharmacokinetic Parameters and Clinical Characteristic of Patients.**

The equation which was resulted from multivariate analysis, linear regression, between dependent variable (half life), and independent variables (creatinine clearance, albumin, and tobiasen index), was ha lflife=2.498+(0.006xClcr)-(0.3xalbumin)-(0.143xtobiasen index). Anova test performed P value = 0.007 (p <0.05), and it considered that the equation can be used. Adjusted R square value was 0.669 (66.9%) showed on Summary Model of multivariate analysis. Linearity in the regression was failure based on linearity test (Dahlan, 2011)

## DISCUSSION

Various factors influenced Cmax value, such as burn area, sepsis, trauma and hypoalbuminaemia (Bauer, 2008; Conil, 2006; Scaglione and Paraboni, 2008). The big variability of weight (42,0 – 74,0 kg (10.1 – 17,8 mg/kg weight) with mean 13,6 mg/kg weight) might influenced the dose receiving by each burn patient (mg / kg weight) in fixed dose 750 mg once daily. Using the individual pharmacokinetic parameter, it also evaluated the use of fixed dose of 750 mg once daily and 15 mg/kg weight once daily in estimating the achievement of targetted $C_{max}$/MIC, and the result was applying dose based on mg/kg weight give a better opportunity to attain therapeutic concentration.

In all patients showed that 24 hours dose interval of 750 mg once daily in burn patients was not sufficient – 24 hours was more than the sum of time for amikacin serum concentration to decline up to 4-8 µg/ml and PAE (PAE = 8 hour). Twenty four hours interval was also estimated using pharmacokinetic individual parameter to find out the possibility of adequation interval if all the patients received various dosage of amikacin (500 to 1500 mg, and 10-30 mg/kg weight. Based on this estimation, it showed that 24 hours dosing interval was inadequate in this various dose, and dose interval of 12 hours was preferred. Eight hours interval was not chose due to adaptive resistance of amikacin. Amikacin has 8-16 hours in vivo adaptive resistance, with complete refractory in 12 hours (Eve, 1980). Expected $C_{max}$ is five times MIC value if the data of safety high dose amikacin has not established (Kashuba, 1998). While, te expected of $C_{min}$ value for 12 hours interval is 5-10µg/, not more that 10 µg/ml (Chambers, 2001; Kastrup, 2001). Based on the pharmacokinetic individual parameter in this study, it was estimated that the range dose of amikacin to attain the expected target (which was 5 times MIC - MIC = 4 µg/ml) with 12 hours interval was 6,7-19,9 mg/kg weight, and suggested to co-administered with other antibiotic.

Based on the correlation test between Pharmacokinetic Parameter and Clinical Characteristic of patients, it showed that there were no correlation between them, except for renal function (creatinine clearance) and half life; and albumin and volume of distribution which had significant correlation. However, there was no correlation between renal function (creatinine clearance), and clearance of amikacin. It might be considered to big variability of volume of distribution value which was altered from normal condition.(McEvoy, 2003; Bauer, 2008; Scaglione, Paraboni, 2008). Eight of 12 total patients had volume of distribution bigger than 0.2 -0.3 L/kg- normal value of amicin volume of distribution (Bauer, 2008).

The normal value of half life can be found in increasing value of amikacin clearance, because of the greater value of volume of distribution. In burn and other hyperdinamic conditions such as sepsis can cause big variability in drug clearance (Scaglion, Paraboni, 2008). Parallel increasing on both volume of distribution and amikacin clearance may cause the unchanged half life, which was on the normal value –in the range 1,5 – 3,0 hour (Conil, 2006; Bauer, 2008). Therefore, clearance of amikacin was not significant correlated with creatinine clearance. This fact was suitable to burn study conducted by Conil in 2006. Electrical burn is a special condition. The destruction of body tissue was caused by electrical wave passing through parts of the body (such as brain, hearth, muscle, and renal) and it made the extensive local or systemic destruction. The destruction was slow but it was certain, and difficult to predict the area of destruction, because it can be affected to vessel system in the body which was suffering from electrical wave (Moenadjat, 2000). Furthermore, hyper metabolic and catabolism, which may be happened until full healing of wounds(: this process may be lasted in 28 – 398 days), contributed on the alteration of pharmacokinetic parameter such as the rate of elimination and volume distribution that may be influenced in $C_{max}$ value of amikacin (Hart, 2000; Morison, 1992, Bonate, 1990, Jaehde, 1995). All of the burn severity index cannot represent the progress of wound healing due to the hemodynamic condition on burn patients.

The equation which was resulted from multivariate analysis, linear regression, was halflife=2.498 +(0.006xClcr)-(0.3xalbumin)-(0.143xtobiasen index). Anova test performed P value = 0.007 ($p < 0.05$). which it was considered that the equation can be used. Adjusted R square value was 0.669 (66,9%) showed on Summary Model of multivariate analysis. Sixty six point nine percent (66,9%) in adjusted R square value showed that the equation was representing 66,9% of the factors related half life and the rest (33,1%) could not be calculated. However, linearity in the regression was failure based on linearity test. Althought it will not invalide the analysis but it weaken it because the extent of the curvilinear relationship cannot be fully captured (Dahlan M.S., 2011). It might be because of the small sample size, so increasing sample size in the future study is needed.

Finally, this equation could not be used to estimate the alteration of amikacin pharmacokinetic parameter to predict the changes in dosage required to attain therapeutic concentration. Since there were no existed covariates that were able to represent the hemodynamic condition on burn patients, so, to ensure the safe usage of amikacin dosage regimen, the patients therapeutic amikacin monitoring should be applied in electrical burn patients.

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# STUDY PROTOCOL: DEVELOPMENT MULTIPLEX DIPSTICK FOR EARLY DETECTION *HUMAN IMMUNODEFICIENCY VIRUS* -1 AND *HEPATITIS B VIRUS* BASED ON COMBINATION OF MULTIPLEX LOOP-MEDIATED ISOTHERMAL AMPLIFICATION AND LATERAL FLOW DIPSTICK

**Narendra Yoga Hendarta[1*], Siti Helmyati[2], Ludhang Pradipta Rizki[3], Asmarani Kusumawati[4], and Tri Wibawa[3]**
*[1]Dept. of Health Analyst, Health Polytechnics of Yogyakarta, Yogyakarta, Indonesia*
*[2]School of Health Nutrition, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia*
*[3]Dept. of Microbiology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia*
*[4]Dept. of Reproduction and Ob-gyn, Faculty of Veterinary, Gadjah Mada University, Yogyakarta, Indonesia*
*[*]Corresponding author: narendrayogahendarta@yahoo.com*

**ABSTRACT**

*Human Immunodeficiency Virus 1* (HIV-1) and *Hepatitis B virus* (HBV) are agents of fatal-contagious diseases and become as endemic and pandemic global diseases. As fatal diseases cause high mortality and economic loss that incidence increase every year. The viruses are transmitted especially horizontally through bodily fluids and vertically mother to the fetus. Incidence of HIV-1 co-infection with HBV increases and makes difficult treatment of disease but there are no effective medicines so preventive effort is important. Early detection of existence the virus become important of awareness for suspect and the surround which most transmission occur because of unrecognizing the viruses. Some techniques of detection are available but mostly complicated and time consuming. Therefore, the aim of our study is to develop a tool for early detection the viruses based on LAMP and LFD that claimed a simple and rapid method. LAMP as a rapid isothermal nucleate acid amplification using simple device is combined to LFD to visual confirmation of specific amplicon that be known of rapidness and simplicity. This study will begin analysis of conserved region sequence of target and followed primer design to amplify that target using special software for LAMP from Eiken Japan. Two set of primers which each consist of 2-3 pairs of primer will be used to optimize reaction to obtain optimum of condition reaction such as temperature and time and reagent component such as dNTP, $MgSO_4$, and Betain. Result will be tested its sensitivity and limited application by visual confirmation with electrophoresis and LFD.

***Keywords:*** HIV-1, HBV, LAMP, LFD, Early detection

## INTRODUCTION

*Hepatitis B Virus* (HBV) and *Human Immunodeficiency Virus* Type 1 (HIV-1) are agents of dangerous transmission disease becoming global endemic and pandemic and causing high death and economic losses. The viruses are transmitted horizontally through body fluid contact and vertically through birth and breast feeding. Early detection is one of most importance which these viruses detected as soon as possible, the suspect and surround would be aware. Most of transmission occurs as caused of unrecognizing of viruses in the suspect.

Methodologies used to detection based on antigen-antibody reaction still have problem with window period of antigen existence in body which need repeated test. On the other hand, detections based on nucleate acid detection and amplification need time-consuming procedures and expensively complicated devices as well as skilled technician. Loop Mediated Isothermal amplification (LAMP) is an isothermal reaction using *Bacillus stearothermophilus* (*Bst*) DNA polymerase with double-strand displacement activity developed by Notomi *et. al* (2000). Comparing to conventional amplification, PCR, this methodology has many benefit such as rapid reaction (30-60 min.), high specifity and sensitivity, use a simple device (e.g. thermobath) (Parida *et. al*, 2008). LAMP reaction uses 2 pair primers namely outer primer (F3 and B3) and internal primer (FIP and BIP) and/or one or two loop primer (LF and LB) to increase reaction. The primers work particular function which outer primer for initiating reaction, internal primer for cyclic reaction, and loop primer access loop region. The mode of amplification with auto double-strand displacement and hybridization of complement sequence give cauliflower-like structure and on agarose gel electrophoresis visualization shows ladder pattern (Notomi *et. al*, 2000; Parida *et. al*, 2008).

Lateral flow dipstick (LFD) is important in diagnostic application which most known in pregnant test. LFD now has high sensitivity, selectivity, and simple using (Posthuma-Trumpie *et. al*, 2008). Combination

of LAMP and LFD for detecting pathogen has proof to work by Nimitphak *et. al* (2008 and 2010), Soliman and El-Matbouli (2008), Kiatpathomchai *et. al* (2008) and Kusumawati and Hendarta (2012). This method works with biotin labeling of LAMP amplicon and hybridization with labeled probe using FITC which recognize target gene. Hybridized amplicon is then drops into sample pad of dipstick strip and flow laterally through conjugate pad. FITC will bind with antibody-anti FITC coating gold particle, goes to test line containing immobilized avidin and give purple color showing positive result. Meanwhile unhybridized FITC labeled probe flow to control line containing antirabbit-antibody and give color (Posthuma-Trumpie et. al, 2008; Milenia Biotec GmbH). LFD takes only 10-15 minutes procedure and it is so rapid comparing confirmation amplicon using agarose gel electrophoresis taking a lot of time. The use of labeled probe recognizing specific target gene become confirmation of spesifity of amplicon (Nimitphak et. al, 2008 and 2010; Soliman et. al, 2008; Kusumawati and Hendarta, 2012).

The aim of study is to development a methodology detecting HBV-1 and HIV based on LAMP and LFD. Due to simplicity, this technique would be expected as diagnostic tool to early detecting virus and applied as routine procedure such as in blood donor screening.

## MATERIALS AND METHODS

1. Samples
   RNA HIV-1 and DNA HBV are extracted from serum using High Pure Viral Nucleic Acid Kit (Roche, German).
2. Primer.
   Primers LAMP consist of basically 2 pairs (F3, B3, FIP, and BIP) with or not loop primer (LF and LB) and are designed using online software, Primer Explore V4, from Eiken Chemical Japan. Sequence target to amplification is based on conserve region of HBV and HIV-1 gene to detect all type of virus especially isolate from Indonesia. To confirmation capability to detect, using a candidate sequence from Indonesian isolate and then aligned using *blastn* in NCBI site.
   HIV-1 LAMP primer, for LFD, 5'end of FIP or BIP will be labeled with biotin which bind to avidin in test line on dipstick (Hybridetect 2T Milenia Biotech German). Meanwhile, HBV LAMP primer, 5' end of FIP or BIP BIP will be labeled with Digoxigenin which bind to anti-Digoxigenin in test line on dipstick.
3. Labeled Probe
   Two DNA probes for hybridization are designed to recognize region each HIV-1 and HBV between FIP and BIP along 15-20 bp and 5'end will labeled with FITC or FAM which bind antibody-anti FITC/FAM coating gold particle on conjugate pad of dipstick (Hybridetect 2T Milenia Biotech German).
4. LAMP reaction
   Optimizations LAMP reaction for HIV-1 and HBV use sequence DNA template product of PCR with primer amplify between F3 and B3 which amplicon is purified (High Pure PCR Product Purification Roche, German). This reaction will be conducted separately for HIV-1 and HBV. LAMP reaction consist of: 2μM internal primer (FIP and BIP), 0.2 μM outer primer (F3 and B3), 1.4 mM dNTP mix (Roche, German), 0.6 M Betain (Fluka Biochemika), 6 mM $MgSO_4$, 8 U of Bst DNA polymerase (Large fragment, NEB, USA) along with 1x reaction buffer. To increase reaction is then added 1 μM loop primer (LF and LB). Template use 1 μM DNA product of PCR and finally add dH2O to reach total volume of 25 μM. Temperature reaction will be conducted at 60, 63, 65°C to get best reaction and stopped with incubation at 80°C for 4 minutes. Concentration of $MgSO_4$ will optimatized from 4mM to 12mM and dNTP from 0,8 to 2mM.
5. Probe Hybridization
   LAMP amplicon hybridized with labeled DNA probe with add 20 pmol probe and incubate 5 minutes at 63°C. Then is took 8 μl into 150 assay buffer in new tube and LFD strip is dipped for 10 minutes. Purple color will rise at test line and control lines which show positive result. Color rise only at

control line as indicating a negative result.

6. Sensitivity Test
   This test will be performed using ten serial dilution of template from 1 ng to 0.1 pg which amplified with LAMP and PCR reaction. PCR technique with F3 and B3 primers is used as comparing. Amplicon will analyzed with 1.8% agarose electrophoresis. This test is also conducted for probe hybridization.
7. Multiplex LAMP Test
   This will be conducted with positive and negative samples. A number of 10-15 samples contain HIV-1 and the same amount one contain HBV including double infection will be detected using multiplex LAMP. For this test, 0,25 U of Amv Reverse Transcriptase is added in LAMP reaction to transcript RNA template of HIV-1. Also 2 set primers of HIV-1 and HBV LAMP are mixed in the same reaction.

## DISCUSSION

Multiplex LAMP and LFD is a methodology detecting a couple viruses especially infectic and pathogenic. Using labeled probe recognizing target gene is very important to confirmation spesifity of amplicon. LAMP product has ladder pattern on agarose gel electrophoresis visualization which difficult to determine the spesifity. Most problem in LAMP method is what called as contamination which generate product amplification although without template. So far, there is no certain explanation and still confusing. Labeled probe is the solution and has proved by Asmarani and Hendarta (2012) which color has not generate on strip if there was contamination although visible on agarose gel visualisation. Moreover, LFD use fabricated strip known as dipstick omitting further complicated and time consuming technique to confirm result of amplification such as agarose gel electrophoresis. LFD has ability to detect similar to agarose gel electrophoresis (Nimitphak et al., 2008 and 2010). The LAMP problem of contamination become careful design of primer for multiplex platform which two set of HIV-1 and HBV primers should not form self annealing and self amplification.

HIV-1 is RNA virus and HBV is DNA virus which in multiplex LAMP able to work in the same tube reaction without addition time and different temperature for transcription of RNA and amplification of DNA. Multiplex LAMP will work in an hour and in the same temperature. This is the advantage of multiplex LAMP comparing multiplex PCR besides using simple device.

## ETHICS

The protocol has been send to Medical and Health Research Ethics Committee Faculty of Medicine Gadjah Mada University which has concluded that approval is accepted and that the project can be carried on as described.

## TRIAL STATUS

Active. Trial effectively started on early August 2012.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

## AUTHOR'S CONTRIBUTIONS

NYH initiated conceptualization, study design and methodology. NYH piloted these work with the help of SH and LPR. AK and TW contribute as consultans. NYH wrote the paper and is responsible for this manuscript. All authors read and approved the final manuscript.

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# VOLTAMMETRIC BEHAVIOR OF THE TRANSFER OF METHYL EPHEDRINE ION ACROSS THE WATER |NITROBENZENE INTERFACE

**Irdhawati[1]*and Hirosuke Tatsumi[2]**
*[1]Department of Chemistry, Faculty of Mathematic and Natural Sciences, Udayana University, Bali Indonesia*
*[2]Department of Chemistry, Faculty of Science, Shinshu University, 3-1-1 Asahi Matsumoto Nagano 390-8621 Japan*
**Correspondence author: irdhawati@yahoo.com*

**ABSTRACT**

The ion transfer of methyl ephedrine ion ($MeEphH^+$) across the water (W) and nitrobenzene (NB) interface was studied by cyclic voltammetry method. Tetrabutylammoniumtetraphenylborate (TBATPB) and lithium chloride (LiCl) were used as supporting electrolytes in organic and water phases. Both of phases were separated by dialysis membrane. When methyl ephedrine ion across the interface through dialysis membrane, peak current was measured in various concentration and scan rates.The result showed $MeEphH^+$ can be oxidized at 405 mV. The anodic peak potential increased and shifted to more positive potential and the cathodic peak potential shifted to more negative potential with increasing of scan rates. The potential difference was applied between two Ag/AgCl electrodes (working and reference electrodes) and the current between working and counter (Pt wire) electrodes was recorded. These results indicated the suitability of liquid-liquid electrochemistry as an analytical approach in drugs analysis.

***Keywords:*** ion transfer, methyl ephedrine, liquid|liquid interface, diffusion coefficient, cyclic voltammetry

## INTRODUCTION

Methylephedrine hydrochloride (MeEphHCl) is a sympathominetic agent with similar action to ephedrine, but has less pressor activity and central nervous system effect. MeEphHCl is often given by mouth in combination preparations for the relief of cough and cold symptoms. Therefore, quantification of this drug in human plasma is desired for pharmacokinetic studies(Zhu et al. 2005). The chemical structure of MeEphHClcan be seen in Figure 1.

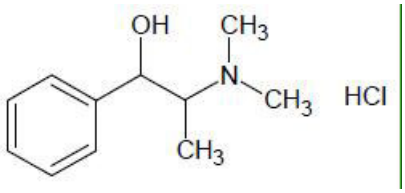


**Figure 1.** Chemical structure of MeEphHCl

An interface between two immiscible electrolyte solutions (ITIES) is formed between two liquid solvents of a low (ideally zero) mutual miscibility, each containing an electrolyte. One of these solvents is usually water, and the other one is a polar organic solvent of a moderate or high dielectric permittivity, such as nitrobenzene or 1,2-dichloroethane, which allows for at least partial dissociation of dissolved electrolyte(s) into ions. Processes are taking place at the interface of a low permittivity dielectric (e.g., liquid hydrocarbon) and an aqueous electrolyte solution (Samec, 2004).

When two immiscible liquid phases are brought in contact, a distribution of the different species, neutral and ionic, spontaneously occurs. The partition of neutral species depends primarily on the nature of the solvents, whereas the partition of ionic species is restricted by the fact that the electroneutrality must be maintained in the two adjacent phases. Furthermore, the partition of ions results in a difference of inner potentials between the two phases, this difference being called the Galvani potential difference. The detailed knowledge of the distributionof acid/base in biphase liquid systems is important to many fields such as solvent extraction, phase transfer catalysis, or drug lipophilicity and its consequences to drug delivery(Reymond et al. 1996).

A view methods have been developed for determination ofMeEph, such as ionic liquid (Liu et al. 2009), ischemia stroke (Imai et al. 2010), reformatsky reaction (Cozzi and Rivalta, 2006), and reduction with lithium aluminium hydride (Terashima et al, 1980).

In this paper, an interface between water and organic was prepared for determination of MeEphHCl by cyclic voltammetry (CV) method. Nitrobenzene (NB) and water are used as organic and water phases, respectively. Ion transfer take place across dialysis membrane between organic and water phases. The peak current enhanced for the oxidation of MeEphHCl. Therefore, liquid|liquid interface can be considered a voltammetric sensor for analysis MeEphHCl in pharmaceutical and clinical samples.

## MATERIALS AND METHODS

### Chemicals

MeEph was purchased from Mitsubishi Tanabe Farma Japan (40 mg/mL). Nitrobenzene and tetrabutylammonium bromide were obtained from Merck.Lithium chloride (LiCl) was purchased from NacalaiTesque. Tetrabutylammonium chloride (TBACl) and sodium tetraphenylborate (NaTPB) were supplied by Wako and Dojindo, respectively.Tetrabutylammoniumtetraphenylborate (TBATPB) was synthesized by previous method(Kakutani et al, 1983). All chemicals were analytical grade, used as received without further purification.

### Apparatus and procedure

Electrochemical measurements were performed on a Hokuto Denko potentiostat with a three-electrode system comprised of platinum wire auxiliary in organic phase, and silver/silver chloride in both of organic and water phases. TBACl was used as inner filling solution for reference electrode in organic phase. TBATPB 0.1 M and LiCl 0.1 M were used as supporting electrolyte in organic and water phases, respectively. All solutions prepared with double distilled water. Freshly MeEphHClsolution was used prior to measurements. CV was used for determination of MeEph ion transfer. CV measurements were performed from 200 to 600 mV with the scan rates of 200; 100; 50; 20; and 10 mV/s, and concentration of 0.1; 0.2; 0.3; 0.4; and 0.5 mM.All measurements were carried out at 25 + 1°C using water flow thermostat.

The experimental setup was as described in Figure 2. CV at various scan rates and concentrations was performed on the following electrochemical cell with full iR compensation.

| | | I | II | III | IV | | |
|---|---|---|---|---|---|---|---|
| Ag | AgCl | 0.1 M TBACl | 0.1 M TBATPB | 0.1 M LiCl x mMMeEphH$^+$ | 0.1 M LiCl | AgCl | Ag |
| | | Water | NB | Water | | | |

**Figure 2.** Scheme of the galvanic cell for the study of electron transfer for MeEphHCl across the W|NB interface

The interface between phases II and III is polarizable. The reference electrode for the NB phase (phaseII) was an Ag/AgCl in 0,1 M TBACl (phase I) electrodewas connected to TBATPB solution directly using asmall glass tube. The reference electrode for theaqueous phase was an Ag/AgCl in 0.1 M LiCl (phase IV) immersed in an aqueous phase (phase III) was separated by membrane glass. The counter electrodes, a Pt wire was immersed in phase II. The organic phaseconnected to the aqueous phase through dialysismembrane. The location and the distance of the electrodes adjusted by using silicone plug.

## RESULTS AND DISCUSSION

An electrochemical process at liquid-liquid interface proceeds as a complex, coupled electron-ion transfer reaction. The ion transfer at the W|NB interface is driven by the electrode reaction. As noted in the previous section, both liquid phases contain an electrolyte with a common ion. In the course of

the voltammetric experiment, the transfer of the common ion accompanies the electrode reaction of the redox probe. Both electrochemical processes are virtually simultaneous, resulting in a single voltammetric response that represents the overall electron-ion transfer reaction. Besides being a transferring ion, the purpose of the common ion is to control the potential difference at the liquid-liquid interface.

To determine the ion transfer of methyl ephedrine at W|NB interface, the peak current was measured at various scan rates and concentration. The best scan rate obtained 100 mV/s. The cyclic voltammogram is shown in Figure 3 below.

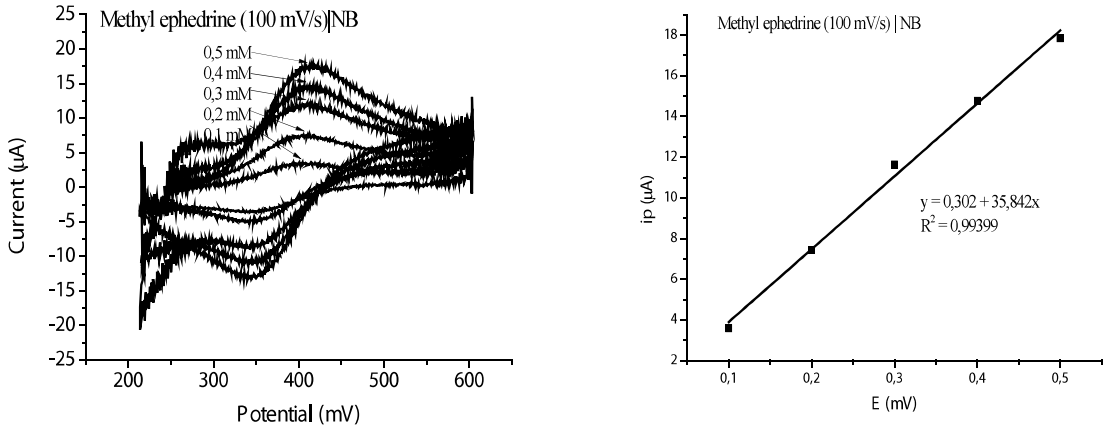


**Figure 3.** Cyclic voltammogram and plot of potential and peak current of methyl ephedrine at 100 mV/s, with various concentration

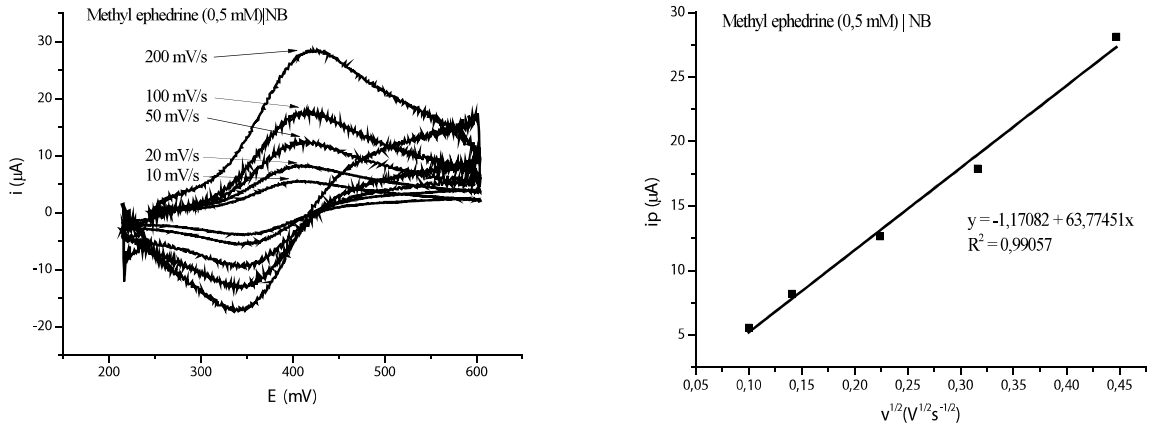


**Figure 4.** Cyclic voltammogram and plot of square root of scan rates and peak currentof methyl ephedrine at 0.5 M, with various scan rates

The voltammetric results in Figure 3 and 4 indicate that the electron-transfer reaction caused by ion transfer mechanism. The forward and backward rate a constant of the homogeneous electron-transfer reaction occurs at the interface between W-NB interfaces, showing the reaction is a reversible process.

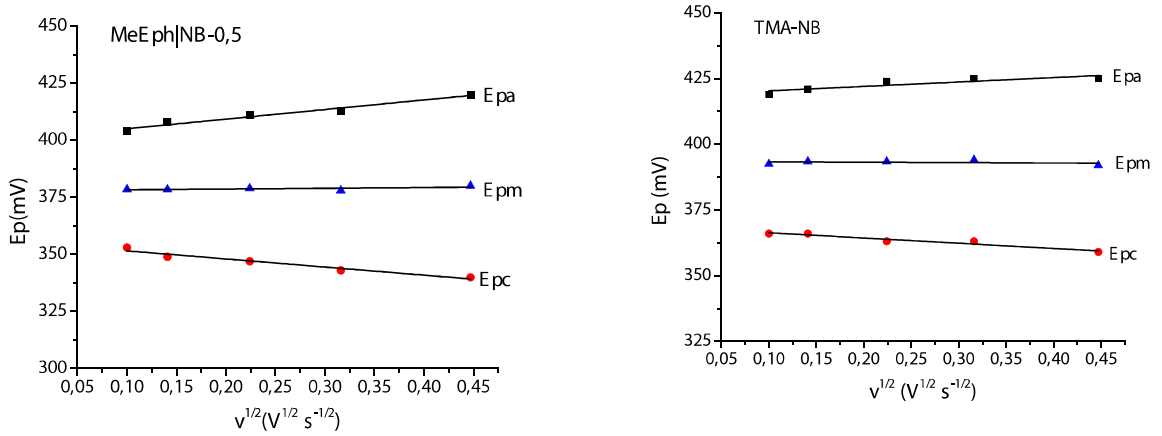


**Figure 5.** Plots of Epa, Epc, and Epm of the transfer of $MeEphH^+$ and $TMA^+$ at W-NB interface against $v^{1/2}$ at C = 0.5 mM

The potential difference between anodic peak (Epa) and catodic peak (Epc) obtained around 60 mV/s, shows $MeEphH^+$ has one electron in oxidation reaction process and confirm that methyl ephedrine ion is a monovalent cation. Furthermore, for reversible reaction, the half wave potential has the same value with the midpoint potential (Epm).

The diffusion coefficient can be determine using Randles Sevcik equation (Bard and Faulkner, 2001).

$$ip = (2.69 \times 10^5)\, n^{3/2}\, v^{1/2}\, D^{1/2}\, AC$$

where ip is peak current, n is the number of electron in redox reaction, v is the scan rate (V/s), D is analyte diffusion coefficient ($cm^2/s$), A is the surface area ($cm^2$), and C is analyte concentration (mM).

The standard for ion transfer potenstial was calculated by,

$$\Delta_o^w f^0\ MeEphH^+ = \Delta_o^w f^0\ TMA^+ - \Delta_o^w f_m\ TMA^+ + \Delta_o^w f\ MeEphH^+$$

The value for the standard ion transfer potential of $TMA^+$ can be seen in the reference Osakai (2006).

And free Gibbs energy,

$$-\Delta G_{tr,MeEph}^{0,o\to w} = z_{MeEph}\ F\ \Delta_o^w f^0_{MeEph}$$

After substitution the data, the diffusion coefficient, the standard for ion transfer potential, and free Gibbs energy was obtained 4.46 x $10^5$ $cm^2/s$, 0,021 V, and 2,026 kJ/mol.

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# ANTIMALARIAL ACTIVITY OF ETHANOLIC EXTRACT OF *Spondias pinnata* (L.f) KURZ. LEAVES

**Ni Putu Ariantari[1*], Ketut Gegel Yoga Widyadana[1], Made Ari Puji Astuti[1], Lidya Tumewu[2], and Aty Widyawaruyanti[2,3]**
[1]*Pharmacognosy and Phytochemistry Laboratory, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Udayana University*
*Jl.Kampus Bukit-Jimbaran, Department of Pharmacy, AF Buiding, Badung, Bali-Indonesia 80364, Telp/Fax: 0361-703837*
[2]*Satreps-JICA Laboratory, Institute of Tropical Disease, Surabaya-Indonesia*
[3]*Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Airlangga University, Surabaya-Indonesia*
**Corresponding author: ari_dedhika@yahoo.com*

**ABSTRACT**

Malaria is parasitic disease, with high prevalence in tropical and subtropical countries. The cases of parasite resistance to current antimalarial drugs are an important problem in controlling malaria. One of effort to overcome this problem is searching for new antimalarial agent from medicinal plants. In present study, we evaluate phytochemical constituents and in vitro antimalarial activity of ethanolic extract of *Spondias pinnata* (L.f.) Kurz leaves, which is traditionally used to treat fever. Dried leaves of *S. pinnata* was extracted with 80% ethanol yielded ethanolic extract, followed by phytochemical analysis using color reaction. Extract was then evaluated for its antimalarial activity against *Plasmodium falciparum* 3D7. Phytochemical analysis of this extract showed the presence of triterpenoids, flavonoids and polyphenols. Result of this study revealed that ethanolic extract of *S. pinnata* leaves was considered to be active against *P. falciparum* 3D7 in vitro, with an $IC_{50}$ value of 0.16 µg/mL. The finding indicates that this extract is potential as antimalarial and follow-up study to identify active component is needed.

***Keywords:*** *Spondias pinnata* (L.f.) Kurz., ethanolic extract, *Plasmodium falciparum* 3D7, antimalarial.

## INTRODUCTION

Malaria still becomes public health problem, especially in developing countries in Africa and Asia, where malaria is endemic. The appearance of parasite resistant strain is major problem in global malaria control program (Rosenthal, 2003). Therapeutic failure of falciparum malaria to recently antimalarial medicine, artemisinin, has been reported in Cambodia, Myanmar, Thailand and Vietnam. If this strain develop and spread to other geographic areas, there will be limit alternative therapy for malaria (WHO, 2013).

Medicinal plants are promising source for developing antimalarial agents (Fotie, 2008). Previous antimalarials, quinine and artemisinin, were firstly isolated and developed from medicinal plants used in traditional medicine for fever, *Chincona sp* and *Artemisia annua*. Recently, most of Asian population still rely on indigenous medicine and every region already had their own indigenous medicine (WHO, 2008). Therefore, exploration of medicinal plant as antimalarial being important in malaria control efforts.

*Spondias pinnata* (L.f.) Kurz. which is belongs to Anacardiaceae, traditionally used in Indonesia for cough, dysentry and fever remedies. This plant is locally named as Kedondong Hutan (Hutapea, 1994). Several studies on pharmacological properties of this plant has been reported. Stembark extract of this plant revealed antioxidant and free radical scavenging activity (Hazra *et al*., 2008). Ethanolic extract of *S. pinnata* pulp has been proved for antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* and antifungal activity against *Candida albican* and *Trichophyton mentagrophyte* (Keawsa-ard and Liawruangrath, 2009). Resin extract of this plant was also found to be active against *Bacillus subtilis* (Gupta *et al*., 2010) and fruit ethanolic extract exhibited strong cytotoxicity (Muhammad *et al*., 2011).

The objective of present study is to investigate in vitro antimalarial activity of ethanolic extract of *S. pinnata* leaves against *Plasmodium falciparum* 3D7. We also evaluate phytochemical constituents in this extract.

## MATERIALS AND METHODS

**Plant material:** S. *pinnata* leaves was harvested from Bukit Jimbaran-Bali, in November, 2011. Plant specimen was authenticated in Purwodadi Botanical Garden, East Java. Leaves were air-dried and then chopped into coarsely powder.

**Parasite:** *Plasmodium falciparum* 3D7 (chloroquine-sensitive strain) was obtained from Institute of Tropical Disease-Surabaya.

**Extraction:** Coarsely air-dried powder of *S. pinnata* leaves (501.76 g) was extracted with 7.5 L ethanol 80% using rotary evaporator at 50ºC for 6 hours. Solvent then evaporated at 50ºC to give 81.7 g crude extract (16.28%). Yield of extract (%) was determined according to the weight of starting material.

**Phytochemical analysis**: Detection for the presence of phytochemical constituents (alkaloid, sterol, triterpenoid, saponin, polyphenols and flavonoids) was done using methods previously described (Departemen Kesehatan RI, 1989, Evans WC, 2000, Hakim *et al*., 2010, Jones WP and Kinghorn AD, 2006). Reaction with Dragendorff and Mayer to detect alkaloid, Lieberman-Burchard for sterol and triterpenoid detection, foam test to detect saponin, $FeCl_3$ reaction to detect polyphenol and Shinoda test to detect flavonoid.

**Assay for in vitro antimalarial:** The assay was conducted according to procedure described by Budimulja AS *et al*. (1997). Parasite culture in RPMI 1640 medium (Gibco) was cultivated in 24-culture well plate. Extract was diluted in DMSO and put in culture plate with series concentration of 0.01, 0.1, 1, 10 and 100 µg/mL. The final concentration of DMSO was 0.5%. No presence of extract for untreated control. Plate then placed in 5% $CO_2$ incubator for 48 hours at 37ºC. Blood smear from each well was made, stained with, 20% Giemsa (Merck) and observed by light microscope under oil immersion (Merck). Parasitemia was counted by number of infected erythrocytes per 1,000 erythrocytes. The antimalarial activity was determined by the concentration of extract which able to inhibit 50% of parasite growth ($IC_{50}$), calculated by Probit analysis using SPSS software.

## RESULTS AND DISCUSSION

Phytochemical analysis by color reactions indicate the presence of triterpenoids, polyphenols and flavonoids in ethanolic extract of *S. pinnata* leaves. Result from antimalarial assay was summarized in table 1 and figure 1. Parasite growth was calculated by substraction of % parasitemia after treatment with each concentration of extract for 48 hours to % parasitemia used for antimalarial assay. Parasite inhibition was determined by comparing parasite growth after treatment relative to the parasite growth of untreated control. Result of this study showed that extract could inhibit the parasite growth in dose-dependent manner. The higher concentration of ethanol extract cause lower parasite growth, which is mean the higher inhibition of parasite. The antimalarial activity of this extract which is expressed as $IC_{50}$ value of 0.16 µg/mL. Extracts considered to have potent antimalarial activity when its $IC_{50}$ is fallen under 50 µg/mL (Ramazani *et al*., 2010). Regarding to this recommendation, ethanolic extract of *S. pinnata* leaves can be stated to possess strong and prospective antimalarial activity. Ramazani *et al.* (2010) also showed that previous antimalarial drug, chloroquine, had $IC_{50}$ of 0.4 µg/mL, against chloroquine-susceptible strain of *P. falciparum* (CY27 strain).

Table 1. Results of antimalarial activity of *S. pinnata* extract against *P. falciparum* 3D7

| Concentration of extract (µg/mL) | Parasite growth (%) | Parasite inhibition (%) |
|---|---|---|
| 0.01 | 2.91 | 32.79 |
| 0.1 | 2.75 | 36.49 |
| 1 | 1.48 | 65.82 |
| 10 | 0.75 | 82.79 |
| 100 | 0.15 | 96.65 |
| Untreated | 4.33 | 0 |

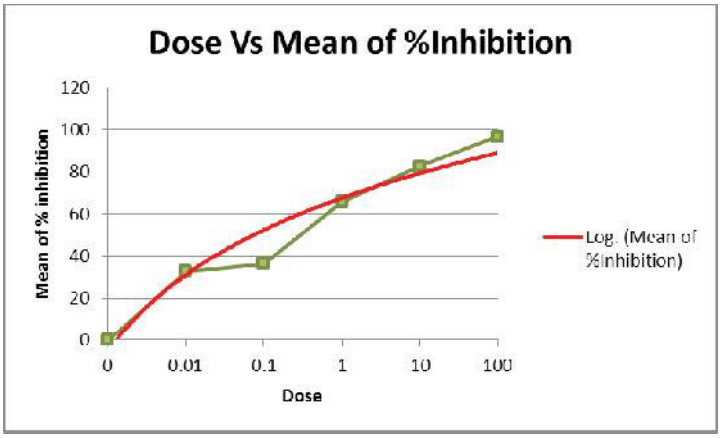


**Figure 1.** Inhibition of parasite after treatment of series concentration of *S. pinnata* extract

Phytochemical constituents in this extract have important role for its antimalarial activity. Type of terpenoids, limonoids, from *Khaya grandifoliola* has been reported to be active against *P. falciparum* (Bickii *et al*., 2000). Several triterpenoids isolated from *Diospyros rubra* were also found to be active as antimalarial (Prachayasittikul *et al*., 2010). Flavonoids are polyphenolic component that responsible for wide range of biological activities (Fotie, 2008). Many flavonoids have displayed their antimalarial activity. Chen *et al*. (1997) reported that Licochalcone A, a class of flavonoid that isolated from Chinese licorice, exhibited strong antimalarial activity of both in vitro against *P. falciparum* 3D7 and Dd2 strain, also in vivo against *P. yoelii* infected mice. Indeed, combination of licochalcone A and artemisinin revealed synergistic antimalarial activity in vitro (Mishra, 2009). Several prenylated flavonoids from *Artocarpus altili*s and *Artocarpus champeden*, both belong to Moraceae family, possessed strong antimalarial activity (Boonphong, 2007; Widyawaruyanti, 2007). Polyphenolic compounds from *Sorindeia juglandifolia* (Anacardiaceae) also exhibited strong antiplasmodial activity both in vitro and in vivo (Kamkuno *et al*., 2012). Crude ethanol 80% extract of *Mangifera indica* from Anacardiaceae family showed moderate antimalarial activity in vitro (Valdes *et al*., 2010).

Result of this research demonstrate that ethanolic extract of *S. pinnata* leaves is potential to be developed as antimalarial. In the next step we will isolate and identify antimalarial components from this crude extract.

## ACKNOWLEDGEMENT

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# STUDY ON PRESCRIBING AND ANTIBIOTIC DOSAGE ACCURACY IN EAR, NOSE AND THROAT CASES PRESCRIPTIONS (CASE STUDIES IN SEVEN PHARMACIES IN DENPASAR IN THE PERIOD OF JANUARY–JUNE 2010)

**Rini Noviyani[1*] and Ida Ayu Alit Widhiartini[2]**
[1]*Department of Pharmacy, Faculty of Math and Natural Sciences, Udayana University*
[2] *Faculty of Medicine, Udayana University*
**Correspondence Author: rini.noviyani@yahoo.co.id*

**ABSTRACT**

Study on prescribing in the case of ear nose throat (ENT) at the 7 pharmacy in Denpasar City has been carried out. The aims of this study were to determine the percentage of incompleteness prescribing administratively, inappropriate dosage of antibiotics, and combination of antibiotic and symptomatic drugs in *pulveres* prescriptions. Data were collected retrospectively and analyzed descriptively on prescriptions ENT doctor served in January-June 2010 period. The ministry of health regulation number 1027 in 2004 and Remington (1990) were used as a standard of prescribing administratively. The antibiotics dose were confirmed to standard IONI book (2008), while combination of antibiotics with symptomatic drugs in *pulveres* prescriptions were also performed. There were 6559 prescriptions found. The incompleteness prescribing administratively were mostly found in patients' Sex (100%), patient's body weight (100%), followed by patient's address (69.17%), drugs dose (24.74%), drug potency (24.7%), doctor license practice (4.34%), prescribing date (3.43%), patient's age (3.33%), doctor's address (2.6%), patient's name (0.38%) respectively. There were 28.50% inappropriate prescription dose of antibiotics oral, consisting of 85.37% *under dose* and 14.63% *over dose*, and topical antibiotics were about 33.93% consisting of 79.86% *under dose* and 20.14% *over dose.* The combination of antibiotic and symptomatic drugs in *pulveres* prescriptions were about 88.27%. The result showed that the incompleteness in prescribing administratively, inappropriate dosages of antibiotic and combination of antibiotic and symptomatic drugs in *pulveres* at the case of ENT were still found.

***Keywords:*** prescription in the case of ENT, antibiotics dosage, *pulveres* prescription.

## INTRODUCTION

The change of orientation in pharmaceutical role from drug-oriented to patient oriented caused the emergence of an idea about pharmaceutical care. This kind of care aims at identifying, preventing and overcoming problems in regards with medicines. Referring to the Governmental Regulation of the Republic of Indonesia No.51/ 2009 concerning pharmaceutical professions, it is stated that pharmaceutical care constitutes the service and direct responsibility of pharmaceutical professions towards patients in relation with pharmaceutical stocks which purpose is to achieve certain results in order to improve the patients' quality of life. In performing pharmaceutical care, any pharmacist is demanded to always improve his/her knowledge, to develop his/her skills and to behave properly so as to be able to communicate well with other medical professions in determining the right therapy to support the rational use of medicament.

One of the various forms of pharmaceutical care in pharmacies is the prescribing monitoring and evaluation. This is done by observing the prescriptions already made by doctors which subsequently become the basic information regarding the accurate and rational use of medicament. The observation on the rational medical prescribing includes the accuracy in indications, medicine selection, usage methods and medicinal dosage, and assessment of the patient's condition. In order to apply the rational prescribing in pharmacies, any pharmacist should perform prescription screening by the rules of administrative prescribing requirements. Problems concerning administrative prescribing are often associated with the results of the omission of information and inappropriate prescribing (Katzung, 1997).

Incomplete prescribing may occur in many cases including in the Ear, Nose and Throat (ENT) prescriptions which are common cases in the population. The frequently occurring ENT case is the Chronic Suppurative Media Autitis (OMSK). In Indonesia, the discovered case of OMSK amounted to 3.8% and the patients constituted 25% of those who were treated in the ENT policlinic of Dr. Sardjito, Yogyakarta in 2004 (Baskett, 2010). Another research in the ENT policlinic of the General Hospital of H. Adam Malik, Medan in 2006 revealed that 26% of the whole patient visits were OMSK patient cases (Aboet, 2007).

The high prevalence of ENT cases results in the high consumption of antibiotics. The outcomes of survey carried out in Dr. Soetomo Hospital, Surabaya and Dr. Kariadi General Hospital, Semarang in 2002 revealed that 83% of the patients obtained antibiotics while the irrational use of antibiotics reached the amount of 60%. The result of assessment on the quality of antibiotic usage at Dr. Kariadi Hospital showed, among others, 9-45% inaccuracy (in terms of dosages, types and duration of use) (Usman, 2008). Inaccurate dosage in the use of antibiotics will increase the occurrence of bacterial resistance against it, causing failure in the treatment (Djuang, 2009).

Another frequently encountered problem in antibiotic usage is the one in which the antibiotics and symptomatic medicines are prescribed in one pulveres. The combination of antibiotics and symptomatic medicines given together in one pulveres becomes irrational in its usage indication because of the difference in each body's needs in order to achieve successful therapy (Hartayu and Aris, 2005). A previous research had been performed in five pharmacies in South Denpasar Regency from April-June 2009 by Prayudeni in regards with a study of pediatric prescribing, which resulted in the percentage of 44.50% in the combined usage of antibiotics and symptomatic medicines in one pulveres.

Based on the above mentioned fact and because of the absence of any research about complete prescribing and inaccuracy of antibiotic dosage as well as combination of antibiotic and symptomatic medicines in one pulveres for ENT cases in Denpasar, it is necessary, therefore, to carry out a research concerning complete prescribing and dosage inaccuracy as well as combination of antibiotics and symptomatic medicines for ENT cases in seven pharmacies in Denpasar.

## MATERIALS AND METHODS

### Materials

The material used in this research consists of all the prescriptions of ENT specialists at seven pharmacies which possess ENT clinics in Denpasar within the period of January-June 2010.

### Methods

The research was performed utilizing descriptive-retrospective approach.

## RESULTS AND DISCUSSION

As an effort in improving the quality of prescribing service in pharmacies, a research has been carried out in regards with the study of prescribing for ENT cases in seven pharmacies in Denpasar. This research was preceded by an investigation concerning the administrative incompleteness of prescribing, then followed by studying the inaccuracy of antibiotic dosage in the already-complete prescriptions and finally judging the existence of the combination of antibiotics and symptomatic medicines in one pulveres. The prescriptions examined within the 6-month period amounted to 6,559 prescriptions which are presented in Table 1.

**Table 1.** Table of the Amount of ENT Case Prescriptions in Seven Pharmacies in South Denpasar

| Total Amount of Prescriptions in 7 Pharmacies | Antibiotic-containing Prescriptions | |
|---|---|---|
| | Single Antibiotic Prescription | Compound Antibiotic Prescription |
| 6559 prescriptions | 5287 prescriptions | 887 prescriptions |

### Administratively Incomplete Prescribing

The study on administrative prescribing based on the criteria of Kep/Menkes/RI/No.l027/Menkes/SK/IX/2004 and Remington (1990) obtained a prescribing figure as presented in Table 2.

**Table 2.** The Study on Administrative Prescribing in Seven Pharmacies in South Denpasar during the period of January-June 2010.

| Criteria | No. | Components of Incomplete Prescriptions | Amount of Incomplete Prescriptions | Percentage |
|---|---|---|---|---|
| Doctor's identity | a. | Name | 0 | 0.00% |
| | b. | Address | 170 | 2.60% |
| | c. | SIP (Practice License) | 285 | 4.34% |
| Date of Prescribing | d. | Date of Prescribing | 225 | 3.43% |
| *Superscripts* | e. | *Superscriptio* (Symbol of R/) | 0 | 0.00% |
| *Inscriptio* | f. | Name of Medicine | 0 | 0.00% |
| | g- | Dosage | 1623 | 24.74% |
| | h. | Potentials | 1620 | 24.70% |
| | i. | Requested Amount | 0 | 0.00% |
| *Subscriptio* | J. | *Subscriptio* (Compounding instructions) | 0 | 0.00% |
| *Signature* | k. | *Signature* (The clear Methods of Usage) | 0 | 0.00% |
| Patient's Identity | 1. | Name | 25 | 0.38% |
| | m. | Age | 219 | 3.33% |
| | n. | Sex | 6559 | 100% |
| | o. | Weight | 6559 | 100% |
| | p. | Address | 4537 | 69.17% |
| Doctor's Signature | q. | Prescribing Doctor's Signature | 0 | 0.00% |

From the table above it can be seen that the highest percentage among the criteria of prescription incompleteness was found in the patient's data inclusion, i.e. the components of patient's sex and body weight which reached 100% respectively.

**Antibiotic Dosage Inaccuracy**

From the ENT (Ear, Nose and Throat) prescription samples observed, antibiotic prescribing was found in most of them. The utilization of antibiotics, as a type of medicine to be used against infectious diseases, must be rational and in accordance with the criteria, depending on the disease's indications, as well as the proper dosage, time interval and duration of therapy. Antibiotic usage is deemed appropriate when the therapy gains maximum effect(s), while the toxic effect(s) in connection with the medicine involved and antibiotic resistance development are minimum. In order to achieve successful antibiotic therapy in bacterial infection cases, the selection of antibiotics must be suitable with the infection-causing micro-organisms and with a dosage that fulfills the supply of medicine inside the human body (Aslam et al., 2003).

Antibiotic therapy can be delivered before obtaining any result of micro-biological examination, based on epidemiologic data, which is called empirical therapy, while antibiotic delivery after micro-biological examination result has been obtained from culture and sensitivity test result is called definitive therapy. The duration of use for empirical therapy is 3-days while the duration of definitive therapy must be as short as possible and not more than 7 days unless there is proof that the use of antibiotics must be prolonged (Suardana et al., 2009). Inappropriate dosage in antibiotic delivery will cause negative effects, such as bacterial resistance against certain types of antibiotics, the increase of medicinal side-effects, or even death. The dosage of antibiotics delivered to any patient is always associated with the size, frequency of delivery, duration of the therapy, and patient's age or body weight. Inaccuracy in prescribing antibiotic dosage is assessed from the size, frequency of delivery and duration of therapy which do not meet the standard, which, therefore, might lead to irrational treatment. In this research, the inaccuracy of oral and topical antibiotic dosages in ENT cases prescriptions as compared with antibiotic dosages recommended by IONI (2008) are categorized into under-dose and over-dose.

The description of antibiotic dose usage in ENT cases as the result of research in seven pharmacies in South Denpasar Regency, which is comparable with antibiotic dosages as recommended by IONI books, can be seen in Table 3.

**Table 3.** Descriptive Table of Antibiotic Use for ENT Cases in 7 Pharmacies in South Denpasar during the period of January-June 2010.

| No | Gol. Antibiotik | Nama Antibiotik | Jumlah Antibiotik Dibandingkan dengan Rekomendasi | | | | | | | | | | | | | | | | Total Antibiotik |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Buku IONI | | | | | | | | | Buku MIMS | | | | | | | |
| | | | UD | | | | TD | OD | | | | UD | | | TD | OD | | | |
| | | | D | F | L | DF | | D | F | L | DF | D | F | L | | D | F | L | |
| 1 | Penisilin | Amoksisilin | 6 | - | - | - | 667 | - | - | - | - | - | - | - | - | - | - | - | 673 |
| 2 | Makrolida | Azitromisin | 7 | - | 5 | - | 124 | - | - | 3 | - | - | - | - | - | - | - | - | 127 |
| | | Eritromisin | 4 | 17 | 2 | 36 | 77 | 1 | - | - | - | - | - | - | - | - | - | - | 140 |
| | | Klaritromisin | 1 | - | - | 2 | 14 | 1 | - | - | - | - | - | - | - | - | - | - | 19 |
| | | Spiramisin | - | - | - | - | - | - | - | - | - | - | - | - | 433 | - | - | - | 433 |
| | | Roksitromisin | | | | | | | | | | | | | 444 | | | | 444 |
| 3 | Kuinolon | Siprofloksasin | - | - | - | - | 103 | - | - | - | - | - | - | - | - | - | - | - | 103 |
| | | Levofloksasin | - | - | 45 | - | 62 | - | 3+2* | - | - | - | - | - | - | - | - | - | 112 |
| | | Ofloksasin | - | - | - | - | 19 | - | - | - | - | 4* | - | - | 13* | 2* | - | - | 23 |
| 4 | Sefalosporin | Cefadroksil | 759 | - | - | - | 128 | 23 | 68 | - | - | - | - | - | - | - | - | - | 1178 |
| | | Sefiksim | - | - | - | - | 16 | 1 | - | - | - | - | - | - | - | - | - | - | 17 |
| | | Sefradin | - | - | - | - | 3 | - | - | - | - | - | - | - | - | - | - | - | 3 |
| | | Sefaklor | 1 | - | - | - | 2 | - | - | - | - | - | - | - | - | - | - | - | 3 |
| 5 | Aminoglikosida dan Polipeptida | Neomisin Sulfat dan Polimiksin B Sulfat | - | - | - | - | - | - | - | - | - | 222* | - | - | 619* | 31* | - | - | 845 |
| | | Framisetin sulfat - Gramisidin | - | - | - | - | - | 20* | - | - | - | - | - | - | - | - | - | - | 20 |
| | | Gentamisin | - | - | - | - | 201* | - | - | - | - | - | - | - | - | - | - | - | 201 |
| 6 | Linkosamid | Linkomisin | - | - | - | - | - | - | - | - | - | 2 | 2 | | 27 | - | - | - | 31 |
| | | Klindamisin | - | 101 | - | - | 126 | - | - | - | - | - | - | - | - | - | - | - | 327 |
| 7 | Amfenikol | Kloramfenikol | - | - | - | - | - | 2* | - | - | - | - | - | - | - | - | - | - | 2 |
| 8 | Sulfonamid dan Trimetoprim | Co-Trimoksazol | - | - | - | - | 10 | - | 3 | - | - | - | - | - | - | - | - | - | 13 |
| 9 | Penisilin dan B-laktam non klasik | Co-Amoksiklav | - | - | - | - | 97 | 60 | - | - | 6 | - | - | - | - | - | - | - | 163 |
| 10 | Amfenikol dan Polipeptida | Kloramfenikol + Polimiksin Sulfat | - | - | - | - | 1* | 14 | - | - | - | - | - | - | - | - | - | - | 15 |
| Total | | | 778 | 118 | 53 | 41 | 2150 | 115 | 76 | 3 | 6 | 228 | 2 | - | 1526 | 33 | - | - | 4297 |

Note: UD = Under-Dose; TD = Therapeutic Dose; OD = Over-Dose; D = Size of Antibiotic Dose; F = Frequency of Antibiotic Delivery; L = Length/ Duration of Antibiotic Therapy; DF = Combination of the Dosage and Frequency of Antibiotic Delivery; (*) Topical Antibiotics (Ear-drop or Nose-drop).

Table 3 indicates that the most frequently prescribed oral antibiotic is from the cefalosphorine category, i.e. Cefadroxil, while the most frequently prescribed topical antibiotic is Polymixine Sulphate, Neomycin Sulphate of aminoglycocide category.

**The Criteria of Antibiotic Dosage Inaccuracy**

**A. Oral Antibiotics**

From research outcomes, it is known that the occurrence of oral antibiotic dosage inaccuracy amounted to 118 antibiotics (22%) out of a total 540 oral antibiotics samples. This obtained figure of 22% oral antibiotic dosage inaccuracy was categorized into 94% of under-dose occurrence details and 6% of over-dose occurrence details. The research outcomes of oral antibiotic dosage inaccuracy were also presented in form of a diagram (Figure 1.).

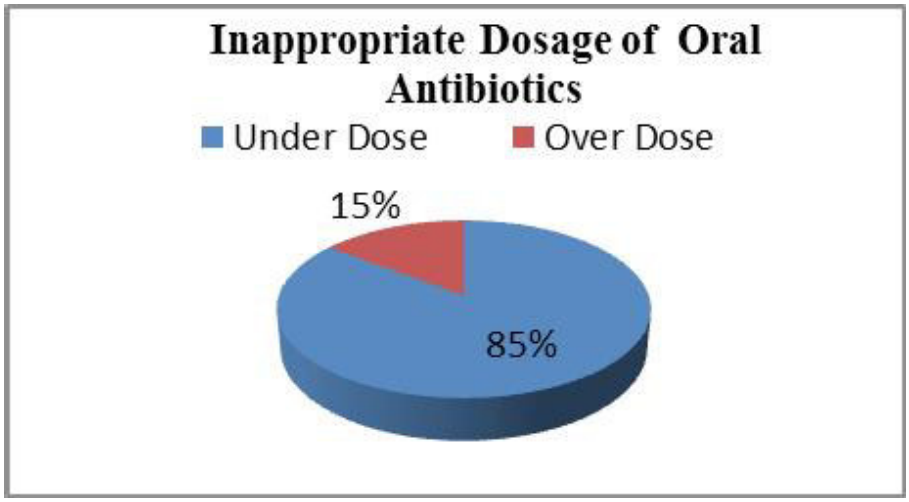


**Figure 1.** Diagram of Oral Antibiotic Dosage Inaccuracy

Figure 1. above shows that the percentage of under-dose category of oral antibiotic dosage inaccuracy is bigger than that of the over-dose category. The under-dose category as was meant by the research was the result of insufficient dosage (involving 778 antibiotics which consisted of Amoxycillin, Azithromycin, Claritromycin, Erythromycin, Cefadroxil and Cefachlor), insufficient frequency (involving 218 antibiotics which consisted of Erythromycin and Clindamycin), insufficient therapy duration (involving 333 antibiotics which consisted of Levofloxacin, Azithromycin and Erythromycin) and insufficient combination of dosage and frequency in antibiotic delivery (involving 41 antibiotics which consisted of Erythromycin and Clarithromycin).

Under-dose medicine delivery might bring failure in therapy. Under-dose prescribing might cause insufficient concentration of medicine in patient's serum than the expected therapeutic range, causing failure in achieving the expected medicinal effects. Delivering antibiotics with insufficient dosage will cause bacterial resistance against them, which in turn will cause great loss to patients (Yasin and Supriyanti, 2009).

Antibiotic over-dose discovered in this research was caused by excessive dosage in delivery (involving 123 antibiotics, which consisted of Clarithromycin, Erythromycin, Cefadroxil, Cefixim and Co-amoxyclave), excessive frequency of delivery (involving 76 antibiotics, which consisted of Cefadroxil and Co-trimoxazole), overlong duration of therapy (involving 3 antibiotics of Azithromycin types) and excessive combination of dosage and delivery frequency (involving 6 antibiotics of Co-amoxyclave types).

**B. Topical Antibiotics**

From the result of the research, the occurrence of topical antibiotic dosage inaccuracy amounted to 18% of 11 antibiotic samples in form of ear-drops. The 18% inaccuracy of topical antibiotic dosage obtained from the research above is all categorized into under-dose occurrence details (100%) with no over-dose occurrence details (0%). The result of the research in topical antibiotic dosage accuracy is also presented in form of a diagram (Figure 2.).

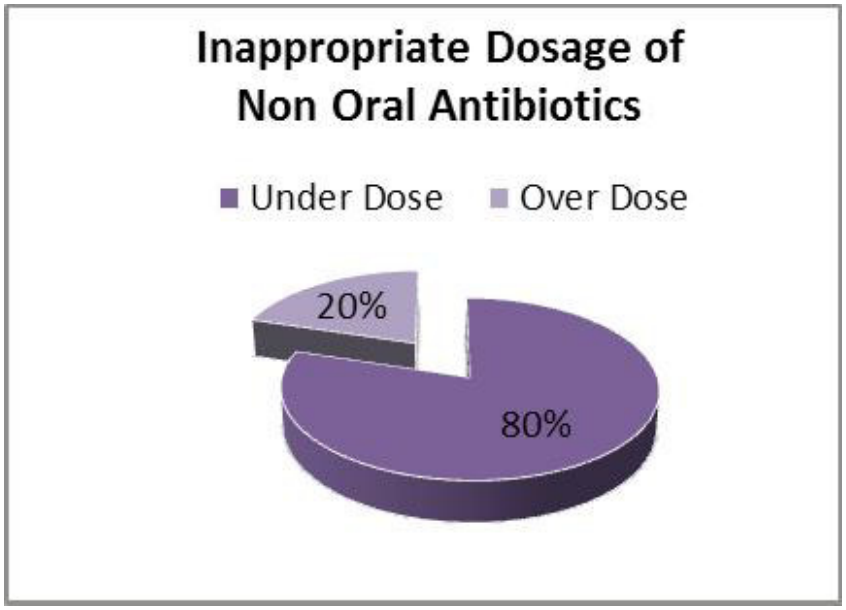


Figure 2. Diagram of Topical Antibiotic Dosage Inaccuracy

The frequently prescribed topical antibiotics in the research of ENT cases prescriptions are Ofloxacin, Neomycin Sulphate and Polymixin Sulphate. Topical antibiotics in form of ear-drops and nose-drops are delivered in order to obtain local effects for bacterial infections which take place around the ear or nose areas. Figure 2. shows that the occurrence of topical antibiotic dosage inaccuracy consisted of under-dose cases because of insufficient dosage (involving 228 antibiotics, i.e. those containing Neomycin Sulphate, Polymixin B Sulphate and Ofloxacin) and over-dose cases because of excessive dosage (involving 33 antibiotics, i.e. those containing Neomycin Sulphate, Polymixin B Sulphate and Ofloxacin).

**The Combination of Antibiotics and Symptomatic Medicines in One Pulveres**

The result of pulveres prescribing calculation in ENT cases research is presented in Table 4 below:

**Table 4.** The Result of Prescribing Calculation for Antibiotic Compounded Medicines and Combination of Antibiotics with Symptomatic Medicines in One Pulveres

| Total Amount of Compound Prescriptions | Non-antibiotic Compound Prescriptions | Antibiotic Compound Prescriptions | |
|---|---|---|---|
| | | Single Antibiotic | Combination of Antibiotics and Symptomatic Medicines |
| 2174 prescriptions | 1555 prescriptions | 104 prescriptions | 783 prescriptions |

From the 887 samples of compound antibiotic prescriptions observed, it was found 88.27% (783 prescriptions) of combined antibiotics and symptomatic medicines in one pulveres. The combination of antibiotics and symptomatic medicines in one pulveres indicates improper methods of usage. The methods of usage are vital for they are associated with the necessary supply of medicine inside the human body in order to achieve therapeutic purposes. Causative medicines, i.e. antibiotics, are needed within a certain time period or duration of therapy; even if the patient's body condition has got better, the consumption of such medicines must be continued so as to maintain its proper supply inside the patient's body. The kind of antibiotic which is always combined with symptomatic medicines in this research is Erythromycin.

For ENT cases, the symptomatic medicines which are frequently used are antipyretics, analgesics, mucolytics and medicines for relieving influenza symptoms. From the result of the research, it is known that symptomatic medicines which were often combined with antibiotics were those for influenza and cough indications. Such symptomatic medicines are only consumed when needed, for instance in the case of antipyretic use for fever, whenever the body temperature has reached the normal level again, its use must be discontinued for it is no longer necessary for the body. If a pulveres contains a combination of causative (antibiotic) and symptomatic medicines which are consumed within the same time period and duration of therapy, this means that the symptomatic medicine's consumption will continue although it is no longer needed by the human body. Such a circumstance might cause unwanted effects and will surely influence the therapy's success.

The high percentage of combination of antibiotics and symptomatic medicines is possibly the result of the factor in which the more medicines are delivered, the less obedient the patients will be in consuming them, so in order to minimize the occurrence of such a case, the prescribing doctor delivered the combination of both types of medicines in one pulveres. In case antibiotic and symptomatic medicines are both needed simultaneously by any patient, the prescribing doctor should deliver both of them separately and the pharmacist who receives the combined medicines' prescription should confirm it to the prescribing doctor.

Nevertheless, it frequently occurs that the pharmacist does not obtain complete data on the patient's condition just as the case in this research, and collecting data retrospectively makes direct improvement difficult to do. in future researches, therefore, prospective observation can be carried out to obtain the prevailing patient's condition at the time when certain medicines are delivered, through direct information and communication among the patient, doctor and pharmacist involved in order to determine certain compound combination responsibly. This process of communication can guarantee the rationality of the patient's treatment therapy.

## CONCLUSIONS

1. The percentage of administratively incomplete prescriptions in ENT (Ear, Nose and Throat) cases in seven pharmacies in Denpasar during the period of January-June 2010 was most frequently found in regards with the components of patient's sex (100%) and body weight (100%), followed by patient's address (69.17%), medicine dosage (24.74%), potential (24.70%), SIP or doctor's practice license (4.34%), prescribing date (3.43%), patient's age (3.33%), prescribing doctor's address (2.60%), and finally patient's name (0.38%). On the other hand, the components of prescribing doctor's name, *superscripts,* medicine's name, medicine's amount, *subscriptio, signatura,* and doctor's signature have all been completely included in the prescriptions.
2. Oral antibiotic dosage inaccuracy in ENT cases prescriptions in the seven pharmacies during the period of January-June 2010 amounted to 28.50%, which is categorized into 85.37% of under-dose cases and 14.63% of over-dose cases, while topical antibiotic dosage inaccuracy amounted to 33.93% which is categorized into 79.86% of under-dose cases and 20.14% of over-dose cases.
3. The percentage of the combined use of antibiotic and symptomatic medicines in one pulveres for ENT cases in the seven pharmacies mentioned above during the same period of time amounted to 88.27%.

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# PRELIMINARY STUDY OF NUTRITION HOUSEHOLD CONSUMPTION MONITORING IN YOGYAKARTA

**Fatma Zuhrotun Nisa**
School of Health and Nutrition, Medical Faculty, Gadjah Mada University, Yogyakarta, Indonesia
Correspondence Author: fatma_znisa@yahoo.com

**ABSTRACT**

Food security remains a problem in Indonesia even in the world. One indicator of the achievement of food security level of the individual / household is nutrition consumption. Monitoring nutrition consumption at the household level is not easy to do so little survey or research on the nutritional intake of the household. This study is a preliminary study on household nutrition consumption which in turn will be used as the basis for the study of household nutrient consumption with broader subjects. The subjects of this study were households in Yogyakarta. Subjects who are willing and interviewed are 56 households. Based on the results it is known that most households (82%) have a family member is less than or equal to 4 members. Almost all family members eat breakfast, lunch and dinner at home. From the consumer survey with 24-hour food recall is known by 41% of households have a low energy intake, 41% of households have sufficient energy intake and 18% of households had a higher energy intake. As for the protein intake is known by 18% of households have a low protein intake, as many as 36% of households have sufficient protein intake and by 46% of households have a high protein intake. Based on these results, a conclusion can be drawn that the nutrition consumption of households in Yogyakarta has not balanced, so we need further studies to better reveal the nutrition consumption of households in Yogyakarta and its influencing factors.

***Keywords***: food security, nutrition, household, Yogyakarta.

## INTRODUCTION

Food insecurity still become problem in Indonesia and it need awareness from stakeholders to think the solution. Food insecurity has been defined by national experts as "limited or uncertain availability of nutritionally adequate and safe foods or limited or uncertain ability to acquire acceptable foods in socially acceptable ways" (Anderson, 1990; Bickel et al., 1999; Nord et al., 1999). One indicator of food security is nutrition consumption especially energy and protein consumption per person. There is correlation between consumption and health. Recent research show that "household energy adequacy per adult equivalent and household dietary diversity score were inversely associated with under nutrition after adjusting for gender, age, puberty, and the interaction between age and puberty. So strategies focused on increasing household energy intake and improving dietary diversity among the most vulnerable households could improve the nutritional health of adolescents" (Cordeiro et al., 2012). Another study reported that "in men, food insecurity was associated with lower intake of energy and energy-adjusted fiber, vitamin C, iron, zinc, and magnesium. In women, food insecurity was associated with a higher intake of carbohydrates and lower intake of fiber, dietary folate equivalent, vitamin C, iron, magnesium, calcium, and vitamin D. For both sexes, when traditional food was consumed, there was a higher intake of protein, protein-related micronutrients, and vitamins A and C and a lower intake of carbohydrates, saturated fat, and fiber and a lower sodium:potassium ratio ($P \leq 0.05$) (Egeland et al., 2011). Monitoring nutrition consumption at the household level is not easy to do so limited survey or research on the nutritional intake of the household. This study is a preliminary study on household nutrition consumption which in turn will be used as the basis for the study of household nutrient consumption with broader subjects.

## METHOD

This study is a preliminary study to describe the general nutrition of households that exist in Jogjakarta. Respondents in the study were residents living in Yogyakarta who is willing to answer questions about nutrition for 24 hours. The number of respondents in this study 56 households. The questionnaire used in this study is kuesiner Nutrition Consumption Monitoring (PKG), which has been used in Indonesia. Interview on intake based on the type and amount of food at the table and the number of family members

who consume these foods. The results of the interview then processed using Food Processor Software to calculate the energy and protein intake of the household. Individual households that consume calories and protein is less than 70% of the average assessed value of a home that food insecurity means problematic in terms of sufficiency of daily food consumption or household deficit of calories and / or protein.

## RESULT AND DISCUSSION

Results in this study include three main variables, namely the number of family members of respondents, the average energy consumption of households and the average household energy consumption.

**Table 1.** Number of Family Members of Respondents

| No | Family Members Categories | Number of Respondents | Percentage (%) |
|---|---|---|---|
| 1 | Less than or equal to 4 members | 46 | 82 |
| 2 | 5 to 7 family members | 7 | 13 |
| 3 | More than 7 members | 3 | 5 |
| | **Total** | **56** | **100** |

Number of family members most of the respondents (82%) are less than or equal to 4 means that most of the respondents belong to the small family (Table 1). Members of the family household consists of a husband, wife, children and other people who live together. Based on the number of household members, the households are grouped into three, namely household small, medium, and large. Smaller households are households whose members number less than or equal to 4 people. Households are households that currently has a membership of between five to seven people, while large households are households with a total membership of more than seven (BKKBN, 1998).

The number of family members has significant effect on the amount of food consumed and the distribution of food consumption among family members. Hunger in large families are more likely to occur than in small families. The majority of households and consumption of food and it is known that the number of poor families with children are much more difficult to meet their food needs, compared to households with children sedikit.lebih further said that households with less food consumption , children under two years more often suffer from malnutrition

The number of family member that got breakfast, lunch and dinner at home was investigated too. It shown that almost all family members eat breakfast, lunch and dinner at home.

**Table 2.** The average energy consumption of respondents

| No | Energi Consumption Categories | Number of Respondents | Percentage(%) |
|---|---|---|---|
| 1 | Less than 70 % | 23 | 41 |
| 2 | 71% - 100% | 23 | 41 |
| 3 | More than 100 % | 10 | 18 |
| | **Total** | **56** | **100** |

The average energy consumption of less than 70% and energy consumption between 71% to 100% by 23 (41%) of respondents. This indicates that most of the respondents had either abnormal energy intake deficiency or excess. If seen from the number of family members of respondents, mostly small family then energy intake should respondents mostly normal or sufficient. This might be due to other factors that affect such income, knowledge of nutrition and food security need to do further study on the factors that influence energy intake of respondents.

**Table 3.** The average protein consumption of respondents

| No | Protein Consumption Categories | Number of Respondents | Percentage (%) |
|---|---|---|---|
| 1 | Less than 70 % | 10 | 18 |
| 2 | 71% - 100% | 20 | 36 |
| 3 | More than 100 % | 26 | 46 |
| | **Total** | **56** | **100** |

The average consumption of protein majority of respondents (46%) more than 100% and only 18% of respondents had a protein intake of less than 70%. Excess protein is not a positive thing and shows that people are resistant food. There is a possibility of the availability of food to other food sources are inadequate, while abundant source of protein. This is not in accordance with the provisions of the general guidelines for balanced nutrition (PUGS) to always consume a diverse diet with balanced amounts.

## CONCLUSION

Based on the results it can be concluded that most households (82%) have a family member is less than or equal to 4 members. Almost all family members eat breakfast, lunch and dinner at home. From the consumer survey with 24-hour food recall is known by 41% of households have a low energy intake, 41% of households have sufficient energy intake and 18% of households had a higher energy intake. As for the protein intake is known by 18% of households have a low protein intake, as many as 36% of households have sufficient protein intake and by 46% of households have a high protein intake. This study indicated that the nutrition consumption of households in Yogyakarta has not balanced, so we need further studies to better reveal the nutrition consumption of households in Yogyakarta and its influencing factors.

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# A COMPARATIVE STUDY ON JEMBRANA DISEASE VIRUS DETECTION METHODS

**Asmarani Kusumawati[1], Tenri Ashari Wanahari[2*], and Issabellina Tampubolon[3]**
*[1]Faculty of Veterinary Medicine, University Gadjah Mada, Yogyakarta, Indonesia*
*[2]Faculty of Medicine, Sebelas Maret University, Solo, Indonesia*
*[3]Biotechnology Study Center, Gadjah Mada University, Yogyakarta, Indonesia*
**Correspondence author: tenriashari@gmail.com*

**ABSTRACT**

Infection of Jembrana disease virus on Bali cattle have caused substantial economic losses for farmers in Indonesia and carrier cattle known as a potential source of transmission. In order to control the spread, examination the health surveillance of the cattle before distribution is should be carried out. In this study we used three different detection methods based on genomic approach, i.e. Reverse Transcriptase-Polymerase Chain Reaction, Reverse Transcriptase-Loop Mediated Isothermal Amplification and Dot Blot Hybridization. Utilization of pGEX-TM, a recombinant plasmid containing env tm gene, as a positive control on detection assay showed that Reverse Transcriptase-Loop Mediated Isothermal Amplification is the most sensitive method. It could detect template concentration up to $10^{-6}$ ng/µl or equivalen to $1.52x10^2$ plasmid copy number, $10^2$ and $10^5$ more sensitive than Reverse Transcriptase-Polymerase Chain Reaction and Dot Blot Hybridization, respectively. Detection assay on infected whole blood also gives the same results as the positive control.

***Keywords***: Jembrana Disease Virus, RT-PCR, RT-LAMP, Dot Blot Hybridization

## INTRODUCTION

Jembrana disease virus (JDV) is a lentivirus associated with an acute disease syndrome on Bali cattle *(Bos javanicus)* in Indonesia. After short incubation, infected cattle show clinical signs of fever, lymphadenopathy and lymphopenia, at which stage high viral titres of $10^8$ infectious units/ml are found in the plasma (Soeharsono *et al.,* 1990; Soesanto *et al.,* 1990). In fatal infection, death is attributed to multisystem involvement. In non-fatal infection regression of lesions commences about 5 weeks post-infection and recovered cattle is resistant to further development of clinical disease (Dharma *et al,* 1991). A recover cattle will develop a delayed antibody respone to the the same or different isolates of JDV but still viraemia with no recurrence for at least 2 years after infection (Soeharsono *et al*., 1996). It roles as a carrier cattle and become a potential source of infection. The etiology of the disease and the mode of transmission in nature are as yet unknown but the disease can be readily transmitted to susceptible cattle by the inoculation of blood, spleen, or lymph node material from infected cattle (Soeharsono *et al.,* 1990). The disease have been spread to other islands in Indonesia such as Sumatra and Kalimantan and it is believed occurred via the distribution of carrier cattle.

In order to control the spread of JDV infection, an examination the health surveillance of Bali cattle before distribution is should be carried out. The method used in JDV detection must give accurate result because it is known there is a possibility to cross react with another bovine lenvirus i.e. Bovine Immunodeficiency Virus (BIV). Several methods have been developed and in this study we describe genomic approach based on method, such as Transcriptase-Polymerase Chain Reaction (RT-PCR), Reverse Transcriptase-Loop Mediated Isothermal Amplification (RT-LAMP) and Dot Blot Hybridization to detect JDV. Those methods are commonly used in detection purpose for many infectious disease. RT-PCR and RT-LAMP works by amplifying the viral template until results in sufficient amount of amplicons which can be detected by electrophoresis while dot blot hybridization works by using a spesific probe, hybridize to certain gene of JDV. The aim of this study is to compare and find which method will give the best result in specificity, sensitivity and simplicity to be applied.

## MATERIALS AND METHODS

### Sample and Material Preparation

Whole blood samples were obtained from carrier cattle on some livestocks in South Kalimantan, Indonesia. Each sample was collected in 5 ml EDTA K2/K3 blood collection tube and storaged in 4°C until

used. In this study we used pGEX-TM, a recombinant plasmid containing JDV env tm gene, for positive control assay. All glassware used were treated with 0.1% Diethyl Pyrocarbonate in water) overnight (12 hours) at 37°C, and then autoclaved or heated to 100°C for 15 min.

**RNA Viral and Positif Control Sample Preparation**

RNA viral was extracted with High Pure Viral Nucleid Acid Kit (Roche®), following the manufacturer's instructions. pGEX-TM was obtained from previous study, propagated in Luria Bertani liquid medium and extracted with GenJet Plasmid Miniprep Kit (Fermentas), following the manufacturer's instructions. RNA and pGEX-TM concentration and quality also were measured by spectrophotometric analysis at 260 and 280 nm. Each RNA sample from whole blood extraction and pGEX-TM was prepared in 10 ng/µl concentration. They were then diluted with double-distilled $H_2O$ RNAse free in a series of 10-fold dilution and used as a template for PCR and LAMP detection.

**Detection by Polymerase Chain Reaction (PCR)**

DNA synthesis from each RNA sample was performed in a 25 µl reaction volume containing 6.75 µl of RNA and 5 µM of B3 primer. RNA mixture was warmed 70°C for 10 min and cooled in ice water for 5 min. A mixture containing 10 mM of dNTP mix (Bioron, Germany), 7.5U of AMV reverse transcriptase (Promega), 1x of AMV reaction buffer, 4U of Protector RNAse Inhibitor (Roche, Germany) and nuclease free water was added to RNA mixture. The mixture was incubated at 37°C for 60 min and terminated at 95°C for 2 min using waterbath (Haake L).

The detection by PCR assay was performed using a DreamTaq™Green PCR Master Mix 2X in a 25 µl reaction volume. The mixture consist of 2 µl each of sample (cDNA or pGEX-TM), 12.5 µl of PCR master mix, 5 µM each of F3 and B3 primer and nuclease free water. The amplification program was performed as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles at 95 °C for 45 s, 58°C for 30 s; 72°C for 45 s and then a terminal extention step of 72°C for 10 min. 5 µ of PCR products then were electrophoresed on 1.8% gel agarose to verify the presence of the expected 211 bp band target.

**Detection by Loop-mediated Isothermal Amplification (LAMP)**

LAMP reaction mixture consist of 1,8 µM each of FIP and BIP primer, 0.2 µM each of F3 and B3 primer, 1.2 mM of dNTP mix, 0.6 M betaine, 10 mM MgSO4, 8 U of *Bst* DNA polymerase, 1x of *Bst* DNA polymerase reaction buffer and 1 µl each of sample (pGEX-TM or RNA). For RNA sample, 0.125U of AMV reverse transcriptase and 4U of Protector RNAse Inhibitor were added on the LAMP reaction mixture. Both LAMP and RT-LAMP was performed in a 25 µl reaction volume, incubated at 60°C for 1 hour and terminated at 80°C for 4 minutes.

**Probe Preparation**

A linierized pGEX TM was used as a template for probe synthesizing with *PCR DIG Labeling Mix* (Roche®). According to the manufacturer's instructions, reaction was performed in 50 µl containing 5 ng pGEX-TM, *PCR DIG labelling mix* (200 µM dNTP, digoxigenine-11-dUTP) (1 ul), 1-5 U Taq DNA polymerase, and 50 pmol each of F3 and B3 primer. PCR program was performed as follows as PCR detection method.

Quantification of probe was performed using *DIG Quantification Teststrips (*Roche). Probes were diluted to obtain 1 ng/µl final concentration. 1 ul of diluted probe were blotted into membrane *DIG Quantification Teststrips (*Roche), dried for 2 m, blocked into blocking solution for 2 m, dipped into antibody solution for 3 m and blocked again into blocking solution for 1 m. The strips then was washed with washing buffer for 1 m, dipped into detection buffer for 1 m and finally incubated in *color-substrate solution* (NBT/BCIP) for 5-30 m. The color signal produced then compared to control quantification on the kit *(*Roche).

**Dot Blot Hybridization and Hybridization Detection**

RNA was diluted into 10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl. Each of sample was denaturated in MOPS buffer, 50% formamide and 2 M formaldehyde at 65°C for 5 m and then placed into ice tube. 1 µl of denatured sample was immobilized on nylon Hybond-N membrane (Amersham Pharmacia). Membran was dried at 50°C for 1 h then crosslinked by UV *crosslinker* for 5 m.

Prehybridization was carried out at 42°C for 3-4 h in prehybridization medium (*buffer* 50% formamide, 2 X SSC, 50 mM sodium phosphate, 2% blocking reagent, 0,1% sodium sarkosyl, 7% SDS), followed hybridization by added 25 ng/ml of DIG labeled probe which had been denaturated at 100°C for 10 min in prehybridization medium. Hybridization was carried out at 45°C overnight. After hybridization, membrane was washed twice with 2X SSC and 0,1% SDS at room temperature for 15 min, and 0,5 X SSC dan 0,1% SDS, at 68°C for 15 min. Hybridization was detected with alkaline phosphatase-conjugated and anti-DIGoxigenin antibody according to the manufacturer's instruction by washed the hybridization membrane with washing buffer I (100 mM maleic acid, 150 mM NaCl pH 7.5, 0.3 Tween 20) for 1-3 min, blocked into *blocking reagent* (100 mM maleic acid, 150 mM NaCl, pH 7,5, 1% *blocking reagent*) for 1 hour, washed twice with *washing buffer* I for 15 min and wash once with *washing buffer* II (100 mM Tris-HCl, pH 9,5, 100 mM NaCl, 50 mM $MgCl_2$) for 15 min. The process was continued by incubation of the membrane into 2 ml *buffer* II containing 7 µL Nitro Blue Tetrazolium (NBT) dan 7 µL solusi 5-Bromo-4-Chloro-3-indolylphosphate (BCIP) (Sigma) in dark room for 2 h to overnight.

## RESULTS AND DISCUSSION

Infection of Jembrana disease virus on Bali cattle have caused substantial economic losses for farmers. Therefore it is very important to identify the presence of this virus to prevent further disease transmission among the Bali cattle or outbreak to other distribution areas. This virus has spread to almost west region in Indonesia (Suharsono *et al*., 1995; Burkala et al., 1999) and has high prevalence more than 50% (Rachmacandran, 1996). In this case, detection method based on genomic approach is an appropriate method because of its accuracy to detect the agent of infection. In this study we described three kind of genomic methods, i.e. Reverse Transcriptase-Polymerase Chain Reaction, Reverse Transcriptase-Loop Mediated Isothermal Amplification and Dot Blot Hybridization, and compared them to each other.

PCR and LAMP assay were carried out firstly by utilization of pGEX TM in 10 fold serial dilution as a control positive template. Both of them give different result. Analyse with electrophoresis on gel agarose showed the presence of the expected 211 bp band target up to concentration $10^{-1}$ pg/µl or equivalen to $1.52x10^4$ plasmid copy number. LAMP products also analysed with electrophoresis on gel agarose but it gives different performace from PCR assay, which known as DNA ladder like pattern. The pattern appear up to concentration 1 fg/ul or equivalen to $1.52x10^2$ plasmid copy number (data not shown). Comparison of both result assumed that LAMP is 100 sensitive more than PCR assay. Application both method on RNA sample give the same results as the control positive sample (Fig. 1 A and B). Quantification of labeling probe showed that probe could detected up to concentration of 3 pg/ul (data not shown). Based on the dot comparison between probe and the standard of the Roche ®, it is obtained that probe have a high labeling efficiency. This information then used to determine the amount of probe in hybridization process. Hybridization was carried out on different concentration of RNA sample, ie. 1 ng/ul, 100 pg/ul and 10 pg/ul. From five sample (II-IV fig. C), probe could detect up to 10 pg/µl for control positive sample and up to concentration100 pg/ul for RNA sample

From all methods, it is known that RT-LAMP is the most sensitive method for JDV detection. The sensitivity up to $10^2$-$10^5$ times with spesific results. Its simple procedure on isotermal temperature, which can carried out on a simple waterbath, make this method is appropriate to be applied in field condition which has minimal facility, replaces PCR as the common method for detection purpose. Beside of its sensitivity, it is known that this method also has high specificity and rapidity. The specificity due to the utilization of 2 or 3 primer pairs in the process, if there is no one of the pair, the amplification would not

work (Notomi et al., 2000), while the rapidity due to there is no time losses during the process because of temperature changing as in PCR (Parida et al., 2008). Dot blot hybridization generate specific results because of utilization of specific probes but this method is time consuming and very laborious.

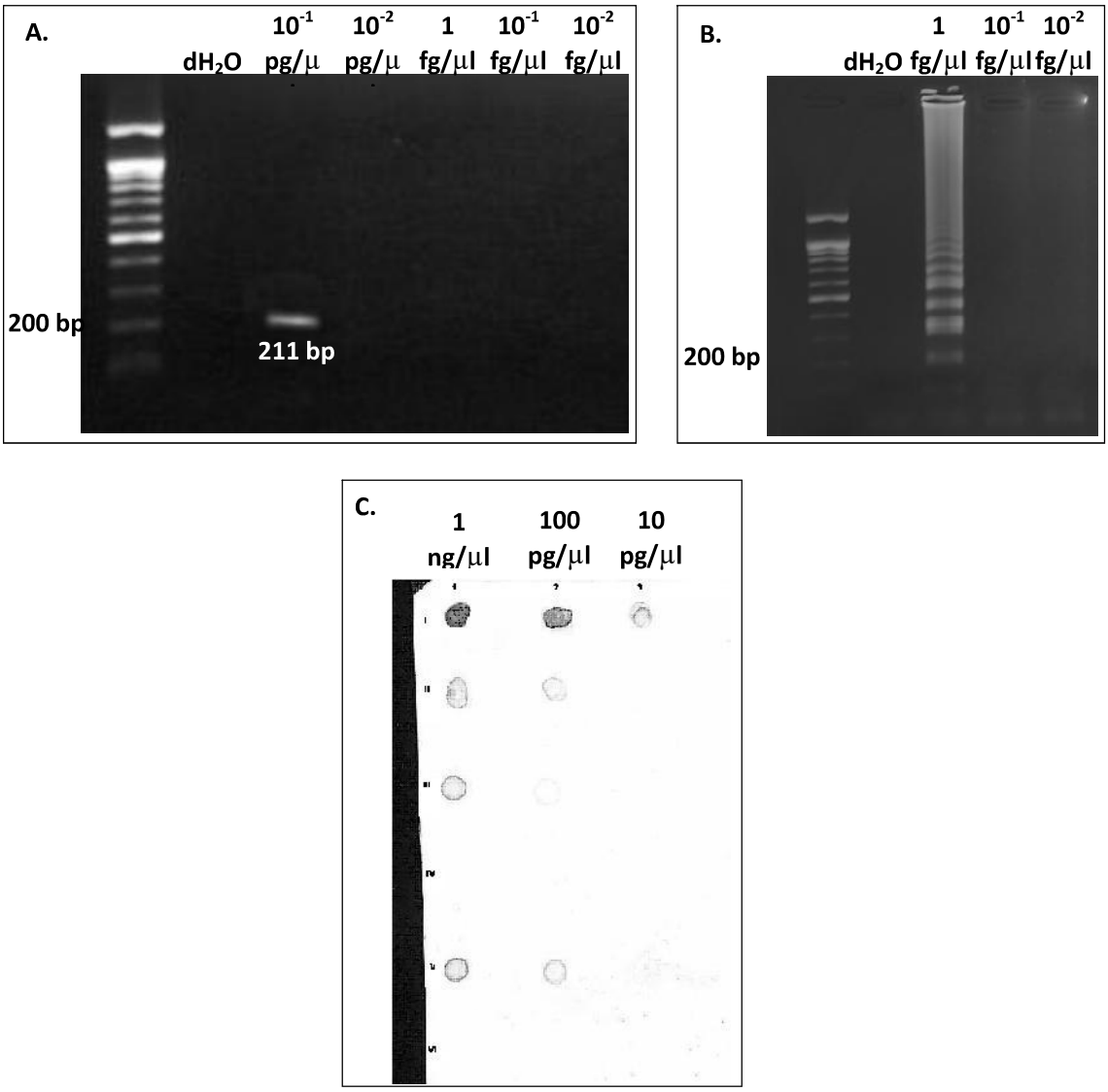


**Figure 1.** Comparison of RT-PCR (A) RT-LAMP (B) and Dot Blot Hybridization (C) on RNA sample

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# *IN VIVO* ANTIMALARIAL ACTIVITY OF ETHANOLIC EXTRACT OF *Spondias pinnata* (L.F) KURZ. LEAVES

**I Wayan Dwisada Purnamayadi[1], Wayan Ria Medisina[1], Ni Made Suartini[2], and Ni Putu Ariantari[1*]**

*[1]Pharmacognosy and Phytochemistry Laboratory, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Udayana University. Address: Jalan Kampus Bukit-Jimbaran, Department of Pharmacy, AF Buiding, Badung, Bali-Indonesia 80364, Telp/Fax: 0361-703837*

*[2]Department of Biology, Faculty of Pharmacy, Faculty of Mathematics and Natural Sciences, Udayana University, Bali-Indonesia*

*Corresponding author: ari_dedhika@yahoo.com*

**ABSTRACT**

Malaria is an infectious diseases caused by *Plasmodium* parasite. The major problem in malaria control is the appearance of parasite resistance to available antimalarial drugs. Any efforts in antimalarial drugs discovery and development are urgently required. One of the resource is natural products. *Spondias pinnata* (L.f.) Kurz. belongs to Anacardiaceae, commonly known as "Kedondong Hutan" has been traditionally used to treat fever. The objective of this study was to determine TLC profile and evaluate *in vivo* antimalarial activity of 80% ethanolic extract of *S. pinnata* (L.f.) Kurz. leaves. The study began with extraction and determination of TLC profile of *S. pinnata* (L.f.) Kurz. leaves extract using silica gel $GF_{254}$ plate as a stationary phase and chloroform:methanol (92,5:7,5 v/v) as a mobile phase. Extract was further assessed for its antimalarial activity in *in vivo* against *Plasmodium berghei* ANKA infected mice. Percentage of parasitemia was observed for seven days. Data was then analyzed using *one-way* ANOVA and Tukey. TLC profile of extract indicated the presence of flavonoids and terpenoids. Spots with Rf of 0.14, 0.18, 0.65 and 0.84 were suspected as flavonoids and spot with Rf of 0.29 was suspected as terpenoid. Result of antimalarial activity study revealed that ethanolic extract of *S. pinnata* (L.f.) Kurz. leaves gave significant reduction in parasitemia, with percentage of inhibition of 23.07%, after treating animals with oral dose of 100 mg/kgBW.

***Keywords:*** *Spondias pinnata* (L.f.) Kurz., 80% ethanolic extract, *Plasmodium berghei* ANKA, antimalarial.

# EXTRACT ETHANOL SEAWEEDS LOWERING LEVEL OF SERUM HMG-COA REDUCTASE ENZYME OF WISTAR RATS HYPERCHOLESTEROLEMIA

**Ketut Sri Marhaeni Julyasih[1*] and I Gede Putu Wirawan[2]**
*[1] Faculty of Agriculture UPN "Veteran" East Java,Indonesia*
*[2] Faculty of Agriculture, Udayana University Denpasar, Indonesia*
**Correspondence Author: smjulyasih@gmail.com*

**ABSTRACT**

Patients with hypercholesterolemia usually require a prolonged treatment; and the newer and more potent generation of antilipid agents are costly. Therefore the use of materials derived from seaweed, as well as good diets, also has other benefits. In Bali there are several types of seaweed that are generally consumed by the local people and known by the local names of *Bulung Boni* (*Caulerpa* spp.) and *Bulung Sangu* (*Gracilaria* spp.). Until this time, the effect of *Bulung Boni* and *Bulung Sangu* extracts to reduce the level of HMG-CoA reductase enzyme are still very limited, and further investigations were considered relevant and needed. This study used completely randomized design, using a total of 24 Wistar rats divided into six sample groups of equal size, all fed with a diet high in cholesterol content. The six sample groups were respectively designated as negative control, positive control, and four treated sample groups and respectively fed orally with a dose of 20 mg and 60 mg extracts of *Bulung Boni* per 100g of body weight per day, and 20 mg and 60 mg extracts of *Bulung Sangu* per 100g body weight per day. Our study showed that rats fed with high-cholesterol diet and treated with oral *Bulung Boni* or *Bulung Sangu* extract at a dose of 20 mg and 60 mg/100 g bw/ day statistically significant decreased serum HMG-CoA reductase enzyme levels ($p < 0.05$) when compared to those fed with high cholesterol diet without being treated with *Bulung Boni* or *Bulung Sangu* extracts.

***Keywords*** : *Caulerpa* spp., *Gracilaria s*pp., enzyme HMG-CoA reductase, hypercholesterolemia

# BIFIDOGENIC NATURE OF INDIGENOUS BALINESE ROOT CROPS

**I N. Sujaya[1,4*], N.L.K. Aquarisnametri[1], K.A. Nocianitri[2,4] and Yan Ramona[3,4]**
*[1]Study Program Public Health, Faculty of Medicine, Udayana University*
*[2]Department of Food Technology, Faculty of Agricultural Technology, Udayana University [3]Department of Biology, Faculty of Math and Natural Sciences, Udayana University*
*[4]UPT Lab. Terpadu Biosain dan Bioteknologi, Udayana University, Denpasar, Bali, Indonesia*
**Correspondence Author: sakabali@hotmail.com*

**ABSTRACT**

Severals methods have been conducted in order to stimulate the growth of Bifidobacteria in the gastrointestinal tract. Food containing prebiotic compunds are of interest targets to develop an healthier gut of human. Tubers and root crops are the natural prebiotics resources in the tropics. Nevertherless, it was limited number of researches have been conducted to explore the prebiotics effects of the natural tubers in Indonesia. This research was aimed to evaluate the potency of indigenous tubers in modulating the health promoting bacterium, *Bifidobacterium breve* JCM 1273. Nine (9) types of Balinese indgienous tubers were studied for their capabilities to promote the growth of *B breve* JCM1273. The tuber extracts were prepared by water extraction then were used as a carbon sources in TOS modified medium. The growth of *B. breve* JCM1273 was measured by determining its turbidity at 660nm, while the hydrolizing products of *B. breve* JCM1173 was determined by TLC. The results showed that the crude extract of *gadung* showed the best fermentation activities and resulted the best growth of *B. breve* JCM 1273. The pH of the medium was 3,97 while the terminal $OD_{660\,nm}$ was 1.1057 and these values did not show significant differences with gucose (control treatment) with its pH and $OD_{660nm}$ values were 3,70 and 1,2670, respectively. The growth of *B. breve* JCM 1273 was better in ethanol extracts obtained from *gadung* than the other tubers. The fermnetaion activities of *B breve* JCM1273 producing a distinct spot on TLC. This implied that *gadung* might be containing poli/oligo sachharide, which could be hydrolyzed by *B breve* JCM1273. Thus, *gadung* might be a source of prebiotics and this fact need to be clarified through *in vivo* experiment.

***Keywords***: *Bifidobacterium,* tuber extracts, Prebiotic, Bifidogenic

# DETECTION OF JEMBRANA DISEASE VIRUS AMPLICON BY LATERAL FLOW (IMMUNO) ASSAY

**Asmarani Kusumawati[1], Issabellina Tampubolon[2*], and Tenri Ashari Wanahari[3]**
[1]Faculty of Veterinary Medicine, University Gadjah Mada, Yogyakarta, Indonesia
[2]Biotechnology Study Center, University Gadjah Mada, Yogyakarta, Indonesia
[3]Faculty of Medicine, University of March, Solo, Indonesia
*Correspondence author: issabel.icha@yahoo.com

**ABSTRACT**

Amplification techniques for detection of Jembrana Disease Virus infection on Bali cattle commonly use agarose gel electrophoresis to detect and identificate the amplicon, but now it can be done simply by using Lateral Flow (Immuno) Assay. It is an assay based on nucleic acid hybridization on the dipstick membrane. A signal amplification system in this assay allows visual detection which can be used to detect amplicon from various amplification procedures. It offers a simple, safe and cost-effective method, an alternative to replace agarose gel electrophoresis or other highly complex detection methods. In this study, Lateral Flow (Immuno) Assay which consist of ligand biotin and gold particle conjugated to anti FITC antibody was used to detect the amplicon of Reverse Transcriptase-Loop-mediated Isothermal Amplification from Jembrana Disease Virus. A biotin labeled primer was used in amplification process so that the amplicon can be detected by the recognition system on the dipstick membrane of Lateral Flow (Immuno) Assay. Amplicon hybridized with a complementer 6-Carboxyfluorescein labeled probe at 61$^{o}$C for 30 minutes, then a dipstick membrane dipped into this solution and incubated for 5 minute. In this process, biotin at one of the hybrid end will captured by the ligand biotin meanwhile 6-Carboxyfluorescein labeled probe will captured by antibody anti FITC, make the colloidal gold particle to concentrate and form a visible line on the test zone as a positive signal hybridization. This method hopefully will increase the availability of genomic screening in resource-limited settings and its applicability in field.

***Keywords***: Lateral Flow (Immuno) Assay, Jembrana Disease Virus, Nucleic Acid Hybridization

# AQUEOUS EXTRACT OF BALINESE PURPLE SWEET POTATO (*Ipomoea batatas* L.) PREVENTS OXIDATIVE STRESS AND DECREASES BLOOD INTERLEUKIN-1 IN HYPERCHOLESTEROLEMIC RABBITS

**I Made Jawi and Agung Wiwiek Indrayani**
*Department of Pharmacology, Faculty of Medicine, Udayana University*

**ABSTRACT**

Hypercholesterolemia can cause oxidative stress, endothelial disfunction and atherosclerosis. Anthocyanin can prevent oxidative stress, it will decrease the inflammation by decreasing the proinflammatory cytokine in animal models. Purple sweet potato in Bali have been proven of having high anthocyanins content. The aim of these study was to prove the ability of aqueous extract of Balinese purple sweet potato (*Ipomoea batatas* L.) in decreasing the blood interleukin-1 level in hypercholesterolemic rabbits. Subjects of this study were 18 male adult rabbits divided into 3 groups with randomized post-test only control group design. Group 1 rabbits were given standard diet as a control group. Group 2 rabbits were given high cholesterol diet. Group 3 rabbits were given aqueous extract of Balinese purple sweet potato 4 ml/Kg.BB/day and high cholesterol diet. After 60 days of treatment, the blood level of total cholesterol, MDA and interleukin-1 were measured as post test examination. The data were analysed by using anova. The results showed significant increases of blood total cholesterol, MDA, and interleukin-1 in group 2 ($p<0.05$). In group 3 the increases of blood total cholesterol and MDA levels were sligthly lower than the group 2 ($p<0.05$). Group 3 also showed significantly lower of blood interleukin-1 levels than those in group 2 ($p<0.05$). From this findings, it can be concluded that aqueous extract of Balinese purple sweet potato can decrease oxidative stress and decrease the level of interleukin-1 in hypercholesterolemic rabbits.

***Keywords:*** Balinese purple sweet potato, oxidative stress, IL-1, hipercholesterolemic rabbits

# ORAL PRESENTATIONS: AGRICULTURE

# THE USE OF CHITINO-PROTEOLYTIC ACTINOMYCETES AS BIOLOGICAL CONTROL AGENS FOR ROOT-KNOT NEMATODES *Meloidogyne* sp.

**Bambangrahayu Trisno Pujiastomo[1], and Donny Widianto[2]**
*[1]Plant Pest and Diseases Department, Faculty of Agriculture, Gadjah Mada University, Bulaksumur-Yogyakarta.*
*E-mail: bambangrahayu@yahoo.com*
*[2]Microbiology Department, Faculty of Agriculture, Gadjah Mada University, Bulaksumur-Yogyakarta*

## INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) parasitize a wide range of annual and perennial crops, often impacting both the quantity and quality of marketable yields (Sasser and Carter 1985; Tsay, 1997; Yan *et al.,* 2011). These pests are also considered to be the most economically important plant-parasitic nematodes. Nowadays, the most used method for root-knot nematodes control is the utilization of chemical agents, causing of significant environmental pollution (Sasser, 1979; Adegbite and Agbaje, 2007). Social concern has generated the search for alternative control systems (i.e., biological control), which contribute to the generation of sustainable agricultural development (Van Gundy, 1985; Kerry, 1987; Sikora, 1992). Interactions among the different organisms are the natural bases of biological control. Biological control is becoming more important because the use of methyl bromide as a soil fumigant for the control of nematodes, in agriculture and horticulture, was banned in 2001 due to its adverse environmental effects (Gortari and Hours, 2008). Substantial economic losses can be caused by root-parasite nematodes. These nematodes invade the root and partially reorganize its function to satisfy their nutritional demands for development and reproduction (Anonim, 2012; Sijmons,1994; Hussey and Grundler,1998; Davis, 2000; Jasmer *et al*., 2003; Hussey and Williamson 1998; Bird, 2000). The nematode egg is an important stage of the root-knot nematodes life cycle both from the perspective of development of the nematodes and the potential target for control strategies. Thus, the structure of the egg is important from both points of view: the development of the parasite, and its control. Depending on the taxonomic order, the eggshell of root-knot nematode is composed of three layers. The outer is protein layer and the inner layer is lipid. The chitin of the middle layer is responsible for the structural strength of the eggs (Bird and Bird, 1991), and it is susceptible to enzymatic degradation. Interestingly, chitin is present only in the eggshell of nematodes, and for this reason it is the target of possible control strategies in this research.

Actinomycetes have important characteristics for biological control of root-knot nematode and plant growth promoting agents, due to their capacity to produce spores, antibiotics, enzymes with chitinolytic, proteolytic and chitino-proteolytic activity, plant growth promoting substances and to act as organic matter decomposers (Keiser *et al*., 2000). These microorganisms have an important role in the degradation of polymers such as chitin (Crawford *et al*., 1993).

## MATERIALS AND METHODS

Materials in this experiment were Root-knot nematode (*Meloidogyne* sp.) eggs from culture, soil samples of paddy rice, compos and scrimb/ petis industry waste. Seed of tomatos local variety Kaliurang Tugu Jogja brand, Yogyakarta. Chemicals, chlorox for root-knot nematode eggs isolation, FAA fixative solution, Lactic acid, glycerine and acid fuchsin lactophenol, aquades. Chitin agar, skim milk agar and "pati agar"

A survey was conducted to determine the Actinomycetes with chitinolytic, proteolytic, and chitino - proteolytic activity in several areas in Yogyakarta, Central Java and East Java. Samples were collected from rhizosphere and shrimp waste. The actinomycetes were grown on chitin agar (Hsu and Lockwood, 1975) at room temperature and were purified. The purified isolates were then kept in "pati agar" (Joetono *et al*., 1980).

The activity of crude actinomysetes extracellular hydrolytic enzymes was tested againts root-knot nematode eggs *in vitro*. Isolates of actinomycetes with hydrolytic activity were selected, and the crude enzymes capable of hydrolyzing eggshell and killing second stage juvenile were further evaluated (*invitro*). The experiment were observed at 16 hrs and 32 hrs.

The experiment under mezocosmos condition conduct for two months. Root-knot nematode identification base on perineal pattern (Taylor and Sasser 1978). Seedling of tomatos were planted on steam sterilized soil in plastic bags, three weeks later were then inoculate with 1000 root-knot nematode eggs per plastic bag and $10^6$, $10^7$ and $10^8$ of selected actinomycetes spores (PSJ-27, TL-8 dan TL-10) as Completely Randomized Design in green house, replicate three time. The rating of root galls base on Zeck techniques (1971), second larvae stage population were extract/isolation from root by Baermann funnel sprey modification technique and second larvae stage from soil (100 ml) population were extract by Whitehead tray modification technique. The data were analyzed by Duncan Multiple Range Test at 95% significant level and the the root damage level were analyzed by non parametric Kruskal Wallis (Conover, 1981)

## RESULTS AND DISCUSSION

A total of 84 actinomycetes isolates were obtained (Table 1.). Which the soil with high organic matter from animal waste are good for actinomycetes habitat and have ability to protein hydrolized (Richa *et al*. 2009). So the soil waste from scrimb/ petis Sidoarjo have protein and chitin matter. That condition as good habitat for chitino proteolytic actinomycetes.

**Table 1.** The total isolates base on ability protein and chitin hydrolysis

| Isolates source | Total isolates | Activities | | |
|---|---|---|---|---|
| | | chitinolytic | proteolytic | chitino-proteolytic |
| 1. *Ganoderma lucidum* waste from Pare, Kediri | 5 | 0 | 0 | 5 |
| 2. Paddy rice soil from north Klaten | 4 | 0 | 0 | 4 |
| 3. Stardec | 6 | 1 | 0 | 5 |
| 4. Compos | 1 | 1 | 0 | 0 |
| 5. Paddy rice soil from Tulung, Klaten | 12 | 0 | 2 | 10 |
| 6. Paddy rice from Ngawen, Klaten | 3 | 0 | 0 | 3 |
| 7. Scrimb/ petis Industrial waste from Prambon, Sidoarjo, East Java | 26 | 5 | 0 | 21 |
| 8. Rhizosphere soil of tomatos from Wonocatur, Yogyakarta | 3 | 2 | 0 | 1 |
| 9. Scrimb soil waste from Sareman, Bantul, Yogyakarta | 10 | 2 | 4 | 4 |
| 10. Paddy rice soil from Kulon Progo | 3 | 3 | 0 | 0 |
| 11. Paddy rice soil from Yogyakarta Special Region | 5 | 5 | 0 | 0 |
| 12. Paddy rice soil from Bantul | 2 | 2 | 0 | 0 |
| 13. Paddy rice soil from Sleman | 2 | 2 | 0 | 0 |
| 14. Trasi (fish-paste) soil waste | 2 | 2 | 0 | 0 |
| J u m l a h | 84 | 25 | 6 | 53 |

Selection based on the ability to hydrolyze chitin and protein in the medium, resulted 14 isolates (Table 2.) which had ratio of clear zone diameter and colony diameter more than two.

**Table 2.** Isolates which had ability to hydrolyze chitin and protein with ratio clear zone (Halo) diameter and colony diameter khitin and protein ≥ 2

| No. | Isolates | Ability to hydrolyze * | |
|---|---|---|---|
| | | chitin | protein |
| 1. | TL-6 | 2 | 2,15 |
| 2. | LUB-8 | 2,33 | 3 |
| 3. | **TL-10** | 2,69 | 3,06 |
| 4. | PSJ-36 | 3 | - |
| 5. | PSJ-28 | 3,2 | - |
| 6. | SD-2 | 3,5 | - |
| 7. | PSJ-47 | 4,29 | - |
| 8. | PSJ-46 | 4,67 | - |
| 9. | IK | 5,6 | - |
| 10. | **PSJ-27** | 5,67 | - |
| 11. | LUB-4 | - | 4,67 |
| 12. | LUB-9 | - | 3,07 |
| 13. | TL-7 | - | 4,23 |
| 14. | **TL-8** | - | 4,85 |

Note:calculated using the formula: φ halos / φ isolate colony
TL= paddy rice soil from Tulung; LUB= Scrimb soil waste from Sareman, Bantul; PSJ= Scrimb/ petis Industrial waste from Prambon, Sidoarjo tanah; SD= stardec; IK= Compos inoculum. The number after a letter are isolate number.

Isolates of actinomycetes PSJ-27, TL-8, and TL-10 had high hydrolytic activities for chitin and protein substrates. Those isolates original come from soil waste produc of industrial petis Sidoarjo, east Java and paddy rice soil in Klaten, midle Java which have high organic matters contain. That condition is good for habitat of actinomycetes which have high ability to hydrolyzed of chitin and protein.

The crude enzymes from those isolates were then tested to show the activity in preventing hatching and killing the juvenile (Table 3.). The result showed some variation among isolates. At 16 hours after inoculation, TL-10 isolate with chitino-proteolytic stimulated the eggs hatching prematurly (30.33%) and killed 50.54% juveniles; whereas PSJ-27 isolate having chitinolytic activity reduced egg hatch (15%), killed 40% juveniles and TL-8 isolate having proteolytic activity caused 19% hatching and killed 45.58% juveniles. Furthermore, at 32 hours after inoculation, TL-10 isolate resulted highest mortality of juveniles (92.40%) followed by TL-8 (61.26%) and PSJ-27 (25.81%). All isolates had ability to inhibit egg hatching.

The PSJ-27, TL-8 and TL-10 isolates give the highest mortality effect among their hydrolytic activities groups (chitinolytic, proteolytic and chitino-proteolytic). Both TL-8 and TL-10 isolates caused eggshell damage, with TL-10 isolate showed higher activity.

Three actinomycetes isolates with high pathogenicity in vitro due to their chitinolytic, proteolyric and chitino-proteolytic activity were further tested their efficacy in the greenhouse. The result showed all isolates were able to reduce root-knot nematodes population and galls formation (Table 4.).

**Table 3.** Efect of crude enzumes to nematode eggs hatching and root-knot nematode juvenile killed

| Isolates | Eggs hatching (%) | | Juvenile stadium-2 (%) | |
|---|---|---|---|---|
| | 16 hrs | 32 hrs | 16 hrs | 32 hrs |
| TL 6 (kp) | 22,00 b | 26,33 e | 0,00 h | 82,29 c |
| LUB 8 (kp) | 21,00 b | 24,66 f | 30,14 d | 90,56 b |
| TL 10 (kp) | 30,33 a | 30,66 d | 50,54 a | 92,40 a |
| PSJ 36 (k) | 11,33 f | 21,66 g | 17,65 g | 21,55 j |
| PSJ 28 (k) | 14,66 d | 42,00 b | 20,46 f | 13,49 i |
| SD 2 (k) | 6,66 i | 48,00 a | 34,98 fg | 13,19 i |
| PSJ 47 (k) | 11,33 f | 42,00 b | 44,13 e | 22,22 fgh |
| PSJ 46 (k) | 11,00 fg | 38,00 c | 45,45 e | 25,44 fg |
| IK (k) | 14,00 d | 42,33 b | 35,71 e | 21,26 gh |
| PSJ 27 (k) | 15,00 d | 41,33 b | 40,00 d | 25,81 de |
| LUB 4 (p) | 9,33 gh | 41,00 b | 21,44 g | 27,64 d |
| LUB 9 (p) | 9,00 h | 22,33 g | 29,55 fg | 38,81 h |
| TL 7 (p) | 13,33 de | 16,66 h | 52,51 c | 60,02 ef |
| TL 8 (p) | 19,00 c | 37,00 c | 45,58 b | 61,26 b |
| Kontrol Aquades | 12,00 ef | 46,66 a | 0,00 h | 0,00 k |

Note: (kp)= chitino-proteolitic; (k)= chitinoklitic; (p)= proteolitic.
Number in the same colom followed by same a letter indicated non significant.

**Table 4.** The degree tomato root damages by root-knot nematodes

| Isolates | Spores density | Damage Scale |
|---|---|---|
| PSJ-27 | $10^6$ | 5± 0,57 |
| PSJ-27 | $10^7$ | 4± 0 |
| PSJ-27 | $10^8$ | 3± 0,57 |
| TL-8 | $10^6$ | 4± 0,57 |
| TL-8 | $10^7$ | 4± 0,57 |
| TL-8 | $10^8$ | 2± 0,57 |
| TL-10 | $10^6$ | 4± 0 |
| TL-10 | $10^7$ | 3± 0,57 |
| TL-10 | $10^8$ | 1± 0,57 |
| Control 1 | Inoculate with nema eggs | 8± 0,57 |
| Control 2 | No eggs and actino spores | 0 |

The rating of root galls base on Zeck techniques (1971)

TL-10 isolate which had chitino-proteolytic activities showed the best performance in controling root-knot nematodes, and reduced root galls formation. Vigor of tomato treated with these isolates were significantly improved. No differences in root weight among all treatments were noted. The higher spores density the more effective in suppressing root-knot nematodes population and root galls development. These findings revealed that TL-10 isolate was effective for biocontrol agents of root-knot nematodes.

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# Micropropagation of *Vanda tricolor* Lindl. var. *suavis* forma Bali Carrying *KNOTTED1- LIKE Arabidopsis thaliana* (*KNAT1*) Gene

**Rindang Dwiyani [1*], Aziz Purwantoro[2**], Ari Indrianto[3***], and Endang Semiarti[3****]**
[1]*Faculty of Agriculture, Udayana University, Denpasar (Bali), Indonesia*
[2]*Faculty of Agriculture, Gadjah Mada University, Yogyakarta, Indonesia*
[3]*Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia*
**rindangdwiyani@yahoo.co.id*
***ronsasm@hotmail.com*
****ariindri@gadjahmada.edu*
*****endsemi@ugm.ac.id*

**ABSTRACTS**

Regeneration rate of orchid's explant in micropropagation is relatively low, that need to be improved. In this research, genetic engineering with the insertion of *KNOTTED1-LIKE Arabidopsis thaliana* (*KNAT1*) gene was conducted to improve the cell totipotency. Objectives of the research was to ensure that the *35S::KNAT1* transformant has highly totipotency cells, so that it can produce higher number of propagules in micropropagation. The *35S::KNAT1* transformant of *V. tricolor* was obtained through *Agrobacterium* - mediated transformation. Organs such as leaves and shoots were sliced and grown on the NP medium added with 5µM 2i-P and 0,15 µM NAA. The number of propagules was observed after 6 weeks. The results showed that the *35S::KNAT1* transformant of *V. tricolor* produced higher number of propagules *in vitro* compared to those of WT. The number of propagules produced from the *35S::KNAT1* transformant increased by 68 times compared that of WT. No propagules was produced from the explant of the based of leaf of WT plant compared to 30 propagules that can be produced from those of the *35S::KNAT1* transformant.

***Keywords:*** Micropropagation, *KNAT1* gene, transformant, Wild Type, propagules

## INTRODUCTION

Regeneration rate of orchid's explants in micropropagation is relatively low, that need to be improved. Kanjilal *et al*. (2009) investigated on *Dendrobium* orchid, the number of propagules per explant was range from 1 until 8 depended on hormone used on the medium. Preliminary research by the author found that low number of propagules was produced per explant either from leaves or shoots on *Vanda tricolor* orchid (data not shown). The current research tried to increase the regeneration rate of explant from *V.tricolor* orchid using genetic engineering i.e. gene insertion.

*KNOTTE1-LIKE Arabidopsis thaliana* (*KNAT1*) gene is a key regulator for other *KNOX* (*KNOTTED1-LIKE HOMEOBOX*) gene at shoot apical meristem (SAM) and together with other *KNOX* gene keep SAM in the meristematic state (Scofield *et al.,* 2008). Overexpression of *KNAT1* gene on *Phalarnopsis amabilis* orchid resulted in multiple shoots coming out from a protocorm (Semiarti *et al.,* 2007). Ectopic meristem was produced on leaves with overexpression of *KNAT1* gene on Nicotiana tabacum (Chuck *et al*., 1996) as well as on *Lactuca sativa* (Frugis, *et al.,* 2001). Overexpressed of *KNAT1* gene on *V.tricolor* orchid was done in the current research in order to increase plant cell totypotency, thus the regeneration rate of the explant in micropropagation was also increased.

Objectives of the research was to ensure that the *35S::KNAT1* transformant has highly totipotency cells, so that it can produce higher number of propagules in micropropagation.

## MATERIALS AND METHODS

The research was conducted in Laboratory of Biotechnology of Biology Faculty, Gadjah Mada University, Yogyakarta. The *35S::KNAT1* of *V. tricolor* and its wild type /WT (both were 12 month after sowing) were used as materials. Those of *35S::KNAT1* of *V. tricolor* were obtained with *Agrobacterium*-mediated transformation which has been done in the previous research by the authors. Organ such as Leaves and shoots were cut in to slices around 0.5 cm length, then they were planted in to the *New Phalaenopsis* (NP) medium (Islam *et al*., 1998) added with 0.15 µM Napthalene acetic acid (NAA) and 0.5 µM 2-

isopentenyladenine (2-iP) for shoot induction. The production of propagules were observed and counted produced 6 weeks after planting. Photographs of those propagules were also periodically taken.

## RESULTS AND DISCUSSION

The number of propagules at 6 weeks after planting was much higher on explants from the *35S:: KNAT1* transformant compared to those of WT (Table 1 and Figure 1), indicating that the regeneration rate of explants from the transformant was higher than those of the WT. These results were in line with the results by Dwiyani, *et al*. (2010) on *Phalaenopsis amabilis* orchid.

**Table 1.** An average number of propagules (shoots) per explant at 6 weeks after planting

| Explant Type | Wild Type ( An average of 10 explants) | Transformant ( An average of 6 explants) |
|---|---|---|
| Slices of shoot | 1 | 68 |
| Base of leaves | 0 | 30 |
| Leaf in a whole | 0 | 11 |

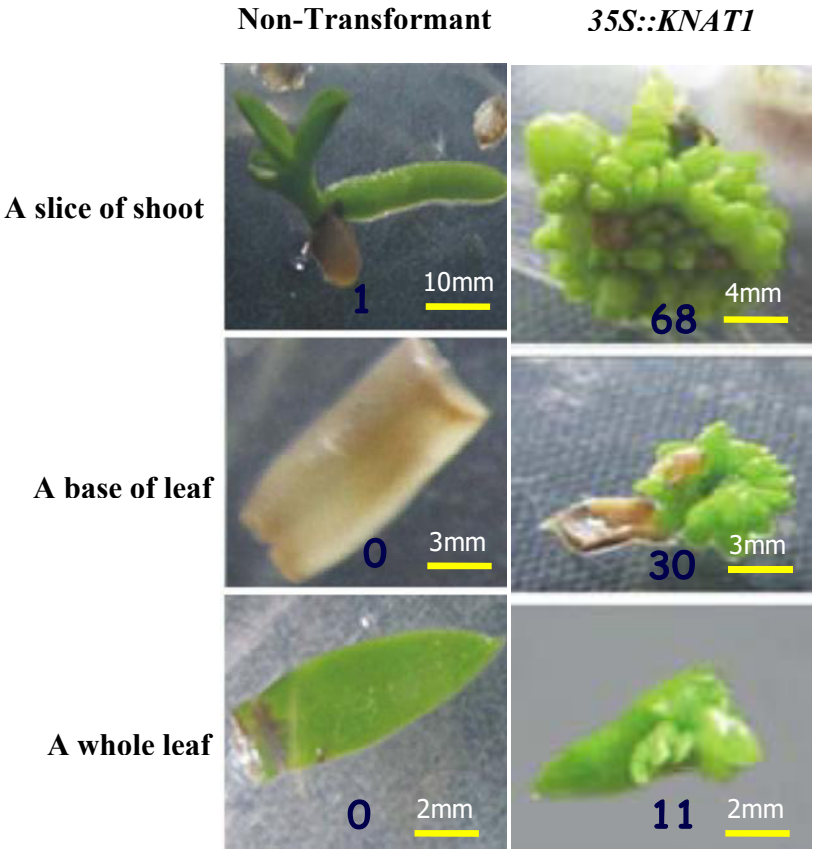


**Figure 1.** The production of propagules (shoots) from various types of explants from transformant and non-transformant at 6 weeks after planting. The number in dark blue colour indicating the number of propagules

Scofield *et al.* (2008) proposed that during vegetative growth of *Arabidopsis thaliana*, *SHOOTMERISTEMLESS* (*STM*) gene is active and keeps the stem cell pool in SAM. The role of *STM* in keeping stem cell pool can only be substituted by overexpression of *KNAT1* gene, but not with other *KNOX* genes. In the current research, constitutive promoter like 35S makes *KNAT1* gene can be expressed

in organs of *V. tricolor* orchid. *KNAT1* gene is a key regulator of activation of other *KNOX* gene such as *STM*. This caused organs of transformant plants become meristematic like in the SAM and lead to higher number of production of propagules in micropropagation compared to those of the WT. Cytokinin (in this case 2-iP) would promote these meristematic cells to form new shoots.

Yanal *et al.* (2005) proposed that *KNOX* protein in *A. thaliana* promotes biosynthesis of indegenous cytokinin of plants. This could explain that propagules was much higher on the *KNAT1* transformant compared to the WT, since Indranto (2003) proposed that cytokinin can induce shoot formation *in vitro*. Frugis *et al*. (2001) found that overexpression of *KNAT1* gene in in *Lactuca sativa* was positively correlated with biosynthesis of indegenous cytokinin.

In the current research, shoot was the type of explant that produced highest number of propagules among other organs, indicating that 'shoot' has highest regeneration rate among others. We suggests that cells in the shoot was more responsive to the addition of 2-iP compared to those of leaf, thus the change of the cell status from indeterminate state to be determinate state was faster.

We can conclude that *V.tricolor* carrying *KNAT1* gene has high cell totipotent, so that it can produce higher number of propagules in micropropagation.

## ACKNOWLEDGEMENT

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# ETHYL METHANESULFONATE DELAYED GERMINATION AND ALTERED SEEDLING MORPHOLOGY OF *Capsicum annuum* L.

**Made Pharmawati[1], I Ketut Suada[2], and Made Ria Defiani[1]**
[1]*Biology Department, Faculty of Mathematics and Natural Sciences, Udayana University, Kampus Bukit Jimbaran, Bali;*
*Email: pharmawati@hotmail.com*
[2]*Agroecotechnology Department, Faculty of Agriculture, Udayana University,*
*Kampus Bukit Jimbaran, Bali*

**ABSTRACT**

*Capsicum annuum* L. is an important horticultural plant in Indonesia. Despite its importance, the productivity of *C. annuum* in Indonesia is low. Global climate change has influenced the productivity of *C. annuum*. To produce variability in *C. annuum*, mutation breeding can be applied. This research aimed to evaluate the germination and growth of *C. anuum* seedling after treated with EMS (Ethyl Methanesulfonate). At germination test, the seeds of *C. annuum* "Smart" were soaked in 0.1%, 0.3%, 0.5%, 0.75%, 1% and 1.5% EMS. As control, seed were soaked in phosphate buffer pH 7. Seeds were sown in petridishes. At polybag experiment, the concentrations used were 0%, 0.5%, 1% and 1.5%. Results from germination test showed that EMS delayed germination. At longer time seeds-treated were able to germinate which mean that seeds develop adaptation. EMS At polybag experiment, plant height decreased in plants generated from EMS treated seed. However the number of leaf varied between control and treatments. This could due to the act of mutagen at random sites of *C. annum* genome. To evaluate the levels of mutation induced by EMS in *C. annuum* "Smart", observation must be conducted at M2 generation.

***Keywords:*** *Capsicum annuum*, EMS, germination, seedling

## INTRODUCTION

*Capsicum annuum* L. is one of vegetable crop that has high economic value in Indonesia. The fruit contains high nutrition and vitamin. *C. annuum* is commonly used as food ingredient or as a component of in processed food such as chilli paste. Beside that it is also used as traditional medicine (Setiadi, 1996). The productivity of *C. annuum* decreased 40% at the end of 2010 (Republica.co.id). The decrease of *C. annuum* productivity contributed as many as 0.22% to inflation in Indonesia in 2010 (Badan Pusat Statistik, 2011).

The decrease of *C. annum* productivity was caused by several reasons. One reason was that climate change such as long period of reany sesason or long period of dry season (Badan Pusat Statistik, 2011). Other causes were high of pest and disease attacks and the occurance of natural disaster such as the volcano eruption of Merapi and Bromo which affected centre of chili production around those regions (Badan Pusat Statistik, 2011).

The used of *C. annuum* varieties that have low productivities has also caused low average yield (Wardani dan Ratnawilis, 2002). Therefore the increase of genetic diversity of *C. annuum* is needed to obtain plants with high resistancy to climate change, pest and diseases, as well as have high productivity. With more variation, well adapted plants and improved varieties can be obtained (Emrani *et al*, 2011). The increase of genetic variation can be obtained by genetic modification of local varieties or varieties that have been developed using induced mutation. Mutation is a tool to learn characteristics and gene functions, and to have raw materials for genetic improvement of economical plants (Adamu *et al*., 2004).

Induced mutation can be conducted using gamma radiation, chemical mutagens or combination of gamma radiation and chemical mutagens. One of the chemical mutagen that commonly used to induce plant mutation is ethyl methansulphonate (EMS). This mutagen works efficiently and has the potential to induce variation in plant (Natarajan, 2005; Bahar and Akkaya, 2009). In this research induced mutation on *C. annuum* was done using different concentration of EMS. The aim of this study was to evaluate the effects of different concentration of EMS on germination and seedling growth.

## MATERIALS AND METHOD

Seeds of *C. annuum* 'Smart' were obtained from agricultural commercial store in Denpasar, Bali. Treatments of seed with EMS were conducted according to Jabeen and Mirza (2004). Seeds were soaked in water for 6 hours, and then were soaked in EMS with concentration of 0%, 0.5%, 0.75%, 1% iand 1.5% in phosphate buffer pH 7 for 6 hours. The treatments were done in room temperature. As control, seeds were soaked in phosphate buffer pH 7 (EMS 0%). Seeds were then washed in water to remove excess of mutagen. Germination test was conducted in petridishes. As many as 30 treated seeds were put on wet filter paper in petridishes and repeated three times. The germination was observed at 3, 5, 7 and 10 days. The inhibition rates (IR) on seed germination at 3, 5 and 7 days were calculated according to the formula of Ahn and Chung (2000): [(Control-EMS concentration)/Control] x 100. IR at 10 days was not calculated because seeds have been adapted to EMS.

In polybag experiment, the seeds were sown individually and then transferred to polybags three weeks after sowing. Each treatment was repeated four times with tree polybags in each replicates. The seedling height was measure every week and morphological changes in morphology were observed and recorded.

## RESULTS AND DISCUSSION

### Germination Test

The effects of EMS on seed germination of *C. annuum* was done with concentrations of 0%, 0.1%, 0.3%, 0.5%, 0.75%, 1% and 1.5%. The percentage of seed germination can be seen in Table 1. Soaking seed with EMS delayed seed germination (Table 1, Figure 1). This was due to EMS is a poisonous chemical. However, after longer time, seeds were able to adapt and germinated although the germination percentages of treated seeds were lower that that of control seeds. The final measurements for germination were done at 10 days after sowing test where at that time the EMS treated seeds were able to germinate and develop adaptation.

Chemical mutagen affected seed coat permeability (Al-Qurainy dan Khan, 2009), therefore water imbibition was inhibited which then lead to delay of germination. Table 1 also indicates survival of seedling. At control and lower concentration of EMS, seedlings were still at high number.

Table 1. The Percentage of Seed Germination of *C. annuum* as Affected by EMS. Numbers are Averages of Seed Percentage ± SE

| Concentration (EMS) | Germination (%) | | | |
|---|---|---|---|---|
| | 3 days | 5 days | 7 days | 10 days |
| 0 | 57±5.5 | 95±2.3 | 100±0 | 100±0 |
| 0.1% | 48 ±4 | 90±3.6 | 100±0 | 100±0 |
| 0.3% | 56±5.03 | 91±1.8 | 95±2.3 | 97±2.3 |
| 0.5% | 36±9.5 | 85±1.7 | 98±1.7 | 98±1.7 |
| 0.75% | 18±10 | 72±7.5 | 95±2.1 | 98±2.1 |
| 1% | 6.3±3.7 | 57±8 | 93±3.8 | 96±4.4 |
| 1.5% | 0 | 43±5 | 72±1.9 | 87±2.3 |

Inhibition rate (IR) of EMS at 3, 5 and 7 days were calculated based on average of seed germination (Table 2).

Table 2. Inhibition Rate (IR) of Germination at 3, 5 and 7 Days

| Concentration (EMS) | Inhibition Rate (%) | | |
|---|---|---|---|
| | 3 days | 5 days | 7 days |
| 0.1% | 15.8 | 5.3 | 0 |
| 0.3% | 1.7 | 4.2 | 5 |
| 0.5% | 36.8 | 10.5 | 2 |
| 0.75% | 68.4 | 24.2 | 5 |
| 1% | 88.9 | 40 | 7 |
| 1.5% | 100 | 54.7 | 28 |

Figure 1. Seed germination experiments in petri dishes of *C. annuum* affected by EMS at different concentration at day 10. First row from left to right : control, 0.1%, 0.3%; second row from left to right: 0.5%, 0.75%, 1% ; third row : 1.5%

The increase of IR in *C. annuum* at higher EMS concentration indicated the occurrence of plant injury. Plant injury at M1 generation such as reduction of seed germination, growth rate of seedling is common effect of chemical mutagen (Shu *et al*, 2012).

**Polibag Experiments**

Another set of experiment was done in polybag system with randomized block design with 4 replicates/block and three polybags in each block. Seedings were transferred to polybag after 3 weeks old. Observation for plant height and number of leaf was done at week four after transfer to polybag (Table 3).

Table 3. Plant Height and Number of Leaf at 5 Weeks After Transfer

| Concentration (EMS) | Plant height | Number of leaf |
|---|---|---|
| 0 | 10.2±0.4 | 6.5±0.37 |
| 0.5% | 7.1±0.32 | 4.1±0.64 |
| 1% | 8.6±0.36 | 6.6±0.32 |
| 1.5% | 8.5±0.51 | 6.8±0.41 |

Table 3 shows that at 5 weeks after transfer, the height of control plant was the highest compared to that of EMS treated plant. This correlated with the results of seed germination test. As the germinated delayed, the plants grew slower so that the height became lower than control plants. However, the lowest plant height was at plant derived from seed treated by EMS 0.5%. The number of leaf also showed the same patterns, where 0.5% EMS caused least number of leaf, whereas the number of leaf was not significantly different between control, 1% and 1.5% EMS treatments. These results could because of random disruption by chemical mutagen (Sikora *et al*, 2011) which leads to alteration at certain concentration.

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# *IN VITRO* ANTAGONISM OF BACTERIAL BIOCONTROL AGENTS ON SEVERAL FUNGAL PLANT PATHOGENS

**Siska Darmayanti[1], Yan Ramona[1,2*], and Sri Anggreni Lindawati[3]**
[1]*Laboratory of Microbiology, School of Biology, Udayana University*
[2]*Integrated Laboratory for Biosciences and Biotechnology, Udayana University*
[3]*Faculty of Animal Husbandry Udayana University*
*Corresponding Author: yan_ramona@yahoo.com

**ABSTRACT**

The main objective of this research was to isolate, screen, identify, as well as to investigate the potential of those isolates to inhibit the growth of several fungal pathogens (*Fusarium oxysporum, Ceratocytis* sp., *Aspergillus flavus* dan *Aspergillus niger*) *in vitro*. Bacillus and Pseudomonas were predominant isolates found in the preliminary identification. Besides that Proteus was also identified in this research. In the dual culture assay, 60% of the isolates inhibited the growth of some fungal pathogens with various degree of inhibition, indicating that they are potential to be developed as candidates of biocontrol agents, although their ability to control these fungal pathogens in glasshouse trials or field trials need to be confirmed.

***Keywords:*** Antagonistic, *Dual Culture Assay, Fusarium oxysporum, Ceratocytis* sp., *Aspergilus flavus, Aspergilus niger.*

# THE EFFECT OF SEED TREATMENT WITH LIQUID PRODUCT OF SHRIMP WASTE FERMENTATION ON CHILI

**Budi Prakoso*, Sakhidin and Rostaman**
*Department of Agrotechnology, Faculty of Agriculture, The Jenderal Soedirman University, Karangwangkal, Purwokerto, Central Java, Indonesia*
**Corresponding author: prabud2001@yahoo.com*

**ABSTRACT**

Damping off disease that caused by *Rhizoctonia solani* Khun is the main disease of chili (Capsicum annum L.) in nursery. In natural condition, the disease is controlled by antagonistic microorganisms and its products. The goal of this research was to study the effect of seed treatment using liquid product of fermented shrimp waste on disease incidence and plant growth of chili. The research was carried out in Plant Protection Laboratory, Faculty of Agriculture and Research Laboratory, Jenderal Soedirman University Purwokerto, from March until June 2012. Shrimps waste was fermented uninoculated or inoculated with Bacillus sp. B298 for seven days. Chili seeds were soaked in the liquid fermented product for 2 hours. Then, the seeds were planted on media uninoculated or inoculated with *R. solani.* Split plot design was used in this experiment. Pathogen inoculation was the main plot and seed treatment using product of fermented shrimp waste was the subplot. Observed variables were disease incidence, plant height, and leaves number. Data were analyzed using F test with error probability levels of 5%, if it was statistically significant, it was followed by the Least Significant Differences (LSD) test with error probability levels of 5%. Result showed that some of seedlings grew on media inoculated by pathogen died due to damping off with disease incidence of 43.21%. In contrast, all seedlings grew on media without pathogen inoculation were healthy. Seed treatment using fermented product (uninoculated or inoculated with Bacillus sp. B298) did not have effect on damping off disease incidence and all components of plant growth of chili yet. In addition, liquid and microbial products of fermented shrimp waste did not have anti fungal activities.

***Keywords:*** Fermentation, Shrimp waste, Chili, damping off.

## INTRODUCTION

Damping off disease caused by *Rhizoctonia solani* Khun is one of many factors hindering chili production. At present, plant disease management includes applying cultural techniques, growing disease-resistant plant varieties, and spraying synthetic pesticides. Increasingly, there is evidence that farmers' pesticide applications are often unnecessary. This leads to outbreak of diseases, harmful to human health and other environmental problems (Benbrook, 2002; Chauhan and Singhal, 2006). Many investigations for finding natural environmental friendly substances and microorganisms that can improve the growth of plants and protecting the plants from pathogen infection and disease development have been done (Baker *et al.,* 2002).

The amount of shrimps waste is around 35-45% of the shrimp products. Only 5% of shrimp waste is actually used in some ways, mostly for animal feed, the remainder is discarded and represents an environmental problem (Cira *et al*., 2002). Chitin is the main structural component of the shells of shrimps, crabs, squid pens and cell walls of some fungi. It is expected that microbe or its product that can degrade chitin or convert chitin into chitosan can be used to control plant pathogenic fungi.

Fermentation is natural process in which microorganism decomposed macromolecules of media for growing. The fermented products compose of solid, liquid and biomass of microorganism. Chitin can be converted into chitosan by fermentation which is environmentally safe as a substitute for producing chitosan using strong acid and strong base that are not environmentally friendly (Lertsutthiwong *et al.,* 2002).

The effect of chitosan solution and fermented product on the plant growth varied. Some researchers found that chitosan solution increased the growth of plants, induced resistant of plant to certain plant disease (Kim *et al*., 2005; Lee *et al.,* 2005; Boonlertnirun *et al*., 2008; Abdel-Mawgoud, 2010).

The goal of this research was to study the effect of seed treatment using liquid product of fermented shrimp waste on disease incidence and plant growth of chili.

## MATERIALS AND METHODS

The research was carried out in Plant Protection Laboratory, Faculty of Agriculture and Research Laboratory, Jenderal Soedirman University Purwokerto, from March until June 2012.

Shrimps waste was bought from local market. It was washed, blended, and fermented uninoculated or inoculated with Bacillus sp. B298 for one to seven days in a bottle. 100 g of waste was mixed with 300 ml of sterilized water.

*Rhizoctonia solani* slant culture was bought from Mycology and Phytopathology Laboratory, Faculty of Biology, the Jenderal Soedirman University. The fungi was reculture in PDA in petridishes for 7 days, 1x1 cm of the culture was mixed with sterilized rice grains + 1% sugar and incubated at room temperature for 14 days. The infected rice grains were used as *R. solani* inoculums. 200 g inoculums were mixed with 1000 g planting media.

Chili seeds were collected from chili variety that has been tested susceptible to the *R. solani*. The seeds were soaked in the liquid fermented product for 2 hours. Then, the seeds were planted on media uninoculated or inoculated with *R. solani.*

Split plot design was used in this experiment. Pathogen inoculation was the main plot and seed treatment using product of fermented shrimp waste was the subplot. Observed variables were disease incidence, plant height, and leaves number. Data were analyzed using F test with error probability levels of 5%, if it was statistically significant; it was followed by the Least Significant Differences (LSD) test with error probability levels of 5%.

## RESULTS AND DISCUSSION

Seed treatment by soaking the seeds in liquid product of fermented shrimp waste for 2 hours did not reduce the disease incident and did not improve the seedling growth. In contrast, pathogen inoculation had a devastating effect on all data recorded (Table 1).

Some of seeds planted on media inoculated by *R. solani* did not germinate, others died after germinated and having damping off symptom, only few seedlings survived until one month. In contrast, all seedlings grew on media uninoculated by *R. solani* were healthy. This resulted in that plant height and leaf number of seedlings grew on uninoculated media were higher than that of seedlings grew on media inoculated by *R. solani* (Table 2).

**Table 1.** The effect of *R. solani* inoculation on germinating media, and seed treatment by soaking the seeds in liquid product of fermented shrimp waste (0 until 7 days of fermentation) on disease incident, and seedling growth.

| No | Variable | I | F | I X F |
|---|---|---|---|---|
| 1 | Disease Incident | ** | Ns | Ns |
| 2 | Seedling height | * | Ns | Ns |
| 3 | Numbers of leaves | * | Ns | Ns |

Remark:
** = very significant (1% probability level)
* = significant (5% probability level)
Ns = non significant (5% probability level)
I = *R. solani* inoculation
F = Fermentation

**Table 2.** Disease incidence, plant height, and leaf number of seedlings grown on media uninoculated or inoculated by *R. solani*

| *R. solani treatment* | Disease Incidence (%) | Plant height (cm) | Leaf numbers |
|---|---|---|---|
| Uninoculated media | 0.0 (4.0550 b) | 10.78 (3.284 a) | 7.41 (2.813 a) |
| Inoculated media | 43.21 (41.5341 a) | 5.59 (2.310 b) | 4.61 (2.142 b) |

Remark:
Numbers in the bracket were arcsin√x+0.5 transformed data
Number followed by same letter was not significantly different at LSD 5%

**Table 3.** The effect of soaking seeds for 2 hours in liquid product of shrimp waste fermentation up to 7 days on disease incidence plant height. and leaf number

| Soaking Seed for 2 hours in | Disease Incidence (%) | Plant Height (cm) | Leaf Number |
|---|---|---|---|
| $H_2O$ | 27.78 | 8.67 | 6.50 |
| Fungicide | 11.11 | 9.22 | 7.00 |
| FA0 | 27.78 | 7.07 | 5.67 |
| FA1 | 5.55 | 9.80 | 7.00 |
| FA2 | 5.55 | 7.87 | 6.50 |
| FA3 | 22.22 | 6.85 | 5.17 |
| FA4 | 27.78 | 7.92 | 6.17 |
| FA5 | 22.22 | 9.17 | 7.17 |
| FA6 | 5.55 | 8.12 | 6.17 |
| FA7 | 33.33 | 6.32 | 5.33 |
| FE0 | 11.11 | 9.73 | 7.17 |
| FE1 | 38.89 | 5.35 | 4.33 |
| FE2 | 33.33 | 7.77 | 5.50 |
| FE3 | 33.33 | 6.50 | 5.00 |
| FE4 | 27.78 | 7.10 | 5.00 |
| FE5 | 27.78 | 7.37 | 5.00 |
| FE6 | 5.55 | 8.70 | 6.67 |
| FE7 | 22.22 | 9.83 | 6.83 |

Remark:
FA (0-7) = liquid product of shrimp waste fermentation (0 up to 7 days) without inoculum
FE (0-7) = liquid product of shrimp waste fermentation (0 up to 7 days) by Bacillus sp. B298

Soaking seeds for 2 hours in liquid product of fermented shrimp waste before planting could not reduce the damping off disease incident of chili. The seed treatment could not improve the growth of seedlings (Table 3). This indicated that plant growth promoting substances and antifungal in liquid product of shrimp waste fermentation up to 7 days very low (Figure 1 a and Figure 1b).

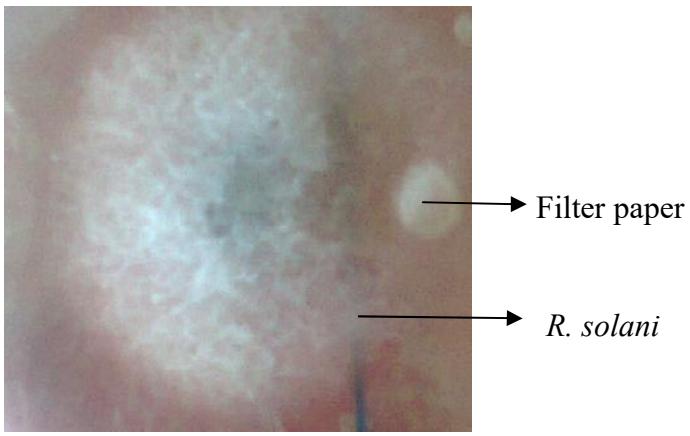


Figure 1a. The effect of liquid product of shrimp waste fermentation by inhabitant microorganisms up to 7 days on the growth of *R.solani* on PDA.

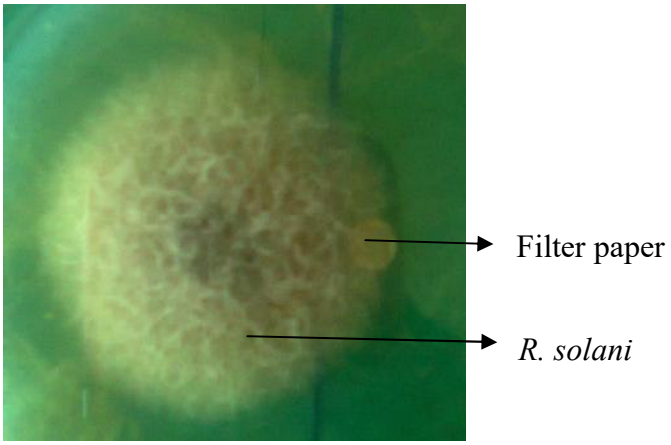


Figure 1b. The effect of liquid product of shrimp waste fermentation by inhabitant microorganisms up to 7 days on the growth of *R.solani* on PDA.

## ACKNOWLEDGEMENT

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# DETECTION OF *toxR*, *tdh* AND *trh* Genes OF *Vibrio parahaemolyticus* OBTAINED FROM WHITE SHRIMP (*Penaeus indicus*) SAMPLES IN JAMBI, INDONESIAN COASTAL AREA

**Marlina[1], Desi Sagita[2], Isamandella Erna[2], Yaya Rukayadi[3], and Son Radu[3]**
*[1]Faculty of Pharmacy, Andalas University, Kampus Limau Manis, Padang, Indonesia.*
*[2] Department of Pharmacy, Sekolah Tinggi Ilmu Kesehatan, Pakuan Baru, 36132 Jambi, Indonesia*
*[3] Department of Food Science, Faculty of Food Science and Technology, and Laboratory of Natural Products (LHS), Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia.*
*Correspondence author: marlina_adly@yahoo.com*

**ABSTRACT**

Six isolates of *Vibrio parahaemolyticus* have been isolated from raw white shrimp (*Penaeus indicus*) samples from Kuala Tungkal, Kampung Laut and Nipah Panjang, Jambi, Indonesia. Isolation has been done by using CHROMAgar *Vibrio* selective media. Identification of *V. parahaemolyticus* were done against all six isolates using simple bi$^{oC}$hemicals test and Polymerase Chain Reaction (PCR) method to amplify the *toxR* gene. *toxR* gene is the specific gene for *V. parahaemolyticus* species. Futhermore, using the same method, amplification against toxin hemolysin gene (*tdh* and *trh*) which is the main virulence factor of *V. parahaemolyticus* has been done. Result showed four out of six samples are positive *V. parahaemolyticus*, the isolates contain the *toxR* gene but not even one isolates contain toxin hemolysin genes either *tdh* or *trh*.

***Keywords:*** *Vibrio parahaemolyticus, toxR, tdh, trh, Penaeus indicus*

## INTRODUCTION

Since early 1950, the number of cases extraordinary events of foodborne disease caused by *Vibrio parahaemolyticus* increased dramatically around the world (Wong, 2003; Hara-Kudo, 2003), Outbreaks occur mainly due to the consumption of seafood, especially raw oysters and shrimp contaminated with *V. parahaemolyticus* (Fuenzalida *et al*. 2005).

*V. parahaemolyticus* is a gram-negative bacterium halofilik normal flora around the coast. This bacterium occurs primarily in summer (McLaughlin *et al*. 2005). Bacteria living symbiotically with shrimp, the *toxR* gene possessed by bacteria *V. parahaemolyticus* is estimated as the originator or regulator toxin-producing genes. The existence of genes *toxR* owned by bacteria *V. parahaemolyticus* is one cause of outbreaks of foodborne infections that cause gastroenteritis (Amizar, 2011).

Shrimp is also a popular food and consumed by many people and is one of the major export products of value that is 20% of the marine products. Sixty percent of whom are from developing countries (Sujeewa *et al.* 2009).

Therefore, there is need for further research to determine the presence of bacterial contamination *V. parahaemolyticus* (Sujeewa *et al*. 2009) was the shrimp. Bacteria detected by the molecular *toxR* gene using Polymerase Chain Reaction (PCR).

## MATERIALS AND METHODS

### Material

CHROMAgar™ Vibrio media, Salt Polymixin Broth (SPB) media, Luria Burtani (LB) Broth, destilled water, Natrium Chlorida (NaCl), 0.5× buffer Tris Base Boric Acid EDTA (TBE), agarose powder (Merck), primer, taq polymerase, 5× buffer PCR, 2.5 mM dNTP's (deoxy Nucleotide Triposphate), ethidium bromide.

All materials and tools used sterilized by autoclaving at 121°C for 15 minutes and all work performed aseptically.

### Isolation of *Vibrio parahaemolyticus*

A total of 10 g sample is introduced into a sterile plastic bag containing 100 ml of medium Polymixin Salt Broth (SPB) homogenized in a Stomacher and incubated in the incubator at 37 °C for 24 hours. After

that, the samples were inoculated into medium CHROMagar Vibrio and incubated at 37 °C for 24 hours. Colonies that purple is *V. parahaemolyticus*.

**Identification of *V. parahaemolyticus***

Identification of *V. parahemolyticus* can be done by using Biochemistry and molecular PCR test.

Biochemical identification

1. Gram staining.
   Bacterial colonies suspected *V. parahaemolyticus* from CHROMagar media taken by 1 loop, and then streaked over the object glass of dry, sterile, and followed by fixation. Crystal violet dripped 2 drops and allowed to stand for 30 seconds. Samples were rinsed with water and dried-aired, and then poured the iodine solution and left to stand for 1 minute. Then washed with running water and wind dried. Then washed with 95% alcohol for 30 seconds, washed with water and wind dried. Were given 2 drops of safranin solution for 2 minutes and rinse again with water flow and wind dried. Then preparations were observed under a microscope.
2. Catalase
   Bacterial colonies are suspected *V. parahaemolyticus* from CHROMAgar taken by 1 loop, and then streaked over the object glass. Hydrogen Peroxide dropped as much as 2-3 drops of the bacterial swabs earlier. The presence of air bubbles indicates the activity of catalase.

**Molecular identification of *V. parahaemolyticus***

**DNA genomic extraction**

DNA Genome of *V. parahaemolyticus* were extracted with Boil Cell Extraction (BCE) method. A total of 1 ml of culture was transferred into Eppendorf tube and centrifuged at 12,000 rpm for 5 min, the supernatant was discarded, the precipitate was suspended in 0.5 ml of sterile distilled water and then vorteks. Heat in a dry bath at 100 °C for 10 minutes. Furthermore incubated 10 minutes in the refrigerator with a temperature of -20 °C. Then centrifuged at 12,000 rpm for 5 min, the supernatant was transferred into new eppendrof and liquids used for the PCR. A total of 2µl template DNA was mixed with PCR reagents is 10x PCR buffer, 25 mM MgCl2, Primary 1 Primary 2, 10 mM dNTPS (deoxy nucleotide triphosphate) and Taq polymerase enzyme in 0.2 ml Eppendorf. Table 1 shows program of PCR machine.

Table 1. Program of PCR Cycles Used in This Study

| | Tempetarure/Time | |
|---|---|---|
| Cycle | *ToxR* (23 Cycle) | *tdh* dan *trh* (33 siklus) |
| Pradenaturation | 96ºC (5 Minutes) | 96ºC (5 Minutes) |
| Denaturation | 94ºC (1 Minutes) | 94ºC (1 Minutes) |
| Annealing | 63ºC (1,5 Minutes) | 55ºC (1,5 Minutes) |
| Polymerase | 72ºC (1,5 Minutes) | 72ºC (2 Minutes) |
| Elongation | 72ºC (7 Minutes) | 72ºC (7 Minutes) |

**Electrophoresis**

Electrophoresis was performed on 1.5% agarose gel using 0.5× TBE at 150 volts voltage for 20 minutes

## RESULTS AND DISCUSSION

From the research that has been done, the shrimp samples were inoculated in medium CHROMagar VibrioTM purple bacterial colonies obtained which showed the presence of bacteria *V. parahaemolyticus* (Figure 1). From the gram staining test results, it is known that the bacteria are gram-negative bacteria (Figure 2).

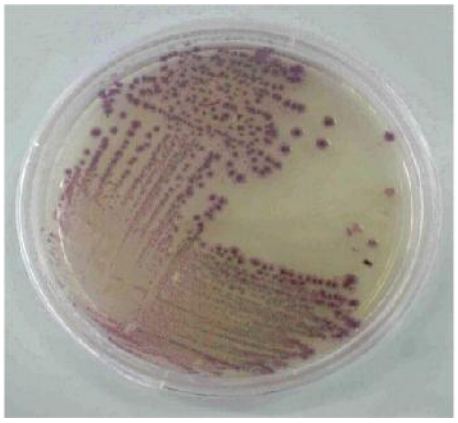

Figure 1. Isolates of *V. parahaemolyticus* on CHROMagar media

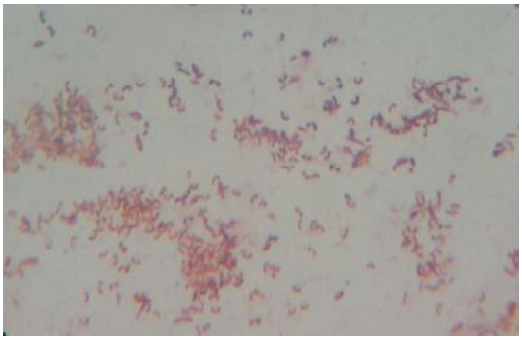

Figure 2. Bacteria colors when using dye safranin after washing with alcohol

From the catalase test results, it is known that bacteria are anaerobic bacteria tested because the formation of air bubbles. From 6 bacterial isolates were detected by polymerase chain reaction method, four isolates that have acquired genes *toxR* DNA band size is 368 kb Vp kt1 Vp kt2, Vp kl1, and Vp kl2 (Figure 3).

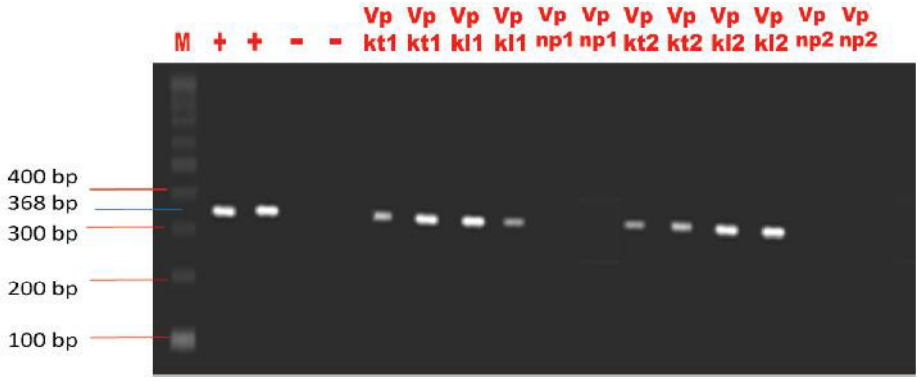


Figure 3. Photo of 1.5% Agarose Gel Results Electrophoresis *toxR* gene DNA from shrimp (*P. Indicus*)

Description:
M : 100 bp DNA ladder marker, a standard tape measure DNA
+ : Positive control genes *toxR* V. parahaemolytcus (368 bp) strain ATCC 1896
- : Negative control distilled water

From the six isolates of *V. parahaemolyticus* non of them showned positive result at 250 and 251 bp (*tdh* and *trh*)

Shrimp is part of the export commodity has a value which is important especially in developing countries (Sujeewa *et al*. 2009). More than 85% of exports of frozen shrimp Indonesia to Europe is a type of the genus Penaeus. Any problems regarding bacterial contamination *V. parahaemolyticus* in seafood reduces the quality of Indonesian exports of marine products (Anonymous, 2010).

*V. parahaemolyticus* is a leading cause of gastroenteritis illness (Velamal *et al*. 2005) are derived from marine products, especially those eaten raw, cooked or contaminated with imperfect raw seafood after ripening (Nair *et al*. 2007).

In this study the bacteria *V. parahaemolyticus* isolated from shrimp samples (*P. Indicus*) were obtained from the waters of Kuala Tungkal, Kampung Laut and Nipah Panjang.

Each sample was added to 100 ml of media add Polymixin Salt Broth (SPB). This medium as it contains antibiotic polymyxin B. Vibrio was resistant to polymyxin B. Polymyxin B is the base polypeptide that is active against gram-negative bacteria (Katzung, 1997) which why no bacterium except vibrio can live.

The bacterial suspension of *V. parahaemolyticus* from the media SPB and then transferred into media that is CHROMagar *Vibrio* selective medium for genus Vibrio. It is known that the genus Vibrio is a bacterium halophilic and not all bacteria can live in an environment of high salinity, NaCl in the medium may decrease or inhibit the growth of other bacteria. Chromagenic material contained in the media is important to identify the species in the genus Vibrio, for the bacterium *V. parahaemolyticus*, chromagenic material will provide the color purple colonies (Hara-Kudo *et al*. 2001; Marlina *et al*. 2008).

Colonies contained in CHROMagar Vibrio media identified by biochemical and molecular test. A simple biochemical test performed to determine the type of stain gram positive and gram negative bacteria. From the results it was obtained red stained bacterial colonies that are gram-negative bacteria. This is because the cell wall of gram-negative bacteria consists of a thin layer of peptidoglycan, this led to the erosion of blue when washed with alcohol (Pelczar, 1986).

Catalase test done to prove whether the colonies were found on CHROMagar media considered aerobic or anaerobic bacteria. Bacteria *V. parahaemolyticus* is a facultative anaerobic bacteria. From the test results obtained, no catalase activity were found.

Colonies of *V. parahaemolyticus* that are purple in the medium CHROMagar then transferred to a medium containing LB Broth Tripton, and NaCl are useful to enhance the growth of pure cultures of bacteria *V. parahaemolyticus*. Pure culture of *V. parahaemolyticus* in Broth LB medium incubated in a rotary shaker incubator for 24 hours at 37 ºC with a speed of 160 rpm. This culture is used in the extraction of bacterial DNA for molecular identification by detecting the *toxR* gene using PCR method.

BCE is the lysis of the bacterial cell wall with the extreme temperature difference from 100 ºC to -20 ºC in order to obtain DNA extract. The process is relatively less expensive, less time-consuming and low cost. However, the DNA extracts BCE can not survive long. In addition, no proteinase and RNAse which can cause contamination in the PCR process. After BCE, DNA extracts were centrifuged in Eppendorf tubes 12,000 rpm for 5 min. The goal is to precipitate cell components other, so that the target DNA present in the supernatant.

Methods Polymerase Chain Reaction (PCR) is a more specific method, simple and fast. PCR is an enzymatic method to multiply exponentially the nucleotide sequence specific manner in vitro (Yuwono, 2006). The basic principle of the PCR process is the multiplication of specific molecules of DNA primers with the help of the form of oligonucleotides (Jamsari, 2007).

Primary primer used was specific to the *toxR*, *tdh* and *trh genes*. With the enzyme Taq Polymerase as polymerizing enzyme, $MgCl_2$ as a cofactor to catalyze the enzymatic process, and dNTP's which serves to supply the bases of nucleotides, and buffer which serves to maintain the pH of the primary, which is a nucleotide base short chain will be extended until the amount equal to the chain of nucleotide bases 1 and 2 and form two perfect genes. The process lasts for one cycle. This cycle will be repeated for as many as 23 cycles for *toxR* genes and 33 cycles for *tdh* and the *trh* gene (Yuwono, 2006; Jamsari, 2007).

The results of amplification were performed using PCR and then separated using elektroforesa. The main principle elektroforesa tool is based on the difference in charge separation on the high voltage of 150 Volt. DNA is negative then the DNA would follow the movement towards the positive phase in elektroforesa. Gel staining is done by immersing the gel into a solution of ethidium bromide for 5-10 minutes, so that the purpose staining DNA bands are shown below the DNA analyst tools and can be photographed. Coloring mechanism is the intercalation of ethidium bromide which binds to the DNA in the gel.

From 6 isolates that have been test, 4 isolates are positive detected *toxR* gene (Vp kt1, Vp kt2, Vp kl1 and Vp kl2). This is evidenced by the bands at 368 bp similar to the positive control. This suggests that 4 samples of shrimp (Vp kt1, kt2 Vp, Vp and Vp kl1 kl2) has been contaminated by bacteria *V. parahaemolyticus*. While no samples were shown at bp 250 and 251 (*tdh* and *trh*).

## ACKNOWLEDGEMENT

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# FIRST REPORT ON OCCURRENCE OF GRAPEVINE LEAF ROLL-ASSOCIATED VIRUS TYPE 3 ON GRAPEVINE IN INDONESIA

**I Gede Rai Maya Temaja*, I Putu Sudiarta, and Dewa Nyoman Nyana**
*Faculty of Agriculture, Udayana University, Denpasar*
**Corresponding author: tderai@yahoo.com*

**ABSTRACT**

In a survey of vineyard fields in Buleleng, Bali, Indonesia, some grapevine cultivars were found showing grapevine leaf roll disease symptomps, i.e.interveinal reddening or yelowing and fall leaves; down rolling of leaves; phloem disruption; and the fruit maturation is delayed. Molecular method was then used to determine the coat protein (CP) sequence. Analysis of leaf samples from diseased plants by reverse transcription-polymerase chain reaction (RT-PCR) using a pair of primer specific to Grapevine Leaf Roll-associated Virus type 3 (GLRaV-3) successfully amplified an expected 477 bp DNA fragment. The sequences of the PCR product confirmed that the grapevine plants were infected by GLRaV-3. This is the first report on occurrence of GLRaV-3 on grapevine plants in Indonesia.

***Keywords***: *Grapevine Leaf Roll-associated Virus type 3* (GLRaV-3), grapevine (*Vitis vinifera*), reverse transcription-polymerase chain reaction.

# POSTER PRESENTATIONS: FOOD SCIENCE AND TECHNOLOGY

# THE INFLUENCE OF SOLVENT TYPES AND pH ON THE ANTIOXIDANT ACTIVITY OF ANTHOCYANIN OF SUPER RED DRAGON FRUIT (*Hylocereus costaricensis*) FLESH EXTRACT

**Ni W. Wisaniyasa*, IN Kencana Putra and Cok. Satria Nugraha**
*Food Science and Technology, Faculty of Agricultural Technology, Udayana University*
**Correspondence Author: wisaniyasa_2007@yahoo.com*

**ABSTRACT**

The objectives of this research are to know the effect of solvent types and pH on the antioxidant activity of anthocyanin of super red dragon fruit flesh extract and to determine the solvent type and pH that produce super red dragon fruit flesh extract with the highest antioxidant activity. This research used randomized block design (RBD). The first factor (the solvent) consisted of two types, namely: methanol and ethanol. The second factors (pH of solvent) consisted of three levels : pH 1.0 ; pH 2.5 and pH 4.0. The treatment was repeated three times so that obtained 18 units of treatments. Variables were observed in this research including: yield, total anthocyanin and antioxidant activity. The research results showed that types of solvent and pH had very significant effect on yield, anthocyanin total, and antioxidant activity of anthocyanin of super red dragon fruit flesh extract. Methanol pH 1.0 produced yield, total anthocyanin and the antioxidant activity were 88.16%, 4.67% and 2.33% respectively.

***Keywords*:** *super red dragon fruit, solvent, pH, antioxidant*

## INTRODUCTION

Dragon fruit is divided into 4 types, there are the white flesh dragon fruit (*Hylocereus undatus*), red flesh dragon fruit (*Hylocereus polyrhyzus*), super red flesh dragon fruit (*Hylocereus costaricensis*) and yellow flesh-white skin dragon fruit (*Selenicereus megalanthus*). From the four types, super red dragon fruit has red colour on the skin and the flesh indicated high levels of anthocyanin. In determining the content of antioxidants can be done in various ways, one of which is the process of extraction using a solvent. The extraction process by using a solvent is influenced by the type of solvent and the amount of solvent used (Utami, 2009).

The type of solvent and the degree of acidity (pH) of a food would affect the stability of some of the compounds such as colors and antioxidant content. So the purpose of this research is to know the influence of solvent types and pH on antioxidant activity of super red dragon fruit flesh extract and to know the type of solvent and pH correct so that the resulting super red dragon fruit flesh extract with the highest antioxidant activity.

## RESEARCH METHODS

### Materials

Materials used in this research were super red dragon fruit (*Hylocereus costaricensis*) purchased in the Orchards of the Dragon, Bajra, Tabanan Bali. Dragon fruit is used in this study is 2 months old are counted starting from flowering. The chemicals used are technical ethanol, technical methanol, ethanol Pa (Brataco chemichal), gallic acid (Sigma), 2.2-dhipenil-1-picryldhydrazyl (DPPH) (Sigma), HCl, KCl, glysin, citric acid.

### Tools

Tools used in this research include: freeze dryer (Ilshin TFD 5505), spectrophotometer (Turner SP-870), vortex (Thermolyne), rotary vacuum evaporator, shaker, pH meter (Istek), retort 250 ml (Pyrex), mortar, sample bottles, test tubes (Pyrex), filter fabric, filter paper, wathman paper No. 1, aluminum foil, blender (Maspion), knives, analytical scales (Metler Toledo AB-204), dropper, eyedropper, drops the sieve, desiccator, beaker glass (Pyrex), and paper labels.

**Experiment Design**

This research used a Random Design Group (RDG) factorial pattern. The first factor was the type of solvent there were ethanol and methanol, whereas the second factor was the pH of a solvent consisting of pH of 1.0 ; 2.5 and 4.0, respectively. So 6 combination treatment is obtained. The whole combination treatment was repeated three times that obtained 18 units attempted. The influences from a variety of treatment carried out analysis of the data obtained and if the treatment shows the influence of the real or the very real to the observed variable then continued with Duncan test (Sastrosupadi, 2000).

**Implementation Research**

**Preparation materials**

Super red dragon fruit (aged 2 months counted from the flowering) have sortation, then cutted by using a knife to split into two so the skin removed. Dragon fruit flesh washed with clean water and destroyed using a blender until it becomes the fruit pulp. Then the dragon fruit on freeze drying for 48 hours until dried. Results of freeze drying crushed by mortar until be powder. For each pH treatment of solvent, we make aqueous buffer to be done making each solvent pH remains stable.

**The making of buffer solution pH 1.0 (Sudarmadji *et al*., 1989)**

0.0745 g KCl dissolved into 50 ml aquadest and 0.89 ml concentrated HCl introduced into 100 ml aquadest. Then mixed 50 ml KCl solution with 97 ml HCl solution so measured by pH meter until it reaches a pH of 1.0 with the addition of a solution of HCl if more than pH 1.0 and if less than pH 1.0 made the addition with NaOH.

**The making of buffer solution pH 2.5 (Sudarmadji *et al.,* 1989)**

0.7505 g glysin dissolved in 50 ml aquadest and 0.89 ml concentrated HCl and introduced into 100 ml aquadest. Then 50 ml solution of glysin is mixed with 28.3 ml solution of KCl. Subsequently the pH measured by the pH meter until it reaches a pH of 2.5.

**The making of buffer solution pH 4.0 (Sudarmadji *et al.*, 1989)**

2.101 g citric acid dissolved in 100 ml aquadest and 2.941 g sodium citrate added in 100 ml aquadest. Then mixed 26.75 ml solution of citric acid with 23.25 ml of sodium citrate solution. Subsequently the pH measured by the pH meter until it reaches pH 4.0.

**The Making of Dragon Fruit Extract**

Before mixing the solvent, settings conducted appropriate pH treatment i.e. 1.0: 2.5: 4.0 for each solvent. Prepared erlenmeyer 250 ml, put the dragon fruit powder into the each retort as much as 10 g, and then put each kind of solvent (methanol, ethanol) with ratio 1: 6 and then was rocked for 24 hours at room temperature. Further filtered by using a fabric filter to get a dragon fruit extract was mixed with the solvent. Extract of the dragon fruit next turn was evaporated using rotary vacuum evaporator at a temperature of approximately 400$^{o}$ C. Termination of the process of evaporation was determined by solvent viscosity and the smell of the super red dragon fruit extract was free of solvents.

**Observed variables**

Observed variables were yield (Sudarmadji *et al.*, 1989), total of anthocyanin (Giusti and Worlstad, 2001) and antioxidant activity method of DPPH (Yun, 2001).

Analysis of total anthocyanin (Giusti and Worsltad, 2001)

1. Take 1 ml of the sample and diluted with pH buffer solution of 1 until 10 ml
2. Take 1 ml sample is taken and diluted with pH buffer solution of 4.5 until 10 ml
3. Read absorbance of samples at a wavelength on 510 nm and 700 nm

The making of buffer solution, pH 4.5 and pH 1 is as follows:

1. pH buffer solution 1.0
   0.0745 g KCl dissolved in 50 ml aquadest ml and concentrated HCl 0.90 ml introduced into 100 ml aquadest. Then mixed with 50 ml KCl solution with 97 ml HCl solution. So measured pH with pH meter until it reaches pH 1.
2. pH buffer solution 4.5
   2.101 g of citric acid dissolved in 100 ml aquadest and 2.941 g of sodium citrate fused into 100 ml aquadest. Then mixed 53.5 ml citric acid solution with 46.5 ml of sodium citrate solution. Subsequently the pH measured by the pH meter until it reaches a pH 4.

Total anthocyanin can be calculated by the formula :

$$C\ (mg/L) = \frac{\Delta A \times MW \times 102 \times DF}{(c \times L)}$$

Keterangan : C = concentration of total anthocyanin
$\Delta A = \{(A\ 510 - A\ 700)pH\ 1 - (A\ 510 - A\ 700)\ pH\ 4.5\}$
c = coefecient of molar extencion (cyanidin-3-glikosida : 29.600 L/mol cm)
L = wide of cuvet ( 1cm)
MW = weight of the molecule (cyanidin-3-glikosida : 449,2 )
DF = dilution factor

Determination of antioxidant activity by DPPH method (Yun, 2001).

Samples taken as much as 0.1 grams diluted with ethanol up to volume 10 ml. To create a DPPH solution, taken 0.004 grams of DPPH and diluted with ethanol up to 100 ml. Sample that has been diluted taken 0.1 ml included in test tubes and added 0.4 ml of ethanol. Later added by 3.5 ml DPPH solution. So vortex test tubes and left open in the air for 20 minutes, then read the absorbance on 517 nm wave length. Determination of the reading DPPH free radicals using standard curve with galic acid concentration (0, 50, 100, 150, 200, 250, 300 ppm) so that the regression equation is obtained (y = ax + b).

Levels of the antioxidant activity of DPPH method is calculated by the formula:

$$\text{Levels of antioxidant activity (\%)} = \frac{X\ x TV\ x FP}{W} x100\%$$

Description: X = the concentration of the sample is obtained from a linear regression equation (mg/L)
FP = dilution factor
TV = total volume (L)
W = weight of the sample (mg)

## RESULTS AND DISCUSSION

### Yield

Spectrum analysis showed that treatment types of solvent and pH have effect very real on yield of super red dragon fruit flesh extract. The calculation of the yield of flesh extract based on the weight of dragon fruit after being dried powder. The results obtained from the weight of the fresh fruit super red dragon was 5% after drying. The average value of yield of super red dragon fruit flesh extract based on types and pH solvent treatment can be seen in Figure 1.

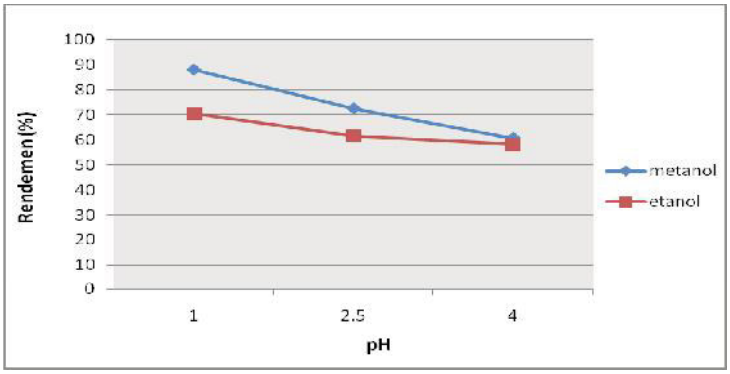


Figure 1. Relationship graph of types and solvent pH on yield of super red dragon fruit flesh extract.

Figure 1 shows the highest average value of yield super red dragon fruit extract was obtain from methanol pH 1.0 treatment, it was 88.16%, while the lowest value was obtain from ethanol solvent pH 4.0 treatment, it was 58.26%. Methanol produce average value of the yield of super red dragon fruit flesh extract is higher than ethanol because the degree of polarity methanol solvent is higher than ethanol. The degree polarity of methanol solvent is 33.60 while ethanol is 24.30 (Sudarmadji *et al.*, 1989).

Figure 1 shows that any increase in pH in each type of solvent, then the yield obtained less. In other words, the more acidic solvent conditions, the more extract yield super red dragon fruit. This is in accordance with the opinion of the Nollet (1996) which says that the lower the pH of the solvent is used will increase number of compounds that can be extracted. Beside that, the more acid condition, the more number broke vacuole cell that increase the yield (Fennema, 1996).

**The Total Anthocyanin**

Spectrum analysis showed that treatment types of solvent and pH have effect very real on total anthocyanin of super red dragon fruit flesh extract. The average value of the total anthocyanin extract of super red dragon fruit can be seen in Figure 2.

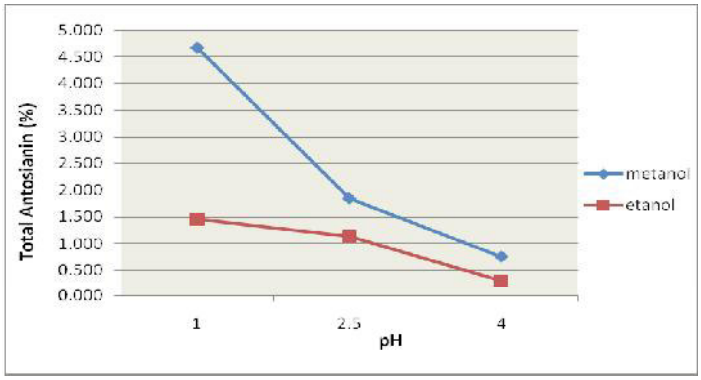


Figure 2. Relationship graph of types and pH solvents on the total anthocyanin of super red dragon fruit flesh extract.

Figure 2 shows the highest average value of a total anthocyanin of super red dragon fruit flesh extract was obtained from methanol solvent pH 1.0 treatment, it was 4.67% . While the lowest value was obtain from ethanol solvent pH 4.0 treatment, it was 0.29%. From those results, total of anthocyanin using the methanol solvent as higher than ethanol solvents. This can be caused of the polarity of anthocyanin pigment has a relatively equal with methanol solvent. Fennema (1996), stated that the anthocyanin plant cell located in the vacuoles as solution like water (aquaeous solution), so anthocyanin is polar.

The process of extraction of natural pigment super red dragon fruit flesh extract by using ethanol produced very small of total anthocyanin. This can be caused the solvent ethanol does not have is the same polarity of anthocyanin pigment extract of dragon fruit. The level of constanta dielektrikum of ethanol is 24.30 smaller than methanol with constanta dielektrikum 33.60 (Sudarmadji *et al.,* 1989).

From Figure 2 also looks that the higher pH on the same type of solvent, the lowerer of total anthocyanin . This is because of the properties of the anthocyanin a more stable in acidic conditions. In addition, anthocyanin are polar compounds are easier extracted in an acid condition (Fennema, 1996). Research results of Lestario *et al.* (2005) show that the fruit duwet extracts with solvents of methanol-HCL 1% produce extracts with the highest levels of anthocyanin.

**Antioxidant activity of super red dragon fruit flesh extract**

The result showed that types of solvent and pH treatment have effect very real on the antioxidant activity of super red dragon fruit flesh extract. The average value of antioxidant activity of super red dragon fruit flesh extract based on solvent types and pH treatment can be seen in Figure 3.

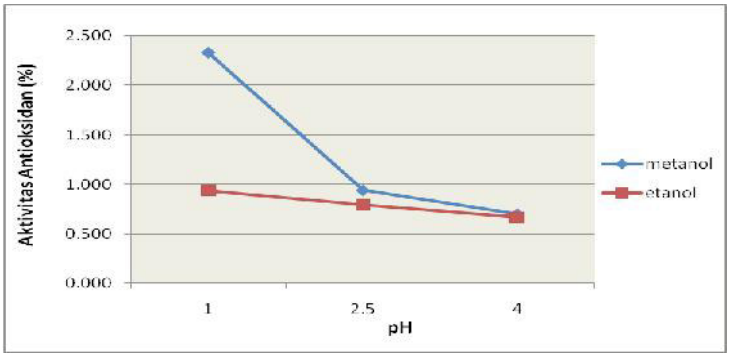


Figure 3. Graph of relation of the solvent types and pH on the antioxidant activity of super red dragon fruit flesh extract.

Figure 3 shows the highest average value of the total antioxidant activity of super red dragon fruit flesh extract was obtained from methanol solvent pH 1 treatment, it was 2.33%. While the lowest value was obtained from ethanol solvent pH 4.0 treatment, it was 0.67% but not significant different with methanol solvent pH 4.0. The principled of measurement of DPPH is based on reduction-oxidation reaction between DPPH and compound antioxidants. The more DPPH radical is reduced, the greater value of antioxidant activity in the sample. The results showed that the methanol pH 1.0 with polarity and acidity easier extract antioxidative compounds of super red dragon fruit flesh extract. This is in accordance with the opinion of the Harbourne (1996) stating that the methanol solvent with HCl 1% match to extract the anthocyanin pigment because it is polar and acid.

## CONCLUSIONS

Based on the results of the study can be summed up things as follows:

1. Type of solvent and pH have effect very real to yield, total anthocyanin and antioxidant activity of super red dragon fruit flesh extract.
2. The results showed that methanol solvents pH 1.0 treatment caused the highest of yield, total anthocyanin and antioxidant activity were 88.16%, 4.67% and 2.33% respectively.

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# EFFECTFIRSTTREATMENTONCHANGEOFANTIOXIDANTACTIVITYOFTAMARILLO (*Chyphomandra betacea S.*) THAT PROCESSED BECAME 'SAOS

**GAK. Diah Puspawati[1*], IM. Indri Hapsari[1], and P. Timur Ina[1]**
*[1]Science and Food Technology, Agriculture Technology Faculty, Udayana University, Denpasar-Bali*
**Correspondence author: dipa_ftp@yahoo.com*

**ABSTRACT**

The aim of the research knew antioxidant activity of tamarillo before and after processed became tamarillo sauces and enchanger after processed. Method of the research was group randomized design by 4 treatments: 1) tamarillo fruit no blanching; 2) tamarillo fruit by blanching; 3) tamarillo sauce from tamarillo fruit no blanching; and 4) tamarillo sauce from tamarillo fruit by blanching. Treatments were repeated 4 times so there were 12 units of experiments. Data were analyzed by analysis of varians with advance analysis by Duncan's Multiple Range Test. The result of research shows that antioxidant activity and phenol total of tamarillo fruit were enchange after became tamarillo sauce. The treatment of blanching could increase antioxidant activity and phenol total of tamarillo fruit or tamarillo sauce. The highest of antioxidant activity and phenol total was tamarillo by blanching that had characteristic such as: antioxidant activity and phenol total by 3.53% and 3.73% respectively. Enchanger of antioxidant activity and phenol total by 1.37 and 3.73% respectively, with sensory acceptable of color : 4,83 (rather like); odor: 4.08 (rather like); texture: 3.25 (neutral); taste: 2.93 (rather dislike); and overall acceptable: 3.92 (neutral).

***Keyword:*** *tamarillo fruit, tamarillo sauce, antioxidant activity, phenol total*

## INTRODUCTION

Tamarillo or tomato tree (*Chyphomandra betacea. Sendth)* belongs to the family solanaceae, a dump tree with red brownish fruit when it's ripe. One of the tamarillo superiority is as a source of vitamin and mineral (Gordon *et al.,* 2007). Kumalaningsih and Suprayogi (2006) reported that tamarillo contains vitamin C and β carotene as provitamin A. β carotene of tamarillo is quite close to β carotene of carrot. Tamarillo is known good for health and also used as a high blood pressure medicine.

Vitamin C and β carotene of tamarillo is characterized as an antioxidant which contains component from phenolic compound such as anthocyanin (derivate of aglicone sianidine, delphinidine, and pelargonidine).

Tamarillo fruit could be processed into a variety of product such as sauce, syrup or juices The present study was undertaken to evaluate the potential antioxidant of tamarillo before and after processed into tamarillo sauce that gave treatments blanching and non blanching.

## MATERIALS AND METHOD

### Materials

Raw material of research such as : tamarillo fruit was collected from Batur Village , Kintamani District, Bali Regency, material additive such as : tapioca starch, sugar, salt, garlic, onion, pepper, sugar wood, acetate acid were collected from Sanglah Market. Material of analysis consists : DPPH, methanol, $NaHCO_3$, follin coacealteu, galat standard, vitamin C standard.

### Methods

Tamarillo fruit was sorted, washed then sliced into two parts. Half of the fruit without steaming and the other part were steamed at 80□C for 5 minutes. The fruits were divided into two parts and the meat was separated from the skin. The meat was blended and added water with the ratio of 1:1 (meat: fruit). The parts were then analyzed for antioxidant activity and phenol total. The rest of the juice were added salt and spices (according to the formula) consists of grounded onion, garlic, red chilies, pepper, and cinnamon, mixed and wrapped with cotton the heated with low flame, after 20 minutes sugar and acetic acid (10%) was added and heated for another 10 minutes and tamarillo sauce was produced and analyzed for antioxidant activity and phenol total.

**Variable analysis**

Analysis was carried out to measure antioxidant activity (Ramamoorthy & Bono 2007, Modified), phenol total (Ramamoorthy & Bono 2007, Modified) and sensory evaluation (Soekarto, 1985). Detectable differences between steam and without steaming of tamarillo sauce on sensory evaluation were detected by a hedonic scale test. Panelist from the students of the Faculty of Food Science and Technology, Udayana University, were selected, based on their familiarity with sensory characteristics of tamarillo sauce as well as their ability to detect slight changes in these properties. The panelist's responses regarding to the acceptability of each samples with respect to color, odor, texture, taste, and overall acceptance were recorded on a seven point hedonic scale where seven represented the highest preference.

## RESULTS AND DISCUSSIONS

**Antioxidant Activity**

The results of antioxidant activity of tamarillo sauce showed a different significance amongst treatments. The average antioxidant activity of tamarillo fruit without steaming is 2.76%, with steaming is 3.53%, while tamarillo sauce without steaming is 1.59% compared to 2.16% of tamarillo sauce with steaming (Table 1). The enchanger antioxidant activity from tamarillo fruits to tamarillo sauce on steaming treatment was more little than without steaming. Steaming will affect on antioxidant activity and more little to decrease antioxidant activity because It could inactive enzyme that disturb phenolic component. They have ability as antioxidant.

**Table 1.** Average of antioxidant activity of tamarillo pulp and tamarillo sauce that was been steaming and without steaming

| Treatments | Average amount (%) |
|---|---|
| A0 (tamarillo fruit without steaming) | 2.76 b |
| B0 (tamarillo fruit with steaming) | 3.53 a |
| A1(tamarillo sauce without steaming) | 1.59 d |
| B1 (tamarillo sauce with steaming) | 2.16 c |

Note : The back alfabet at the same coloum show no significant effect (P>0.05)

**Total Phenol**

The total phenol of tamarillo fruit without steaming is 6.03% compared to 7.48% of tamarillo fruit with steaming. The same trend also occurred on tamarillo sauce, in which tamarillo sauce without steaming is 2.80%, while steamed tamarillo sauce is 3.76% (Table 2). In this case steaming has a significant effect on total phenol.

**Table 2.** Average phenol total of tamarillo pulp and tamarillo sauce that was been steaming and without steaming

| Treatments | Average amount (%) |
|---|---|
| A0 (tamarillo fruit without steaming) | 6.03 b |
| B0 (tamarillo fruit with steaming) | 7.48 a |
| A1(tamarillo sauce without steaming) | 2.80 d |
| B1 (tamarillo sauce with steaming) | 3.76 c |

Note : The back alfabet at the same coloum show no significant effect (P>0.05)

**Sensory Evaluation**

The sensory scores for tamarillo sauce prepared from steaming and without steaming shows no detectable differences such as : color 4.00 (neutral) and 4.83 (rather like); odor 4.00 (neutral) and 4.08 (neutral); texture 3.25 (rather dislike) and 3.58 (neutral); taste 2.82 (rather dislike) and 2.93( rather dislike) and overall acceptance 3.83 (neutral) and 3.92 (neutral) representatively (Table 3).

**Table 3.** Results of Sensory Evaluation

| Sample | color | Odor | texture | Taste | Overall acceptance |
|---|---|---|---|---|---|
| A1 (tamarillo sauce without steaming) | 4.00 a | 4.00 a | 3.25 a | 2.82 a | 3.83 a |
| B1 (tamarillo sauce with steaming) | 4.83 a | 4.08 a | 3.58 a | 2.93 a | 3.92 a |

Note : The back alfabet at the same coloum show no significant effect (P>0.05); (7= very like; 6= like; 5= rather like; 4= netral; 3= rather dislike; 2= dislike; 1= very dislike)

## CONCLUSIONS

1. The treatment steaming and without steaming had an effect on antioxidant activity and total phenol of tamarillo fruit and tamarillo sauce and no significant difference on sensory evaluation on tamarillo sauce
2. The highest antioxidant activity and phenol total on tamarillo fruit by steaming with characteristic such as : antioxidant activity and phenol total by 3.53% and 7.48% respectively and on tamarillo sauce with steaming have antioxidant activity and phenol total such as 2.16% and 3.78%
3. The enchager antioxidant activity and phenol total of tamarillo fruit effect process became tamarillo sauce on tamarillo fruit with steaming such as 1.37% and 3.70%, but on tamarillo fruit without steaming such as: 1.17% and 3.23%

## RECOMMENDATION

To produce tamarillo sauce, it is better to steam the fruit at 80$^{o}$C for 5 minutes.

## ACKNOWLEDGEMENT

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# CHANGES OF TEXTURE AND RESPIRATION RATE OF ZALACCA FRUIT IN MODIFIED ATMOSPHERE USING POLYETHYLINE PLASTIC PACKAGING AT VARIOUS PERFORATION

**Ida Ayu Rina Pratiwi Pudja**
*Department of Agriculture Engineering, Faculty of Agricultural Technology, Udayana University*
*Email: dayu_rina@yahoo.co.id*

**ABSTRACT**

The aim of this reaserch were to study changes of texture of Zalacca fruits from consequence of modified atmosphere packaging and to study of respiration rate of Zalacca fruit to preserve at modified atmosphere packaging.

The treatment of this reasearch was packaging with polyethyline plastic package or passive atmosphere with thickness 0.02 mm and perforation diameter hole is 0.6 cm. The various perforation is 0, 4, 8 and 12 hole. The storage time was set at 0, 2, 4, 6, 8, 10, 12 day in room temperature.

The result showed that the changes of texture of Zalacca fruit was increased during the storage time. The changes of texture rate was slowly at 8 hole polyethyline plastic perforation packaging. At the begining of the storage time, the respiration rate have been increased while in the midle have been decreased. Finally, at the end, the storage time have been increased. The respiration rate was slowly at 8 and 12 hole polyethyline plastic perforation packaging.

***Keywords:*** Modified Atmosphere, Polyethyline plastic perforation packaging, Zalacca Fruit

## INTRODUCTION

There is inclination that consumer be apt to food product which are fresh, practical and high quality (Daugherty, 1990; Labuza and Breene, 1989; Myers, 1989). In last decade in the U.S.A., it was noted that consumption of fresh food product increase in 12%, frozen food product increase by 10%, but canning food product decrease by 10%. Therefore, Indonesia has good opportunity of exporting fruits and vegetables. In order to increase the export value of agro-industry, fruits and vegetables should be cared and handled very well.

There is a problem that zalacca fruit is perishable and have short of time keeping. Senescence will occur after maturation and continued with damage due to the decrease of microbe defenses. Damage is usually caused by mechanical, physical, microbe, and physiological. Mechanical damage is frequently caused by chafe, peeled off, and bruised. Microbe damage is caused by infection and microbe activity. Physiological damage occurs due to internal metabolism reaction resulting in putrefaction.

Zalacca (*Salacca edulis*, Reinw) is Indonesia native fruit. Production was high, reaching 662,546 tons in 1995 (Anon, 1995 in Agung *et. al*., 1999). The cultivation areas of zalacca in Indonesia are Bandung (Batujajar), Tasikmalaya (Manonjaya), Jogjakarta (Sleman), Malang, North Sumatera, South Sulawesi, Central Java (Ambarawa and Magelang) and Bali.

The increasing production and fulfillment of demand should be balanced with post harvest treatment. This should be considered becausezalacca as other fruits is easy to get damage. The negative environment factors along the storage cause of fast decreasing quality resulting in short storage time.

In order to increase the storage time and to avoid damage, delaying the mature is performed by decreasing oxygen absorption and release of $CO_2$ of zalacca. This can be done with atmosphere modification. Low oxygen and high $CO_2$ in modified atmosphere give influence in decreasing respiration rate, ethylene production rate, retard the maturation and others relate to mature such as soften of fruit, change of color, lose of sugar and retard the putrefaction (Brecht, 1980; Kader, 1980).

An appropriate post-harvest treatment of zalaccamay give result in added value of long storage time which means more profit for the farmer, seller and other parties.

## AIMS

The aims of the research are

a. Studying changes of texture of fruit as result of storage in modified atmosphere.
b. Studying respiration speed of zalacca which are stored in modified atmosphere as function of $O_2$.

## METHODS

The research steps are preparation, respiration speed quantification, changes of the texture of fruit as result of storage in modified atmosphere, descriptive and graphical analyze.

A. Treatments

The treatments are polyethylene plastic cover with thickness of 0.6 cm (positive modified atmosphere) and storage time. The perforation of polyethylene plastic cover are 0 hole (without perforation), 4 holes, 12 holes and the storage time are 0, 2, 4, 6, 8, 10, 12 days. The samples treatment and one which is uncovered and used as the control group are stored in room temperature.

B. Respiration rates

a. In tight close container

Close system is used in this experiment (Hangar *et al*., 1992 and Lee, 1997 in Song *et al*., 1992). Three samples are put in glass container/jarwith volume of 2.126 liters. Glass container is closed tightly (with glue/paint/vaseline to avoid leak).

In the middle of the lid, a hole cover with rubber is set to take the gas sample. This container is put in room temperature. Gas samples are collected every 2 days in 12 days. $O_2$ and $CO_2$ concentration are detected with cosmotector. Type XP-314 cosmotector is used for $O_2$ and XP318 for $CO_2$. The relieved volume in the container is also counted. These treatments are repeated for three times.

b. In polyethylene plastic

Three samples are put in a sealed 6.80 liters volume of polyethylene plastic. A hole cover with rubber is set in the bottom part of the polyethylene plastic to make easy for taking the gas sample. The edge of the hole is sealed with glue/paint/vaseline to avoid leak. And then is put in room temperature. Gas samples are collected every 2 days in 12 days. $O_2$ and $CO_2$ concentration are detected with cosmotector. Type XP-314 cosmotector is used for $O_2$ and XP318 for $CO_2$. The relieved volume in the container is also counted. These treatments are repeated for three times.

c. Change of Texture

The probe with diameter of 0.52 mm of FT 327 model of Hand Penetrometer Effegi is used to test physically the stringent of Bali's zalacca to get data of texture of Bali's zalacca. A small area of skin of sample was peeled and then holds by one hand while the other hand pushed the sample with Hand Penetrometer Effegi. The number in $kg/cm^2$ read from the tool shows the texture value of the fruit. The test was repeated for four times for each sample and it was done for some samples. The values were then calculated for the average number.

## RESULT AND DISCUSSION

1. Changes of Texture

Texture is a decisive factor of fresh fruits. Changes of texture of Bali's zalacca showed that there was a variation of texture of Bali's zalacca in storage. The graph of texture changes can be seen in Figure .

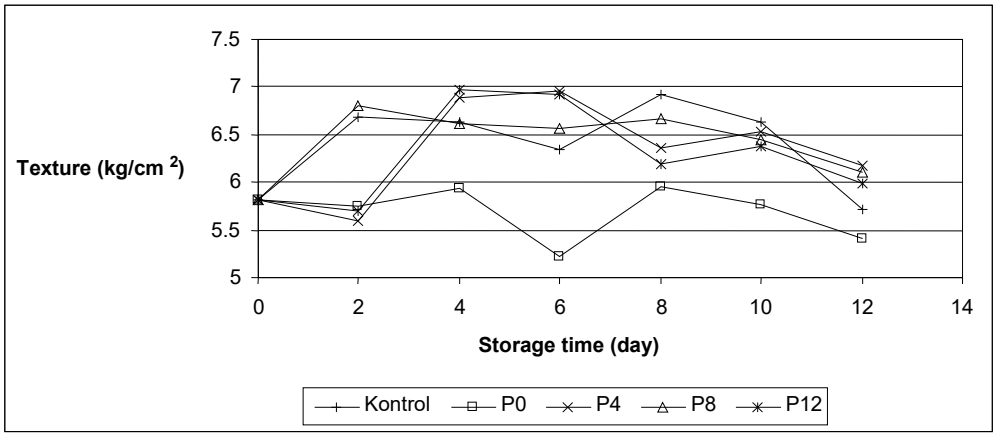


Fig 1. The changes of texture of Bali zalacca fruits during the storage time

Figure 1 showed that the changes of texture level of control of Bali's zalacca and all the Bali's zalacca treated with different perforation of polyethylene plastic container decrease during the storage. The decrease intextureis caused by saturated decomposing propectin to pectin enzyme activity. The solid state rate is caused by the change of cell size and turgor. The reason of texture become lossis related with high level of respiration activity and transpiration of the fruit causing loss of liquid. Therefore, cell size and pressure of inside cell to the cell wall decrease (Suter, 1988).

2. Respiration Rate

The data of $O_2$ respiration rate of Bali's zalacca in storage is presented in Figure 2. Figure 2 showed that in every treatment, respiration rate of $O_2$ changed rapidly, then were going slowly, and finally in a certain time changed rapidly until the last period of storage.

Figure 2shows that $O_2$ respiration rates were increase in the beginning of storage, then decrease, and increase again till the last period of storage. Decrease of respiration rate is caused by a decrease in the substrate for respiration. As addition, the $O_2$ in the container is used by the fruits for respiration and substrate oxidation. With limitation of $O_2$ in the container causing the delay of chlorophyll degradation, low production of $C_2H_4$, decrease in production rate of ascorbic acid, change in composition of unsaturated fat and degradation of pectin were not fast as in nature. These things were reflected as the delay of mature of fruit and, therefore, storage capacity of the fruit is long (Amiarsi *et al*., 1996).

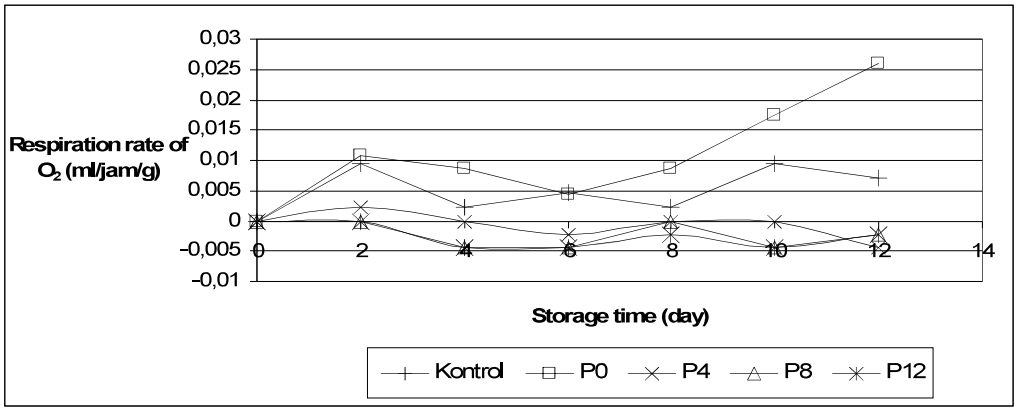


Fig 2. $O_2$ Respiration rate of Balizalacca fruits during the storage time

## CONCLUSION

- The weight of Balizalacca fruit was decreased during the storage time.
- At the beginning of storage time, the result showed that the respiration rate of O2 have been increased while in the middle have been decreased.
- We can use the polyethylene plastic packaging at perforation with 12 holes in modified atmosphere.

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# THE EFFECT OF PULP AND TAMARIND LEAVES RATIO (*Tamarindus indica* L) AND COOKING TIME ON THE ANTIOXIDANT ACTIVITIES

**Sri Mulyani*, A.A.P.Agung Suryawan W., Bambang Admadi, H., and Gracea Pabiola Florencia T**
*Department of Agroindustrial Technology, Faculty of Agricultural Technology,*
*Udayana University, Kampus Bukit Jimbaran - Bali*
**Correspond: moel_pstp@yahoo.com*

**ABSTRACT**

The objective of this research was to determine the effect of pulp and tamarind leaves ratio (*Tamarindus indica* L.) and cooking time tow the antioxidant activity and to determine the ratio of pulp and tamarind leaves that produces the highest antioxidant. Completely Randomized Design two-factor was used in this research with factor 1: pulp fruit and leaves tamarind with ratio (0:100; 25:75; 50:50; 75:25 and 100:0)% and factor 2: cooking time (2.5 and 5 minutes) this treatment was repeated for 3 times. Research showed that the treatment of tamarind leaves and pulp ratio, time cooking and their interactions significantly affected: the total phenols, antioxidant capacity and vitamin C. 100% tamarind leaves with the cooking time of 2.5 minutes gave the best antioxidant activity with total phenol = 0.7 mg/100g, antioxidant capacity = 0.053 mg eq ga/100g, vitamin C = 0.252 mg/100g and the highest ability to inhibit the formation of MDA.

***Keywords:*** tamarind pulp, tamarind leaves, antioxidants, cooking time.

## INTRODUCTION

Antioxidants have an important role in preventing damage on food that contains fat. In food industry synthetic antioxidants such as BHT, BHA, and TBHQ were widely used to prevent damage in food that contains fat (Santosa *et al.*, 2002). However, natural and synthetic antioxidants are also able to protect cells from oxidative damage and minimize cell damage. Antioxidants can reduce the aging process and prevent degenerative diseases such as heart disease, diabetes mellitus, and cancer. Using synthetic antioxidants in a long time and in excessive doses it can cause carcinogenic or cancer in the body (Santosa *et al.*, 2002).

Concerning about the safety of synthetic antioxidant usage has resulted in utilization of natural antioxidants. Mulyani *et al.* (2006) proved that turmeric and tamarind extract contains antioxidants that can inhibit the oxidation of fat and its antioxidant capacity approach the capacity of synthetic antioxidant *butylated hidroksitoluene* (BHT). Mulyani and Mahaputra (2008) proved that the antioxidant capacity of turmeric and tamarind beverages (*kunyit asam*) is 0.11 mg eq ga/100g. Bioactive components from plants more widely studied because of the bioactive potential to reduce the risk of degenerative diseases. Some bioactive components from these plants are: vitamin C, vitamin E, carotenoids, phenolic groups, particularly polyphenols, and flavonoids (Prakash, 2001; Okawa *et al*., 2001).

Pulp and leaves of tamarind are commonly used for traditional drink to maintain a healthy body or as a functional beverage. Turmeric and tamarind beverages (*kunyit asam*) has been proven contains bioactive component, the flavonoid which is able to inhibit the oxidation process in fat (Mulyani *et al.*, 2006). Leaves and pulp of tamarind are rich of flavonoids, phenols, pectin, saponin (Mursito, 2004). The pulp of tamarind also contains anthocyanin (Nair *et al.,* 2004). Flavonoids are potential antioxidants to prevent free radical formation, as well as natural anti-bacterial (Raharjo, 2005). Heating the tamarind pulp will produce xyloglycans acid, tannins, saponins, sesquiterpenes, alkaloids, and phlobatamins (Pauly, 1999). Xylose and xyloglycans in the tamarind pulp are as cosmetics and medical ingredients (Pauly, 1999), while anthocyanins can inhibit the enzyme cyclooxygenase (COX) which prevents the release of prostaglandin (Nair *et al.*, 2004). Tannins, saponins, sesquiterpene, alkaloids, and phlobataminsin tamarind leaves are useful for calming the mind and reducing psychological pressure (Pauly, 1999). Tamarind leaves also can be used as a high-cholesterol-lowering drug with the chemical content of saponins, flavonoids and tannins.

Temperature is one factor that affects the activity of the antioxidants. Lee *et al.*, (1986) said that warming ginger extract at a temperature of $100^0$C for 10 minutes can reduce the antioxidant content of nearly 20%. Cooking turmeric tamarind beverage (*kunyit asam*) in a high pressure (50-90) within 0.5

minutes KPa retains the antioxidant activity (Mulyani and Mahaputra, 2008). Based on the research done by Mulyani *et al.*, (2006), tamarind pulp and leaves have antioxidant activity, but the ratio which produces the highest antioxidant level is unknown. Therefore, more research in this area needs to be conducted.

## MATERIALS AND METHODS

### Materials

Tamarind fruits and leaves (*Tamarindus indica* L) is a local variety derived from Karangasem Regency, Bali. Tamarind leaves were separated from stems and cleaned, while the pulp was separated from its seed then cooked based on the treatment. Chemicals used in this study were distilled water, ethanol Pa (Brataco Chemicals), buffer-2.2-1-picryldhydrazyl diphenyl (DPPH) (Sigma).The tools used in this study are: Spectrophotometer (Turner SP-870), Centrifuge (EC HN-S II 0-9000 rpm), Vortex (Thermolyne) Oven (Blue M), Incubator (Memmert, model 500), Hitachi Spectrophotometer 912 (Roche Diagnostics, Gmbh.), pH meters and other glassware.

### The design of experiments

Completely Randomized Design (CRD) factorial was used in this study. It consists of two factors, the ratio of the tamarind pulp and tamarind leaves (K) and time of cooking (T)

Factor I: The ratio of the tamarind pulp and tamarind leaves (K)
K1 = 100% tamarind pulp and 0% tamarind leaves
K2 = 75% tamarind pulp and 25% tamarind leaves
K3 = 50% tamarind pulp and 50% tamarind leaves
K4 = 25% tamarind pulp and 75% tamarind leaves
K5 = 0% tamarind pulp and 100% tamarind leaves

Factor II: Time of cooking (T)
T1 = 2.5 minutes
T2 = 5 minutes

The treatment was repeated two times to obtain 20 experimental units. The data was analyzed to see its diversity and continued to be analyzed by using Duncan multiple comparison test

### Implementation Research

Cleaned tamarind pulp and leaves then weighed according to the treatment with the ratio of: 0%: 100%; 25%: 75%; 50%: 50%; 75%: 25%; 100%: 0% in a total weight of 100 g. Then added water with a ratio of 1: 4 (w / v) and heated on a water heater for 2.5 hours and 5 minutes after it boils with some occasionally stirring. Cooled down the mixture and filtered, then the filtrate was analyzed. The analysis included was: 1) the antioxidant capacity of DPPH method, (Yun, 2001); 2) Antioxidant activity assays with the TBA method (Kikuzaki and Nakatami, 1993); 3) total phenol (Sakanaka *et al.*, 2003); 4), vitamin C level with 2.6 D method (Sudarmaji *et al.*, 1984)

## RESULTS AND DISCUSSION

### Total Phenols

The treatments have a significant effect on total phenols (Table 1). The amount of phenol is affected by the pH level in tamarind and turmeric mixture, a level of synergism will occur at a certain pH level achieving the highest level of total phenol (Suwariani and Suhendra, 2008). In this study, the pH was achieved in treatment of 75% tamarind leaves and 25% tamarind pulp. Valko *et al.*(2007) stated that time

cooking will lead phenolic compounds to decrease. Phenolic compounds are compounds that are easily damaged, especially at high temperatures. The results showed the highest total phenol obtained on treatment of 75% tamarind leaves and 25% tamarind pulp with 2.5 minutes cooking

Table 1: Total phenols (mg/100g) from tamarind pulp and leaves treatment and cooking time

| Ratio (K) | Time cooking (T) | |
|---|---|---|
| Tamarind leaves (%): Tamarind pulp (%) | 2.5 minutes (T1) | 5 minutes (T2) |
| 0 : 100 (K1) | 0.76 b | 0.42 cd |
| 25 : 75 (K2) | 0.46 c | 0.31 de |
| 50 : 50 (K3) | 0.72 b | 0.22 e |
| 75 : 25 (K4) | 1.02a | 0.51 c |
| 100 : 0 (K5) | 0.75 b | 0.41 cd |

**Antioxidant Capacity**

The results show that treatment and its interaction has a significant effect on the antioxidant capacity (Table 2). Antioxidant capacity is proportional to the total phenols. Tamarind leaves have a higher antioxidant activity than the pulp because the content of flavonoid in the leaves is higher than in the pulp. High level of tamarind leaves in the treatment, increases the antioxidant capacity. Nair *et al.* (2004) stated that the tamarind leaves contain saponins, flavonoids and tannins. According to the study done by Lee *et al.* (1986), cooking time causes antioxidant capacity to decrease. Treatment with the highest antioxidant capacity is generated from a treatment with a composition of 75% and 100% tamarind leaves in 2.5 minutes cooking time.

Table 2. Antioxidant capacity (mg eq ga/100g) from tamarind pulp and leaves treatment and cooking time

| Ratio (K | Time cooking (T) | |
|---|---|---|
| Tamarind leaves (%): Tamarind pulp (%) | 2,5 minutes (T1) | 5 minutes (T2) |
| 0 : 100 (K1) | 0,033 ef | 0,032 f |
| 25 : 75 (K2) | 0,045 bc | 0,038 def |
| 50 : 50 (K3) | 0,039 cde | 0,044 cd |
| 75 : 25 (K4) | 0,051 ab | 0,043 cd |
| 100 : 0 (K5) | 0,053 a | 0,051 ab |

**Vitamin C**

The results show that treatment and its interaction has a significant effect on the vitamin C (Table 3). The highest vitamin C is generated from the treatment with 100% tamarind pulp in 2.5 minutes cooking time. Tamarind pulp contains fruit acids such as tartaric acid, malic acid, citric acid, succinic acid, asenat and ascorbic acid with vitamin C content of 0.6 mg/100g (Thomas, 1989). Tamarind leaves has a little content of vitamin C, since the largest compound on leaves are phenols which has a function as an antioxidants (Nair, 2004). Cooking causes damage to vitamin C because vitamin C is water soluble, easily oxidized and cannot stand the heat (Harper *et al.*,1986).

Table 3. The level of vitamin C (mg/100 g) from tamarind pulp and leaves treatment and cooking time

| Ratio (K) | Time cooking (T) | |
|---|---|---|
| Tamarind leaves (%): Tamarind pulp (%) | 2,5 minutes (T1) | 5 minutes (T2) |
| 0 : 100 (K1) | 0,704 a | 0,440 b |
| 25 : 75 (K2) | 0,373 bc | 0,198 e |
| 50 : 50 (K3) | 0,352 bcd | 0,307 cd |
| 75 : 25 (K4) | 0,681 b | 0,439 bc |
| 100 : 0 (K5) | 0,252 de | 0,176 e |

**Antioxidant activity by the TBA test**

Inhibition trend of MDA (malodialdehyde) formation during the observation can be seen in Figure 1. Studies show that all treatments can inhibit the formation of MDA. The results also show that treatment of 100% tamarind leaves in 2.5 minutes cooking time has the highest ability in inhibiting MDA formation, which means it has the highest of antioxidant activity. It also means that the antioxidant activity of phenolic compounds is better in the inhibition of MDA formation rather than vitamin C.

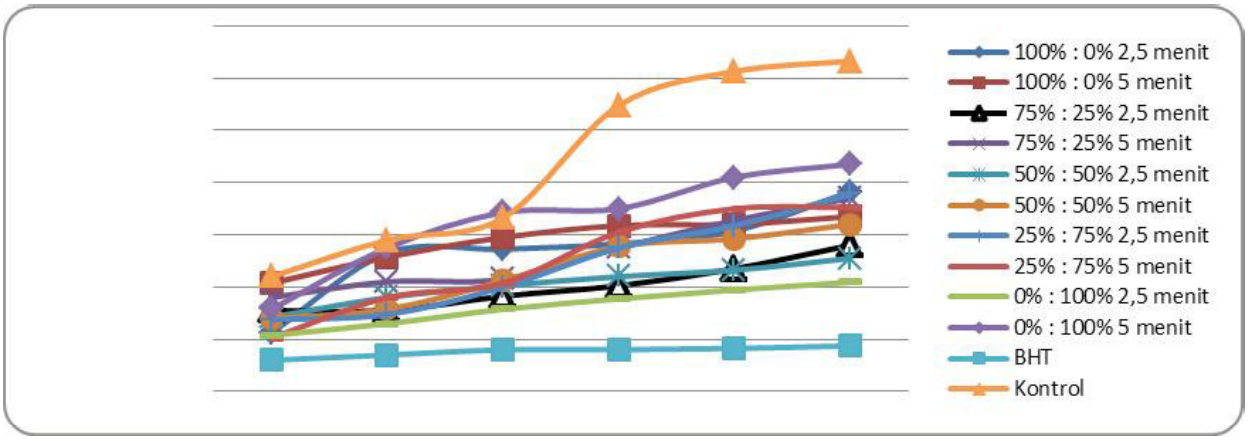


Fig 1:Curve effect of the treatment on the inhibition of MDA formation.

## CONCLUSIONS AND SUGGESTION

**Conclusion**

1. Tamarind leaves and pulp ratio treatment, cooking time and their interactions had significant effect on total phenols, antioxidant capacity and vitamin C.
2. Treatment 100% tamarind leaves with 2.5 minutes cooking time give the best antioxidant activity with the total phenols = 0.75 mg/100g, antioxidant capacity = 0.053 mg eq ga/100g and vitamin C = 0.252 mg/100g, and has highest ability inhibiting the MDA formation

**Suggestion**

Further research needs to be done to obtain tamarind leaves processing technique which is able to maintain antioxidant activity.

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# EFFECT OF CMC (*Carboxy Methyl Cellulose)* CONCENTRATION ON CHARACTERISTIC OF PINEAPPLE (*Ananas comosus* L. *Merr)* JUICE

**Timur Ina[1], GAK. Diah Puspawati[1], and IW. Dedi Juliana[3]**
*[1]Department of Science and Food Technology, Agriculture Technology Faculty,*
*Udayana University,Kampus Bukit Jimbaran-Bali*
*Email: timur_ina@yahoo.go.id*

**ABSTRACT**

The aim of the research was to know the effect of CMC concentration on the characteristic of pineapple juice and find out the best concentration of CMC to pineapple juice with the best characteristic. The research used completely randomized block design consisted of 5 treatments concentration of CMC, such as control (0 %, 0.1%, 0.2%, 0.3% and 0.4%). The results shown concentration of CMC significantly affected of vitamin C, sugar total, pH, total solute solublel, stability and thickly on pineapple juice. The best characteristic of pineapple juice was on 0.4 % CMC with characteristics on vitamin C 29,16 mg/100g, solute soluble total: 13.60 $^{o}$Brix**,** sugar total: 3.09 %, pH 3.98 and stability 338.951 seconds, color: yellow, odor: specific of pineapple and like, texture: rather thickly and like, taste: like, and overral acceptance: like

***Keywords:*** *concentration, CMC, characteristic, pineapple, juice*

## INTRODUCTION

Pineapple is one of fruits that commonly consume. It has very good taste and odor , the color is very nice and contaiened vitamins C and sugar. Moreover it is easy to get when we needed. But It is a perishable product. it needs technology to handle on post harvest. One of methods is processing bécame new product. That is pineapple juice

Pineapple juice is kind of beverage which is liked by children every mens. Recently juice is accepted as healty drink .

Pineapple juice is very easy to be digested, prevent cáncer specially colon cáncer.

There are many problems in processing of pineapple to be pineapple juice, among case problema as the stability of the juice. Between solute soluble and solute insoluble was very easy to seperate. It is there for need methods that maintain stability of solute. C*arboxy Methyl Cellulose* (CMC) could use as stabilizer

CMC is white particel, colorless, tasteless, when it mixe in the wáter, it become coloid ang clear liquid. That way it usefull to increase the stability and viscosity of soluble. Beside that it can prevent sendiment (Winarno 2002). CMC is stabilizer that easily to find in and has a goo charácter tan other stabilizer such as gum and xanthan.

At present, there is no information about CMC as stabilizer in pineapple juice. That way this research is to know concentration of CMC as stabilier in pineapple juice.

## MATERIALS AND METHODS

### Material and Tools

The material of the reseach were pineapple (queen) from Batu Kandik Market, citroen (Gajah), sugur (Gulaku), mineral water, CMC (sumber Urip shop), Amilum, NaOH (merk), $H_2SO_4$ ( merk ), $I_2$ (Iodine), KI (merk), HCL (merk) and $Na_2CO_3$ (merk).

The tools of the research were stainlesstel knife, spoon, cutting board, stirer, blander (Phillips), stove (Hitahci), aluminium foil (Klin Pack), scale glass (Pyrex), erlenmayer (Pyrex), beaker glass, fillter paper , *Hand Refraktometer,* glass volume, pipet and pH meter.

**Design of research**

The research use Completily Randomize Block Designs contained of 5 treatment concentration. They are :

CO : ( 0 % concentration of CMC)
C1 : (0,1% concentration of CMC)
C2 : (0,2% concentration of CMC)
C3 : (0,3% concentration of CMC)
C4 : (0,4% concentration of CMC)

Each of treatments are repeated twice so there were 15 treatment units. The data are analized with analysis of varians, if it significant, it continue with Duncan Multiple Range Test.

**Research Procedure**

**Making of pineapple juice**

1. First, Sortation of pineapple, after that pill of skin
2. Wash them, then cut intp small pieces and weight amount 500 g.
3. Put in the blender and add wáter (2:1) and blender it until bécame pineaplle puree
4. The pineapple puree was added 14% sugar and CMC as designned for treatments.
5. After that they are dough and boild at temperatur 40º C for 3 minutes
6. In the last boiling, they were added 2% citroen acid.
7. Finally pineapple juice was placed into glass bottle. Then pasteurize at 65 oC for 30 minutes.

**Variabel concent**

The research variables were vitamins C (Sudarmadji *et al*.,1982), total solute soluble (Apriyantono *et al*., 1989), suger total by Luff Schoorl methods ( Sudarmadji, *et al*, 1984), pH (AOAC, 1994), stability, sensory evaluation (color, odor, texture : skor test and hedonic test; taste and overall acceptance : hedonic test) (Soekarto, 1985).

## RESULTS AND DISCUSSIONS

**Vitamin C**

The result of varians analysis show that the treatments have significant effect (P<0.05) on vitamins C content. The mean of vitamin C conten of pineapple juice are shown at Table 1.

Table 1. The mean of vitamin C of pineapple juice (mg/100g).

| Treatments | Vitamin C (mg/ 100g) |
|---|---|
| CO (0% concentration of CMC)) | 31,86 a |
| C1 (0,1% concentration of CMC) | 31,42 ab |
| C2 (0,2% concentration of CMC) | 30,74 b |
| C3 (0,3% concentration of CMC) | 29,69 c |
| C4 (0,4% concentration of CMC) | 29,16 c |

Note: The back alfabet at the same coloum show no significant effect (P>0.05)

Table 1 show that vitamin C content of pineapple juice decreased as the concentration of CMC increased. The higest of vitamin C content is on CO (0 % concentration of CMC) that was: 31,86 mg/100g and the lowest was on C4 (0,4% concentration of CMC) treatment that was: 29,16 mg/100g. Increasing of CMC concentration decreased vitamin C of pineapple juice. This is due to fact that the CMC has strong basa properties and vitamin C is not stable on basa condition. Increasing CMC could be decreasing vitamin C content.

**Sugar Total**

The result of varians analysis show that the treatments had significant effect (P<0.05) on sugar total. The mean of sugar total of pineaaple were shown at Table 2

Table 2. The mean of sugar total of pineapple juice (%)

| Treatments | Sugar Total (%) |
|---|---|
| CO (0% concentration of CMC) | 3,28 a |
| C1 (0,1% concentration of CMC) | 3,19 b |
| C2 (0,2% concentration of CMC) | 3,17 b |
| C3 (0,3% concentration of CMC) | 3,17 b |
| C4 (0,4% concentration of CMC) | 3,09 c |

Note: The back alfabet at the same coloum show no significant effect (P>0.05)

Table 2 show that sugar total of pineapple juice decreased as the concentration of CMC increased. The higest of sugat total is at CO treantment that was 3,28 % and the lowest was at C4 (0,4% concentration of CMC) that was 3,09 % . Sugar has soluble in hot water and CMC also has soluble in hot water. This is due to the fact that the free water that tied sugar decrease, The result sugar total of pineapple juice decrease

**Acidity degree (pH)**

The result of varians analysis show that the treatments had significant effect (P<0.05) on pH The mean of pH of pineaaple juice were presentation at Table 3

Table 3. The mean of pH of pineapple juice

| Treatments | pH |
|---|---|
| CO (0% concentration of CMC) | 3,87 d |
| C1 (0,1% concentration of CMC) | 3,89 c |
| C2 (0,2% concentration of CMC) | 3,90 bc |
| C3 (0,3% concentration of CMC) | 3,92 b |
| C4 (0,4% concentration of CMC) | 3,98 a |

Note: The back alfabet at the same coloum show no significant effect (P>0.05)

Table 3 show that pH of pineapple juice increased as the concentration of CMC increased. The higest pH was at C4 (,4% concentration of CMC) that was pH 3.98 and the lowest pH was at C0 ,that was pH 3.87. Increasing of CMC concentration cause the pH increase. It caused CMC has strong basa and weak acid properties. Increasing of CMC concentration could pH increase. It relation with vitamin C that was decrease.

**Total solut soluble**

The result of varians analysis show that the treatments had significant effect (P<0.05) on total solut soluble. The mean of total sulute soluble of pineaaple juice were presentation at Table 4

Table 4. The mean of total solute soluble of pineapple juice

| Treatments | Total solute soluble (% Brix) |
|---|---|
| CO (0% concentration of CMC) | 13,00 c |
| C1 (0,1% concentration of CMC) | 13,20 b |
| C2 (0,2% concentration of CMC) | 13,33 b |
| C3 (0,3% concentration of CMC) | 13,53 a |
| C4 (0,4% concentration of CMC) | 13,60 a |

Note: The alfabet after the same coloum show no significant effect (P>0.05)

Table 4 show that the higest of total solute soluble at C4 (0,4% concentration of CMC) that was 13,60% and the lowest was at CO, that was 13,00%. It was caused CMC could cover of solid surface. It is there for the partical-partical dispertion could not combine and total solute soluble could be stable and increase.

**Stability**

The result of varians analysis show that the treatments had significant effect (P<0.05) on stability. The mean of stability of pineaaple juice were shown at Table 5.

Table 5. The mean of stability of pineapple juice

| Treatments | Separete time (seconds) |
|---|---|
| CO (0 % concentration of CMC) | 1.685 e |
| C1 (0,1% concentration of CMC) | 7.945 d |
| C2 (0,2% concentration of CMC) | 12.624 c |
| C3 (0,3% concentration of CMC) | 137.261 b |
| C4 (0,4% concentration of CMC) | 338.951 a |

Note: The back alfabet at the same coloum show no significant effect (P>0.05)

Table 5 show that the highest of stability was at C4 (0.4% concentration of CMC) treatment, that was 338951 seconds and the lowest was at C0 treatment, that was 1685 seconds. It was caused CMC as stabilizer material. Increasing of CMC concentration because that stability of pineaple juice could more stable. CMC had hidrofilic properties, because that CMC could soluble in water and swollen of granula. Water that out side could not free move because that soluble of juiece could be stable (Fennema., *et al*.1996).

**Sensory evaluation**

The result of varians analysis show that the treatments had significant effect (P<0.05) on viscosity and did not had significant effect on odor, taste and overall aceptance . The mean of viscocity of pineaplle juice were shown at Table 6. The odor, taste and overall aceptance of pineaaple juice were presentationat Table 7 .

Table 6. The mean of viscocity of pineapple juice

| Treatments | **viscocity** |
|---|---|
| CO (0% concentration of CMC) | 2,93 e |
| C1 (0,1% concentration of CMC) | 3,60 d |
| C2 (0,2% concentration of CMC) | 3,80 c |
| C3 (0,3% concentration of CMC) | 3,73 b |
| C4 (0,4% concentration of CMC) | 4,06 a |

Note: The back alfabet at the same coloum show no significant effect (P>0.05)

Table 7.The mean of color, odor, taste,and overall aceptance of pineaplle juice (hedonic test)

| **Treatments** | | | |
|---|---|---|---|
| | **Odor** | **taste** | **Overall aceptance** |
| CO | 5,26 a | 5,13 a | 5,33 a |
| C1 | 5,66 a | 5,06 a | 5,53 a |
| C2 | 5,26 a | 5,06 a | 5,40 a |
| C3 | 5,46 a | 5,40 a | 5,33 a |
| C4 | 5,20 a | 5,26 a | 5,40 a |

Note: The back alfabet at the same coloum show no significant effect (P>0.05)

**Viscosity**

Table 6 shows that the highest of viscocity was at C4 (0.4% concentration of CMC), that was 4.06 (rather viscous) and the lowest of viscocity was at C0 (control) that was 2.93 (netral). Increasing of CMC concentration could increase viscocity of pineapple juice. It was caused CMC could increase stability of total solute soluble and could absorb water that outside surface. The free water at outside surface could not free to move and soluble became stable.

**Odor**

Table 7 show that the odor of the higest concentration (0.4%) of CMC was 5.20 (rather like).

**Taste**

Table 7 show that the higest concentration (0.4%) of CMC was 5.26 (rather like).

**Overall acceptance**

Table 7 shows that the higest concentration (0.4%) of CMC was 5.40 (rather like).

## CONCLUSIONS

1. Increasing of CMC concentration at pineapple juice had significant effect on vitamin C, sugar total, pH, total solute soluble, stability and viscocity and no significant effect on odor, taste and overall acceptance
2. Additional CMC at 0.4% concentration show the best characteristic of pineapple juice
3. Characteristic of pineapple juice such as vitaminc C, sugar total, total solute soluble, pH, stability, viscocity, odor, taste and overall acceptance with 29,16 mg/100g, 3,09 % ,13,60 $^{o}$brix, pH 3,98, 339.951 seconds, 4.06 (rather viscous), 5.20 (rather like), 5.26 (rather like) and 5.40 (rather like) respectively.

**Suggestion**

In producing pineapple juice was suggest addition concentration of CMC at 0.4% concentration.

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# STUDY ON STORAGE METHOD OF INSTANT *LEDOK*

**I Ketut Suter[1*], I Made Anom Sutrisna Wijaya[2] and Ni Made Yusa[1]**
[1] Traditional Cousine Research Center, Udayana University, Jl.PB.Sudirman, Denpasar, Bali.
[1,2] Faculty of Agriculture Technology, Udayana University, Bukit Jimbaran, Badung, Bali. Phone/Fax. (0361) 701801
*Correspondence Author: suter_ketut@yahoo.co.id

**ABSTRACT**

Ledok is a traditional food from Nusa Penida, Bali, in form of non-rice porridge. The main ingredients of ledok were corn and cassava, and other ingredients were peanut, red bean and spinach. These ingredients are locally available. Instant ledok is a product developed from ledok traditional. The study was conducted in order to find out the storage method of instant ledok. Experiments was conducted by Random Block Design, with six storage methods and replicated three times. The storage methods were: (1) Package by polypropylene, ingredients and spices were mixed. (2) Package by polypropylene, ingredients and spices were not mixed. (3) Package by polyethylene, ingredients and spices were mixed (4) Package by polyethylene, ingredients and spices were not mixed. (5) Package by aluminum foil, ingredients and spices were mixed (6) Package by aluminum foil, ingredients and spices were not mixed. Instant ledok were storage four months in room temperature. The characteristics of instant ledok observed were sensory characteristic and its nutrient contents. The result of the study showed that after four months storage, the best storage method was package by polyethylene and all of ingredients of instant ledok were mixed together.

***Keywords***: Instant ledok, polypropylene, polyethylene and aluminum foil

## INTRODUCTION

*Ledok* is one of traditional food from Nusa Penida, Klungkung regency which uses corn and cassava as main ingredient without using rice. Others ingredient such as long bean, read bean and *kemangi* are used as additional ingredient. Instant *ledok* is product developed from *ledok* traditional. Study to improve the nutritive value instant *ledok* added with white tuna (*tenggiri*) has reported by Sugitha, *et al*. (2007) and added with seaweed and mackerel has reported by Suter, *et al*. (2011).

Packaging is intended to preserve food against spoilage and contamination and extend its shelf life (Vaclavik, 1998 and Morris, *et al*., 2004). Three kinds of packaging materials include polypropylene (PP), polyethylene (PE) and aluminum foil (AF) common used in food (Syarief, *et al*., 1989), but if it's used to packed instant *ledok*, the effect of its packaging materials to instant *ledok* during storage did not known yet. The aim of this study is to find out the kind of packaging materials and storage method of instant *ledok*.

## MATERIALS AND METHODS

### Materials

The *ledok* materials consisted of white corn, yellow cassava, red bean, peanuts, spinach, *kemangi*, *salam* leaf, onion, chili, salt, lime, and mackerel. The materials for analysis consisted of $H_2SO_4$, NaOH, tablet Kjeldhal, boric acid, HCL, alcohol, petroleum benzene, methyl red, and methyl blue, PP, PE and AF.

### Experimental design and Formulation

Experiments was conducted by randomized block design, with six storage methods as a treatment and replicated three times. The storage methods were: (1) Package by polypropylene, ingredients and spices were mixed (PP1). (2)Package by polypropylene, ingredients and spices were not mixed (PP2). (3) Package by polyethylene, ingredients and spices were mixed (PE1) (4) Package by polyethylene, ingredients and spices were not mixed (PE2). (5) Package by aluminum foil, ingredients and spices were mixed (AF1), (6) Package by aluminum foil, ingredients and spices were not mixed (AF2). Instant ledok were storage four months in room temperature.

The formulation of raw materials was white corn (110 g), cassava (55 g), red bean (55 g), peanuts (55 g), spinach (9.0 g), *kemangi* (3.0 g), *salam* leaf (5.0 g), *lengkuas* (5.0 g ), water (4 x total weight of raw materials), salt (1 g), spices (15 g), and mackerel (30 g) (Suter, *et al*., 2011).

**Experimental Steps**

a. Preparation and Storage of instant *ledok*

The preparation of instant *ledok* included: 1) yellow cassava was firstly pealed, then washed and chopped into size of 0.5 x 0.5 x 0.2 cm. Then the chopped cassava was steamed at 100 $^{o}$C for 35 minutes and finally after cooling the steamed cassava was dried at 70$^{o}$C in oven dryer; 2) Peanuts and red beans was boiled at 100$^{o}$C, and then dried at 70$^{o}$C in oven dryer. The ratio of peanuts or red bean and water used in boiling was 1 : 3; 3) White corn was boiled at 100$^{o}$C, for 37 minutes and then dried at 70$^{o}$C in oven dryer. The ratio of white corn and water used in boiling was 1 : 3; 4) Spinach, *kemangi* and *salam* leaf blanched at 85$^{o}$C for 5 minutes, and then continued by drying at 70$^{o}$C in oven dryer until dried; 5) Lengkuas was minced and then dried in oven drier at 70$^{o}$C until dried. Mackerel was steamed at 100 $^{o}$C, and then dried in oven drier at 70$^{o}$C until dried. Preparation for spices involved the activities as follows: firstly the raw materials of spices such as onion, red chili, salt, and lime were weighed as formulation, and then blended using mixer. The blended spices were dried into the oven drier at 70$^{o}$C until dried. Finally, all of materials weight as formulation, mixes together and than package as treatments. The instant ledok storage in room temperature for four months.

b. Analysis

The analysis performed into the instant *ledok* included: 1) Sensory analysis (color, aroma, taste, texture, and overall preference) using Hedonik Test (Larmond, 1977) and 2) Analysis of nutrition fact (carbohydrate, protein, fat, ash, and water content) using the *Proximate analysis* methods (Apriyantono, *et al.*, 1989). Crude fiber using hydrolysis method (Sudarmadji, *et al*., 1984), Iodine using spectrophotometer methods and vitamin C using iodine titration method (Jacobs, 1962).

## RESULTS AND DISCUSSION

1. Fresh Instan *Ledok*

The nutrition fact of ready to serve instant *ledok* before storage (fresh) was water 79.60 %, ash 0.74 %, protein, 3.17 %, fat 3.85 %, carbohydrate 12.64 %, crude fiber 3.43 %, vitamin C 21.15 mg/100 g, and Iodine 0.26 mg/kg. The results of sensory test was: color 4.93 (neither like nor dislike), aroma 4.53 (neither like nor dislike), taste 5.40 (like slightly), texture 5.47 (like slightly) and overall preference 5.13 (like slightly). Instant *ledok* ready to eat and instant *ledok* during storage (Figure 1).

2. Instan *Ledok a*fter Four Months Storage

a. Nutrition fact of instant *ledok.*

The nutrition fact of ready to serve instant *ledok* was tabulated at Table 1. It can be seen from Table 1 that the kind of packaging materials and storage method of instant *ledok* were not significantly affecting the water, ash, protein, fat, carbohydrate, crude fiber and iodine content, however, significantly affecting the vitamin C content. Instant *ledok* PP2 had the highest vitamin C (32.00 mg/100 g), however, instant *ledok* PP1 had the lowest vitamin C (19.59 mg/100g).This might be caused by the deterioration of vitamin C in spices.

Table 1. Nutrition fact of instant *ledok* after four months storage

| Treatment | Water (%) | Ash (%) | Protein (%) | Fat (%) | Carbohydrate (%) | Crude fiber (%) | Vitamin C (mg/100 g) | Iodine mg/kg |
|---|---|---|---|---|---|---|---|---|
| PP1 | 75.85 a | 0.84 a | 4.26 a | 3.34 a | 15.73a | 6.61 a | 19.59 c | 0.29 a |
| PP2 | 74.91 a | 0.91 a | 4.80 a | 3.18 a | 16.21 a | 5.72 a | 32.00 a | 0.26a |
| PE1 | 76.94 a | 0.90 a | 3.25 a | 2.95 a | 15.96 a | 6.49 a | 31.60 a | 0.29a |
| PE2 | 75.55 a | 0.81 a | 4.17 a | 3.18 a | 16.29 a | 7.03 a | 29.91 ab | 0.33a |
| AF1 | 74.96 a | 0.85 a | 3.00 a | 3.15 a | 18.05 a | 6.73 a | 28.17 ab | 0.28a |
| AF2 | 75.05 a | 0.87 a | 3.69 a | 3.42 a | 16.99 a | 4.84 a | 26.51 b | 0.25a |

b. Sensory characteristics of instant *ledok*

The sensory characteristics of ready to serve instant *ledok* was tabulated at Table 2. It can be seen from the Table 2 that the kind of packaging materials and storage method of instant *ledok* were not significantly affecting the preference on color, aroma, taste, texture, and overall preference. That is mean all of the instant *ledok* acceptable with score range 4,2 – 4,4 (neither like nor dislike).

Table 2. Sensory characteristics of instant *ledok* after four months storage

| Treatments | Color | Aroma | Texture | Taste | Over all preference |
|---|---|---|---|---|---|
| PP1 | 4.73a | 4.07 a | 4.27a | 3.93 a | 4.33a |
| PP2 | 4.60a | 4.07 a | 4.27a | 4.07 a | 4.20a |
| PE1 | 4.67a | 4.13 a | 4.47a | 4.07 a | 4.27a |
| PE2 | 4.67a | 4.00 a | 4.53a | 4.33 a | 4.40a |
| AF1 | 4.60a | 4.20 a | 4.27a | 4.00 a | 4.20a |
| AF2 | 4.93a | 3.67 a | 4.53a | 3.80 a | 4.40a |

*) Score 1 = dislike very much; 2 = dislike moderately; 3 = dislike slightly; 4 = neither like nor dislike; 5 = like slightly; 6 = like moderately and 7 = like very much.


**Figure 1.** Instant *ledok* ready to eat and instant *ledok* during storage

## CONCLUSION

The results of the study showed that after four months storage the best storage method is package by polyethylene, ingredients and spices were mixed (PE1) and instant *ledok* promise to consume.

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# THE INFLUENCE OF SOLVENT TYPE AND RATIO OF MATERIAL WITH SOLVENT ON ANTIOXIDANT ACTIVITY OF TAMARILLO EXTRACT (*Chyphomandra betacea* Sendth)

**Ni M. Yusa[1], G.A.K. Diah Puspawati[1] and I W. Dirga[1]**
[1]*Faculty of Agriculture Technology Udayana University, Bukit Jimbaran, Badung, Bali*
*Telp.Fax: (0361) 701801.*
*Correspondence Author: nimadeyusa@yahoo.co.id*

**ABSTRACT**

This research was aimed to determine the influence of the solvent type and ratio of materials with solvent on antioxidant activity of tamarillo extract and to determine the solvent type and ratio of materials with solvent that would produce pulp tamarillo extract with the highest antioxidant activity. This research used Completely Randomized Design by factorial pattern with two factor. The first factor was solvent type that contained three levels such as methanol, ethanol, and water. The second factor was the comparison between the materials and the solvent that contained three levels such as 1 : 4, 1 : 6, and 1 : 8. The treatments were repeated twice and resulted in eighteen units of experiments. The observed variables were yield, phenol total, and antioxidant activity. The research showed that the interaction influenced the yield but did not influence the phenol total and antioxidant activity. The solvent type influenced the yield, phenol total, and the antioxidant activity. The ratio of the materials with solvent influenced the yield but did not influence the phenol total and the antioxidant activity. The best results were obtained from the ethanol solvent on a ratio of materials with solvent 1 : 4 with a yield of 3.34 %, phenol total of 7.75 % and antioxidant activity of 1.83 %

***Keywords:*** *Pulp tamarillo extract, methanol, ethanol,, and antioxidant activity.*

## INTRODUCTION

Tamarillo is a kind of plant with oval shaped fruits that goes sharper on its ends, the fruit color becomes brownish red once it is ripen. This fruit is local to Bali island and is specially grown in Bangli regency and Bedugul area. The usage of tamarrilo is still limited, mostly are sold fresh and used as an ingredient of *rujak*. Tamarrilo is rich with vitamin A, vitamin C, minerals and important fibers to help keeping a healthy body (Gordon *et al,* 2007). The brownish red on its color and flesh once it is ripen indicates the rich content of anthocyanin. Anthocyanin is a water-soluble pigment that appears naturally in various plants. Vitamin C is also a non-enzymatic antioxidant which solves in water. Extraction process that uses solvent is influenced by solvent types and the amount of solvent used (Utami, 2009). This researched is aimed to determine the influence of solvent type and the ratio of solvent material on the activity of antioxidant within the tamarillo pulp extract.

## MATERIALS AND METHODS

The research was conducted at Food Analyst Laboratory, Nutrition and Biochemical Laboratory, and Food Processing Laboratory of Agriculture Technology Faculty, University of Udayana, in March 2011 until May 2011. The material used is tamarillo type red with optimum ripeness, which is showed by its red color going even all over the fruit surface.

The research used factorial pattern of Completely Randomized Design which consisted on two factors which were the treatment of solvent type and the ratio of solvent material:

1. First factor is solvent (P), consist of : (P1 = Methanol; P2 = Ethanol and P3 = water solvent).
2. Second factor is the ratio of solvent (A) which were: A1= ratio of material and solvent 1:4, A2= ratio of material and solvent 1:6, and A3= ratio of material and solvent 1:8).

The treatment is repeated twice and resulted in 18 units of experiments. Data obtained is analyzed by variance analysis and when the treatment showed any real influence, it would the be continued with DMRT test to observed the difference between the combination of treatments (Steel and Torrie, 1993).

## 1. Execution of the research

### 1.1. The making of tamarillo pulp extract

The making of tamarillo pulp extract followed the maceration method. A fine juice of tamarillo is weighed at 50 g then inserted into Erlenmeyer 500 ml (wrapped in aluminum foil), then added HCl 1 % until the pH turned 2.5. Then it was continued by maceration method by mixing material with solvent in the shaker at 130 rpm for 24 hours at room temperature.

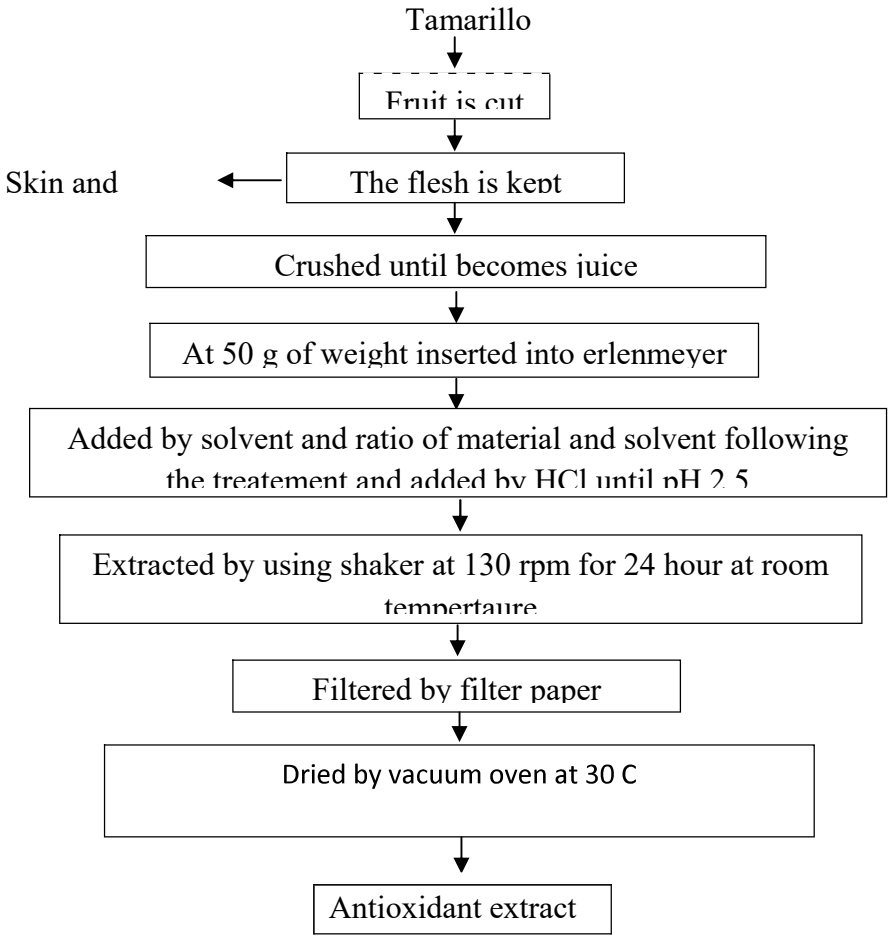


Figure 1. Diagram of tamarillo extraction process (Saati, 2002) modified

Afterward the result was filtered by regular filter paper so then a tamarrilo extract obtained, still mixed with the solvent. The solvent then vaporized by a vacuum oven at ± 30°C. Diagram of the tamarillo extraction process can be seen at Figure 1.

## 2. Observed variable

Variables observed covered yield (Sudarmadji *et al*., 1989), phenol total by using modified method of Folin-Ciocalteu phenol (Ramamoorthy and Bono 2007), and antioxidant activity by using modified method of DPPH (Yun, 2001).

### 2.1 Yield

Tamarilo extract yield was obtained by a calculation (Sudarmadji, *et al*., 1989). As following :

$$\text{Yield (\%)} = \frac{\textit{extract obtained}}{\textit{juice early weight}} \times 100\ \%$$

### 2.2 Total phenol determination

Total phenol analysis was using phenol Folin-Cioccalteu reactor. The solvent absorbance was measured by spectrophotometer at 760 nm. The number obtained then determined as the phenol total using gallic acid as the standard with concentration of 0, 40, 60, 80, and 100 ppm.

### 2.3 Antioxidant activity

Absorbance was read at 517 nm. The determination of ability to capture DPPH free radical used vitamin C standard curve with concentration 0, 40, 60, 80, and 100 ppm. The antioxidant activity was measured by the following formula:

Antioxidant activity (%) = $\frac{X \times TV \times FP}{W}$ x 100%

where :

X: sample concentration (mg/l)

TV: solvent volume total (l); FP: dilution factor; W: sample weight (mg)

## RESULTS AND DISCUSSION

### 1. Yield

Average values of tamarillo yield can be seen Table 1.

Table 1. showed that average values of tamarrilo yield range at 2.24 % - 3. 34%. Treatment of solvent type that resulted in the highest yield was ethanol at 3.34%, meanwhile the solvent type that resulted in the lowest yield was water at 2.2.4 %.

Table 1. Average values of tamarillo yield (%)

| Solvent type | Ratio between material and solvent | | | average |
|---|---|---|---|---|
| | 1:4 | 1:6 | 1:8 | |
| Methanol | 3.64 | 2.49 | 2.32 | 2.82 ab |
| Ethanol | 3.91 | 3.17 | 2.93 | 3.34 a |
| water | 2.59 | 2.31 | 1.83 | 2.24 b |
| average | 3.38 a | 2.66 ab | 2.36 b | |

Remark: average values that were followed by similar letter at the same column or row showed unreal difference (P<0.05)

Table 1. also showed that the average values of tamarillo extract yield with the treatment of ratio material with solvent range from 2.36% to 3.38 %. The treatment of ratio between material and solvent that was able to produce the highest yield was ratio of 1:4 which was at 3.38%, meanwhile the lowest yield is at the ratio of 1:8 at 2.36%

### 2. Phenol total

The experiment result on phenol total can be seen at Table 2. The illustration of interaction of solvent type and ratio between material and solvent can be seen at Figure 2.

Table 2. Total phenol average (%) of tamarillo extract in accordance with gallic acid standard treatment.

| Solvent type | Ratio between material and solvent | | | average |
|---|---|---|---|---|
| | 1:4 | 1:6 | 1:8 | |
| Methanol | 9.68 a | 6.80 bcd | 6.22 de | 7.56 b |
| Ethanol | 7.75 bcd | 8.37 abc | 5.48 e | 7.20 b |
| Water | 9.35 ab | 9.66 a | 10.34 a | 9.78 a |
| Average | 8.93 a | 8.27 a | 7.35 b | |

Remark: different letters at the columns and lines interaction, average of solvent, and average showed significant difference (P<0.01)

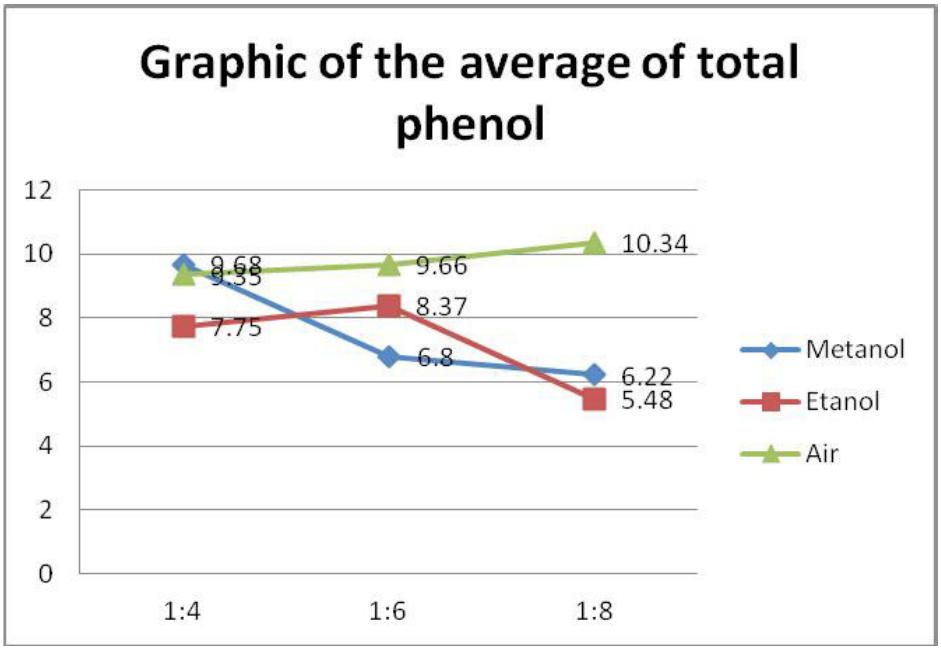


Figure 2. Graphic of average value of total phenol

Table 2. showed that the interaction between solvent type and ratio of material and solvent over the phenol total of tamarillo extract range from 5.48 % to 10.34 %.

Figure 2 explained that the highest phenol total was found at the interaction of water solvent with the ratio 1:8 was 10.34 %, meanwhile the lowest phenol total was found at the interaction of ethanol solvent with ratio 1:8.

Table 2 also explained that average value of phenol total of tamarillo extract with treatment of ratio between material and solvent ranged between 7.35 % to 8.93 %.

**3. Antioxidant activity**

Data of the experiment on the antioxidant activity can be seen at Table 3. The illustration between the solvent type with ratio between material and solvent can be seen at Figure 3.

Table 3. Average value of antioxidant activity (%) of tamarillo extract based on vitamin C standard.

| Solvent type | Ratio material and solvent | | | Average |
|---|---|---|---|---|
| | 1:4 | 1:6 | 1:8 | |
| Methanol | 0,68 b | 0,62 b | 0,73 b | 0,67 b |
| Ethanol | 1,83 a | 1,07 b | 0,76 b | 1,22 a |
| Water | 0,62 b | 0.77 b | 0,73 b | 0,70 b |
| Average | 1,04 a | 0,82 a | 0,74 a | |

Remark: different letters on the columns and lines of interaction, average of solvent type, average of ratio material and solvent indicated significant differences. ($P<0.01$)

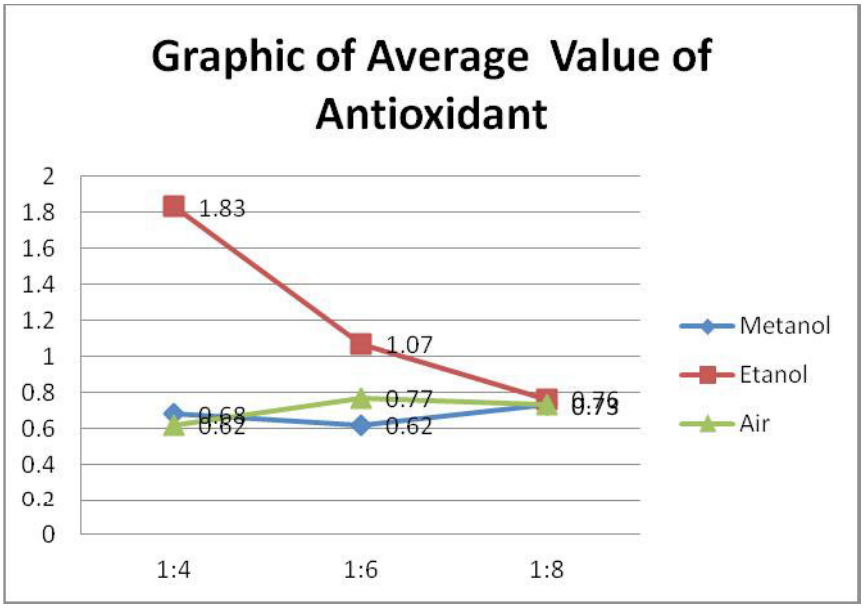


Figure 3. Graphic of antioxidant activities.

Table 3. Showed that the interaction between the solvent type with the ratio between material and solvent on tamarillo extract antioxidant activity range between 0.62 % to 1.83 %. On the table 3 also was showed that the average values of antioxidant activities of tamarillo extract on the treatment of solvent type range between 0.67 % to 1.22 %

Figure 3 showed that the highest antioxidant activities was found on interaction with ethanol solvent with ratio of 1:4 at 1.83%, meanwhile the lowest antioxidant was found on the interaction with methanol solvent and water which was respectively 1:6 and 1:4 at 0.62 %

From this experiment can be concluded that ratio between material and solvent influenced significantly on yield and phenol total but did not influence tamarillo extract antioxidant activities. The best results was obtained from the treatment of ethanol solvent on ratio between material and solvent 1:4 with yield results 3.34%, phenol total 7.75% and antioxidant activities at 1.83%

Further research is needed on the length of time and the temperature of extraction of tamarillo extract in order to find out the optimum ability of capturing free radicals.

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# DETERMINATION OF POTATO SWEET VARIETIES AS DIET FOOD OF DIABETES MELLITUS PATIENTS

**Bambang Admadi Harsojuwono, I Gusti Ngurah Agung and Sri Mulyani**
*Faculty Of Agricultural Technology, Udayana University*

**ABSTRACT**

The research aim was to know the effect of potato sweet varieties to resistant starch, anthocyanin content and a blood glucose enhancement after consumption. Besides that to determine the potato sweet variety that contain resistant starch and anthocyanin is highest but the impact of blood glucose enhancement after consumption is lowest.

Research using completely randomized design with 10 treatments was repeated 3 times. The potato sweet varieties that were experimented include Daya, Borobudur, Prambanan, Kalasan, Mendut, Selat, Cilembu, Gunung Kawi, Papua and Japan variety. In this study, the tests are made using bioassay by SD rats. Observations included resistant starch and anthocyanin content and also an increase of blood glucose.

The results showed that the effect of potato sweet varieties was significant to the resistant starch content and a blood glucose enhancement after consumption, while anthocyanin only detected in Gunung Kawi variety. The Gunung Kawi variety has the highest content of resistant starch and anthocyanin but the lowest impact of blood glucose enhancement after consumption. The Gunung Kawi variety has resistant starch content of 14.538%, anthocyanin of 530.544 mg/100g and the impact of blood glucose enhancement after consumption of 9.667 mg/dL.

***Keywords***: the potato sweet varieties, resistant starch, anthocyanin, blood glucose enhancement

## INTRODUCTION

Efforts to handle diabetes mellitus disease, among others through the management of diet and proper food selection (Marsono, 2002). But strictly regulated diet with a fixed menu and a set quantity of intake resulted in people with diabetes mellitus was bored. This condition is likely to cause breach of the diet, so it is very dangerous to health. In relation to the need to find suitable food, preferred and able to control the rise in blood glucose. Lately, sweet potato has been government and public attention in the fulfillment of nutrition and food security as it is easy to grow and high productivity (Anonymous, 2003). According Hasbullah (2010) there are five main varieties of sweet potato in Indonesia, namely Daya, Prambanan, Borobudur, Mendut, Kalasan and also the preferred local varieties such as Selat, Cilembu and Gunung Kawi (Anonymous, 2010). According Suprapti (2008), each sweet potato variety having own specification. Orange color of sweet potatoes such as Data, Prambanan and Borobudur contains carotenoids, the yellow varieties such as Kalasan contains provitamin A, and the color purple as the Selat containing anthocyanin (Hartoyo, 2007). Sweet potatoes with red color skin containt fiber is higher than white, but lower starch levels (Hartoyo, 2007). According Nainggolan (2006), sweet potato has low calories and protein but high fiber content. Particular yam contains beta-carotene, lutein and zeaxantin that can prevent cell damage caused by free radicals (Apriaji, 2009). Meanwhile, Suda et al (2008) informs that sweet potato contains vitamins (B1, B2, C and E), minerals (Ca, K, Zn), fiber, carbohydrates and antioxidants such as flavonoids, beta-carotene and anthocyanins which are capable of controlling antihiperglikemik blood glucose. Further, Suda et al., (2008) suggested people with diabetes to consume food containing anthocyanins and indole alkaloid compound called ipomine A, which is an acid glycoprotein and acts as antidiabetic (Kusano, Abe and Takamura, 2008).

Ludvik et al, (2009), stated that a diet with sweet potato 4 g / day for 6 weeks can lower blood glucose by enhancing insulin function with no effect on insulin secretion. This is due to the type of antidiabetic active ingredient ipomine A (Yuan et al, 2009) and anthocyanins that inhibit the enzyme alpha-glucosidase (Suda et al, 2008). According to Kusano and Abe (2007), sweet potato has great potential in the treatment of diabetes mellitus, because it has an effect on the reduction of blood glucose by enhancement insulin secretion and function. Giving sweet potato in patients with type 2 diabetes mellitus beneficial meet daily

nutritional adequacy and obtain the active ingredient that serves as a drug hypoglycemia (Saloranta et al., 2007). Other information that sweet potato is known to contain higher levels of resistant starch (low glycemic index) than other tubers such bentul, Uwi, forest taro, taro and potatoes (Harsojuwono et al, 2009).

The above description shows that the potential of sweet potato as a food diet diabetes mellitus. The problem is not all sweet potatoes suitable as a diet food because one of them is highly dependent on sweet potato varieties.Varieties influence the content of resistant starch and antiglikemik compounds such as anthocyanins. By Anonymous (2011b), MSU-yielding varieties of sweet potato 03028-10 and 03065-03 with RIS harvest 125 days each containing anthocyanin 590.8 mg/100 g and 510.8 mg/100 g, while varieties Antin-1 containing 33.89 mg/100 g. According Hartoyo (2007), white sweet potatoes contain starch higher than other colors. Meanwhile, according to Antarlina (2001), sweet potato with optimal harvesting age resistant starch containing anthocyanin and higher than the harvest is less or too old. Further explained sweet potato MSU-03028-10 to harvest 125 days containing anthocyanin 590.8 mg/100 g. In this regard it is necessary to find the right varieties of sweet potato to obtain food diet for people with diabetes mellitus.

The research aim to determine the effect of sweet potato varieties resistant starch and anthocyanin content and the blood glucose enhancement as well as get sweet potato varieties that contain resistant starch and anthocyanins are high but the impact of the lowest blood glucose levels enhancement after kosumisinya.

## METHOD

Research using completely randomized design with treatment of 10 varieties, namely Daya, Borobudur, Prambanan, Kalasan, Mendut, Selat, Cilembu, Gunung Kawi, Papua, Japan, which is repeated three times. Testing conducted using animal bioassay SD rats as much as 30 experimental units are divided into 10 treatment groups, each consisting of three rats as replication. The step of the study as follows:

**1) Identify the nutritional content of sweet potato**

Nutrient levels identified include carbohydrates, fats, proteins, vitamins, minerals, ash, and water resistant to sweet potato starch raw and cooked. Raw sweet potato is prepared as follows: sweet potato (as varieties) peeled, washed and crushed in a blender and analyzed proximate. Meanwhile mature sweet potato is prepared as follows: sweet potato (as varieties) peeled, washed, steamed and then crushed in a blender and analyzed proximate.

**2) Preparation of standard feed**

Made with a standard feed mixture ratio: 620.69 g corn starch, casein 140g, 100g sucrose, 40 g of soybean oil, 50 g CMC, 35g mineral mixture, 10 g of a mixture of vitamins, 2.5 g kholin bitartrate and 1.8 g L -cystin. The mixture was diadon by adding a little hot water if necessary until a dough clay. The dough is then printed with the clay molding machines feed / pellet with a long cylindrical shape then dried in an oven at a temperature of 50$^{o}$C for 12 hours.

**3) Preparation of diabetic rats**

A total of 30 SD rats were 3 months old with an average weight of 200 g were injected with 80 microliters so conditioned alloxan diabetes, and placed individually in special cages were divided into 10 treatment groups for each group there are 3 rats and each mutually exclusive. Rats were placed in a clean place at room temperature with adequate ventilation and lighting.

**4) Treatment of standards feed**

Unconditioned diabetic mice that have been placed in special cages then given standard feed each 5 g per serving to be given to drink ad libitum (without limitation) for 6 days.

**5) Preparation of feed treatment and measurement of resistant starch and anthocyanin content**

Sweet potato varieties suitable treatment, cleaned by washing and peeling the skin and cut into pieces the size of + 200 g and steamed until cooked. Furthermore, appropriate treatment yam cooked, ready

to serve as feed mice treated with a dose has been determined. Meanwhile, most of the sample cooked sweet potato starch content measured resistance and anthocyanin.

6) **Fasting treatment of rats and blood glucose measurement**
The mice that had been fed with standard fodder for the next 6 days fasted for 12 hours in a clean cage at room temperature with adequate ventilation and lighting. At the end of the period of fasting blood glucose levels of the mice were measured using a Blood Glucose Test Meter.

7) **Feeding treatment and measurement of blood glucose** enhancement
Mice that had been fasting for 12 hours and have measured fasting blood glucose levels, further fed treatments (sweet potato with different varieties and harvesting) was mature, 5 g per serving by giving ad libitum drinking (no limit). Furthermore, blood glucose levels of the mice were measured after 30 minutes after a meal.

8) **Measurement of resistant starch, anthocyanin, blood glucose enhancement**
Measurements and observations made on the raw and cooked sweet potato as described above is basically composed of the nutrients include carbohydrates, fats, proteins, water, minerals, vitamins, resistant starch and anthocyanin. It also made measurements of rat blood glucose levels during fasting and after eating using Glucose Blood Test Meter aimed to calculate the blood glucose enhancement levels when their diabetes condition. The blood glucose enhancement is a blood glucose level after meal reduced blood glucose levels during fasting.

The resulting data were analyzed variance and followed by Duncan's multiple comparison test. Treatment of sweet potato varieties resistant starch content and have high anthocyanin and causing an increase in the lowest blood glucose enhancement is best varieties as food diet for people with diabetes mellitus.

## RESULT AND DISCUSSION

**Nutrient content some raw and cooked sweet potato varieties**

Results of analysis of the nutrient content of some raw sweet potato varieties (not steamed), which includes moisture, ash, fat, protein, vitamins and carbohydrates shown in Table 1. In the table, the range of the average water content of some varieties of sweet potato between 77.772 to 89.427%, ash content ranged from 0.007 to 0.013%, the fat content from 0.090 to 0.481%, the protein content of 0.673 to 1.592%, vitamin levels from 0.123 to 0.837% and the levels of carbohydrates from 17.620 to 20.267%. Descriptively apparent nutritional content of sweet potato varies depending on the variety.

Meanwhile, the results of the analysis of the nutritional content of some cooked sweet potato varieties are also shown in Table 1. The table shows the range of average water content between 78.686 to 80.797%, ash content of 0.004 to 0.011%, the fat content of 0.075 to 0.367%, the protein content of 0.627 to 1.236%, vitamin levels from 0.114 to 0.603% and carbohydrate levels from 17.942 to 19.582%. Like the raw conditions, variations in nutrient content appears also depends on the varieties of sweet potato. Sweet potato varieties closely related to physiological and morphological properties. Allegedly some sweet potato varieties have the ability to absorb high potassium. Potassium role in the formation and transport of carbohydrates, as well as a catalyst in the formation of proteins, neutralizing the reaction in the cell, especially organic acids, raising the meristem tissue growth, strengthen the enforcement of the stem so it does not collapse. Besides potassium also served to increase the quality of the bulbs, activating the enzyme either directly or indirectly, and helps root development (Rosmarkam and Yuwono, 2011).

Table 1. Average nutrient content of some varieties of sweet potato in raw and cooked conditions

| SWEET POTATO VARIETY | WATER CONTENT (%) | | ASH CONTENT (%) | | FAT CONTENT (%) | | PROTEIN CONTENT (%) | | VITAMIN CONTENT (%) | | CARBODYDRATE CONTEN (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RAW | COOKED | RAW | COOKED | RAW | COOKED | RAW | COOKED | RAW | COOKED | RAW | COOKED |
| **Borobudur** | 77.772 | 78.686 | 0.011 | 0.010 | 0.481 | 0.367 | 1.347 | 1.236 | 0.123 | 0.120 | 20.267 | 19.580 |
| **Mendut** | 78.479 | 79.793 | 0.011 | 0.010 | 0.232 | 0.182 | 1.592 | 0.717 | 0.631 | 0.543 | 19.056 | 18.754 |
| **Selat** | 78.953 | 80.254 | 0.012 | 0.011 | 0.142 | 0.140 | 1.592 | 0.641 | 0.521 | 0.467 | 18.779 | 18.486 |
| **Gn Kawi** | 79.945 | 80.797 | 0.010 | 0.009 | 0.104 | 0.101 | 0.714 | 0.641 | 0.349 | 0.120 | 18.878 | 18.332 |
| **Prambanan** | 80.427 | 80.516 | 0.010 | 0.010 | 0.310 | 0.301 | 0.796 | 0.627 | 0.837 | 0.603 | 17.620 | 17.942 |
| **Daya** | 79.930 | 79.948 | 0.013 | 0.011 | 0.181 | 0.146 | 0.876 | 0.717 | 0.444 | 0.367 | 18.557 | 18.813 |
| **Papua** | 78.686 | 79.525 | 0.012 | 0.008 | 0.369 | 0.314 | 1.459 | 1.144 | 0.794 | 0.569 | 18.680 | 18.440 |
| **Kalasan** | 78.500 | 78.996 | 0.007 | 0.004 | 0.207 | 0.205 | 1.751 | 0.800 | 0.702 | 0.412 | 18.833 | 19.582 |
| **Jepang** | 78.944 | 79.245 | 0.007 | 0.007 | 0.198 | 0.146 | 0.673 | 0.643 | 0.526 | 0.489 | 19.652 | 19.470 |
| **Cilembu** | 79.460 | 79.638 | 0.007 | 0.006 | 0.090 | 0.075 | 1.151 | 1.119 | 0.228 | 0.114 | 19.064 | 19.047 |

Comparison of the nutrient content of sweet potato varieties descriptively between cooked and raw showed that enhancement water content while other nutrients such as ash, fat, protein, vitamins and carbohydrates decreased. The change is only due to changes in the composition of the comparison between the weight of each nutritional component to the total weight due to an increase in the amount of water vapor in the inclusion of sweet potato during steaming. Water vapor diffuses into the material and then binds to the components of the material resulting in an increase in water content (Abia et al, 2003).

**Levels of anthocyanin, resistant starch and the blood glucose enhancement**

The analysis of variant shows that the effect of sweet potato varieties was significant to the resistant starch content of cooked sweet potato and the blood glucose enhancement after consumption, but does not affect the raw sweet potato. Meanwhile, Duncan multiple comparisons indicate different levels of resistant starch in cooked sweet potato, as well as an blood glucose enhancement after consumption of cooked sweet potato varieties were compared but showed no difference in raw sweet potato.

The cooked sweet potato cooked contain resistant starch ranged from 9.070 to 14.538%, while the increase in blood glucose ranged from 9.667 to 28.000 mg / dL. While the resistant starch content of raw sweet potato ranged from 6.134 to 7.812% anthocyanin levels however can only be detected in sweet potato varieties Gunung Kawi both raw and cooked conditions whose levels respectively 553.342 and 530.554 mg/100g mg/100g as shown in Table 2. This variation is due to differences in the nutritional content of the different nature of the physiology and morphology of these sweet potato varieties (Rosmarkam and Yuwono, 2011).

Table 2 also shows that all the resistant starch content of sweet potato varieties tends to increase after cooked (after steaming) than when raw. This is due to gelatinization and starch into resistant starch retrogradasi result of using heat treatment. According to Englyst and Cumming, (1987) in Marsono, (1998) the formation of resistant starch during processing of starchy materials is influenced by various factors, such as ways of processing, material moisture content, pH, heating temperature, the amount of penglukosangan heating and cooling, freezing and drying. This is consistent with the statement Goni, et al., (2006), the amount of resistant starch is affected by the treatment of the sample such as milling and cooking. Therefore, the content of resistant starch in foods can be manipulated through the food processing and breeding practices (breeding) in plants with high amylose content such as breeding varieties of cereals / legumes (Rahman, et al., 2007).

Table 2. The average of anthocyanin and resistant starch content of sweet potato varieties as well as some increase in blood glucose after consumption

| SWEET POTATO VARIETY | ANTHOCYANIN CONTENT (mg/100g) | | RESISTANT STARCH CONTENT (%) | | BLOOD GLUCOSE ENHANCEMENT (mg/dL) |
|---|---|---|---|---|---|
| | RAW | COOKED | RAW | COOKED | |
| **Borobudur** | ttd | ttd | 7,323 a | 12,234 b | 23,000 c |
| **Mendut** | ttd | ttd | 7,556 a | 14,395 d | 11,000 a |
| **Selat** | ttd | ttd | 6,756 a | 13,393 bc | 20,333 bc |
| **Gn. Kawi** | 553,342 | 530,554 | 7,812 a | 14,538 d | 9,667 a |
| **Prambanan** | ttd | ttd | 6,367 a | 12,415 b | 22,000 c |
| **Daya** | ttd | ttd | 7,112 a | 13,506 c | 19,667 bc |
| **Papua** | ttd | ttd | 6,912 a | 12,508 bc | 20,333 bc |
| **Kalasan** | ttd | ttd | 6,242 a | 12,151 b | 22,000 c |
| **Jepang** | ttd | ttd | 6,776 a | 13,709 cd | 17,667 b |
| **Cilembu** | ttd | ttd | 6,134 a | 9,070 a | 28,000 d |

Description: The same letters behind the numbers in the same column indicates no significantly different at 5% error level

High levels of resistant starch sweet potato varieties to be contained Japan, Mendut and Gunung Kawi, while the low-conceived Cilembu sweet potato varieties. Meanwhile, the low enhancement of blood glucose that occurs after consumption of Gunung Kawi sweet potato varieties, and were higher after consumption of Cilembu sweet potato varieties. This seems related with the high content of anthocyanin and resistant starch of the Gunung Kawi sweet potato varieties so that decomposition resistant starch into simple glucose in a slow digestion, especially of the enzyme alpha-glucosidase that breaks down starch inhibited by anthocyanin. As described by Suda et al. (2008) anthocyanin is a compound that can inhibit the enzyme alpha-glucosidase and prevents hydrolysis of disaccharides into monosaccharides in the intestinal body of people with diabetes mellitus.

The description explains that sweet potato varieties Gunung Kawi is the best sweet potato varieties because it contains two compounds that play an important role in suppressing the blood glucose enhancement in the body, especially for diabetes mellitus patients.

## CONCLUSIONS AND RECOMMENDATION

### Conclusion

1) Sweet potato varieties significantly affect to the resistant starch content of cooked sweet potato and the blood glucose enhancement after consumption, but had no effect on levels of resistant starch raw sweet potato.
2) Sweet potato Gunung Kawi is the best varieties used as food diet, the raw characteristics containing the resistant starch of 7.812%, anthocyanin of 553.342 mg/100 g and cooked characteristics containing the resistant starch of 14.538%, anthocyanin of 530.554 mg/100 g and the impact of blood glucose enhancement after consumption of 9.667 mg / dL.

### Recommendation

Further research needs to be done to get food diet of raw sweet potato Gunung Kawi that provides a high level of consumption but can suppress the blood glucose enhancement after consumption.

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# ANTIBACTERIAL ACTIVITY OF FERMENTED *PANGIUM EDULE* REINW. SEEDS EXTRACT AGAINST FOODBORNE PATHOGENS

**Nurul Syazwani Bt. M. Zainin, Alfi Khatib, and Yaya Rukayadi***
*Department of Food Science, Faculty of Food Science and Technology, and Laboratory of Natural Products, Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM),*
*43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
*Tel: +603-8946-8519; Fax: +603-8942-3552*
**Corresponding author: yrukayadi@food.upm.edu.my*

**ABSTRACT**

*Keluwak* or *kepayang* or *picung* (*Pangium edule* Reinw.) is a fruit from a tall tree native to Southeast Asia and content high hydrogen cyanide that harmful for consumption. The hydrogen cyanide of *P.edule* seed can be removed by boiling and fermentation process resulting edible *P. edule*. This study is conducted to determine or evaluate antibacterial activity of fermented *P. edule* seed extract against several foodborne pathogens namely *Escherichia coli*0157:H7*, Listeria monocytogenes*ATCC 15313*, Proteus mirabilis*ATCC 21100*,* and *Staphylococcus aureus*ATCC 29737*.* Susceptibility of the extract was tested against bacteria mentioned above using disc diffusion method and followed by determine its MIC and MBC, according to the CLSI guidelines. Time-kill curves were constructive to assess the concentration between MIC and bactericidal activity of the extract at concentrations ranging from 0×MIC to 4×MIC. The extract showed susceptible to *E. coli*, *S. aureus* and *P. mirabilis*. MICs of extract were 25 mg/ml, 12.5 mg/ml and 12.5 mg/ml for *E.coli, P.mirabilis* and *S.aureus,* respectively. Meanwhile, the MBCs of the extract for *E. coli, P. mirabilis* and *S.aureus* were 50 mg/ml, 25 mg/ml, and 25 mg/ml, respectively. Time-kill curve shows that *E.coli* was killed with concentration of 2×MIC after 30 minutes, while *S.aureus* was killed by the extract with concentration of 4×MIC after 2 hours of incubation. The extract of fermented *P. edule* seed displayed potent activity against some foodborne pathogens and therefore might have potential application as natural food preservative.

***Keywords:*** antibacterial, fermented food, foodborne pathogen, *keluwak*, *Pangium edule*

## INTRODUCTION

Bacteria are present in soil, ponds, thermal areas, ice, in the deeps or live together with other organisms, so that any food, whether animal origin or not, can harbor bacteria.Animal base commodities which are meat, poultry and seafood are more prone to carry the pathogen because all the nutrients content that they have. Foodborne pathogens such as *Campylobacter jejuni, Escherichia coli O157:H7, Listeria monocytogenes, Salmonella spp., and Staphylococcus aureus can cause illness and a lot of outbreaks occur because of them. The emergence of foodborne pathogens has become major public health concerns and* consumers are more aware about it (Nurmahani *et al*., 2012).Therefore, there is increasing demand for antibacterial agents for public safety.

The demand for antibacterial agents as preservatives is increasing especially the agents that come from natural sources because the safety of food that containing synthetic preservative has been questioning by consumers (Shan *et al*., 2007). There is an interest by scientist to develop an effective and non-toxic antibacterial agents from natural sources such as plant or plant parts. Nowadays, various researches have focused on the antimicrobial activity of plant extracts such as grape, mango, passion fruit, and *longan* seed extracts to overcome the sides effects of the synthetic antimicrobial agents such as the emergence of resistant bacteria. Many compounds present in plants have been reported to be biologically active, antimicrobial, allophatic antioxidant and have bioregulatory properties (Negi *et al*. 2005). This is supported by Fook and Kheng (2009) which state that the major compound in plant that act as antimicrobial are alkaloids, dienes, flavonols, flavones, glycoside, lactones, organic acid, phenolic compounds, and protein – like compound. Thus, in this study, the seeds of *Pangium edule* have been chosen to determine its antibacterial activity against foodborne pathogens.

*P. eduleis* most famous for the properties of its fruits and seeds. Currently the status of this species is a minor and found mostly in villages and rarely the seeds can be observed in the markets. It is used to make a number of distinctive dishes and used widely as preservatives because this indigenous fruit have been found to have valuable effect as antibacterial agents. It is used throughout Sabah and Sarawak community

to preserve meat and fish as the refrigerators usage is seldom among natives(Hanum *et al*., 2007. Besides that, it is already processed commercially into *keluwak* in Indonesia.The seeds of this fruit contain naturally high level of hydrogen cyanide as its anti-nutritional factors. According to Fook and Kheng, (2009), there is a highly toxic hydrocyanic acid produced from reaction of fat with a glucoside called gynocardin which release perrusic acid, and it is contained in all parts of the plant, with the highest concentration in the seeds of the fruit (Lejuni *et al*., 2001).

The seed kernels are edible after some treatment and removal of cyanogenic glucoside. It is called *dage* after the boiled seeds are removed from its kernels and soaking in water for 2-3 days. Apart from that, the seeds can be ferment by buried it under the ground for sometimes until the seeds turn black. The seeds are edible to eat if there are no bitter taste after consume which is mean all of cyanogenic glucoside have been degraded. This fermented seeds product has been used as a spice for soup in Java and South Sulawesi (Andarwulan *et al*., 1999).According to the research by Department of Agriculture (DOA) Sarawak, *P. edule* has high energy level which is 227 kcal/100g because of its oily kernels. Besides that, this fruit also high in protein content and other proximate composition and minerals (Voon and Kueh, 1999). Fardiaz and Romlah (1992) found that the fatty acid profile of *P. edule*Reinw. seed oil showed a predominance of oleicand linoleic acids. Besides the chemical properties, the seed kernels also have been used to preserve the freshness of meat and fish in remote area and also by fisherman because there is lack of ice Apart from being a spices in Indonesia, *P. edule* seed kernel also used as raw material for edible oil, colorant or as fish preservative (Widyasari *et al*, 2006). Considering the value of its physicochemical and biological properties, the present study is conducted to explore the antibacterial activities of *P. edule* seed extract against several foodborne patgogens which are *Escherichia coli*0157:H7*, Listeria monocytogenes*ATCC 15313*, Proteus mirabilis*ATCC 21100*,* and *Staphylococcus aureus*ATCC 29737.

## MATERIALS AND METHODS

### Preparation of seed extract

The fermented *P. edule* seeds was ground and extracted twice with 400 mL of methanol for 48 hours at room temperature followed Rukayadi *et al*. (2008). After 48 hours, the seeds were concentrated by using rotatory evaporator (60-70° C) at Biochemical Laboratory, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang. The seeds extract was dissolved in 10%dimethylsufoxide (DMSO) to obtain stock solution.

### Bacteria strain and inoculum preparation

Pure culture of bacteria strain were obatained fromInstitute Bioscience Laboratory, Universiti Putra Malaysia, Serdang Each of these pure culture were grown on the Mueller Hinton agar plates (37°C, 1 day) and maintained at 4°C. The bacteria strains are *Escherichia coli*0157:H7*, Listeria monocytogenes*ATCC 15313*, Proteus mirabilis*ATCC 21100*,* and *Staphylococcus aureus*ATCC 29737. The inoculum was prepared by inserting 100 µl of bacteria suspension into 10 mL Mueller Hinton broth. Each of the inoculum was incubated at 37° C for 24 hours. *L. monocytogenes*has slightly different in their growth temperature. The optimum temperature for *L. monocytogenes* to grow is 30° C during 48 hours incubation.

### *In vitro* susceptibility test

### Disc diffusion method

The fermented seed extract were tested for antibacterial activity using disc-diffusion method. Two fold dilution seed extract was prepared using sterile distilled water. Each bacteria strain was spread on Mueller Hinton agar plate using sterile cotton swab and 25 µl of two fold dilution of extracts was placed on the bacteria. The seed extract was let to dry before incubated at 37° C for 24 hours and 30° C and 48 hours for *L. monocytogenes*. The plates were observed for any clear zone and perfomed two times in duplicate two verify the results.

**Minimum inhibitory concentration(MIC) and minimum bactericidal concentration (MBC) evaluation**

The minimum inhibitory concetration (MIC) of fermented seed extract for each bacteria was perfomed according to method described in the guidelines of Clinical Laboratory Standard Institute M7-A6(3). This test was done in a 96-well round bottom microtitration plate for all bacteria strain except *L .monocytogenes*. The inoculum with initial density; 3.16 x $10^6$ cfu/ml of *S. aureus*, 1.4 x $10^5$ cfu/ml of *P. mirabilis*, 2.36 x $10^6$ cfu/ml of *E. coli* O157:H7, and 1.4 x $10^6$ cfu/ml of *E.coli* #2 were mixed with two fold dilution of fermented*P. edule* seeds extract stock solution in MHB medium and incubated aerobically at 37°C. The highest concentration of the seed extract was at column 12 and the lowest concentration was at column 3. Column 2 used as positive control (only medium and inoculum or antimicrobial agent-free well) and column 1 used as negative control (only medium, no inoculum and no anti-microbial agent). The endpoints were read visually after 24 hours. The MIC was the lowest concentration of seeds extracts that shows complete inhibition of visible growth.

Minimum bactericidal concentration (MBC) was defined as the lowest concentration of antimicrobial agent at which no growth occur in the agar plate. MBC was determined by spreading the suspension (10 µl) from each wells on MH agar plate. All the wells include positive control (column 2) and negative control (column 1) were spread on the agar plate. The plates then incubated at 37° C for 24 hours or until growth seen at positive control. MBC was tested for all bacteria strains.

**Time-kill curve**

Time-kill curve was done for Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*S. aureus*). Mueller Hinton broth (MHB) was used in order to perform this test. The inoculum with initial concentration 1.5 x $10^6$ cfu/ml for *E. coli* #2 and *S. aureus* was obtained after diluted with 1:10 MHB. Seed extract with final concentration (0×MIC, 1×MIC, 2×MIC) for *E. coli* and (0×MIC, 1×MIC, 2×MIC, 4×MIC) for *S. aureus* were prepared. Mixture of cultures and seed extract with final volume 2 mL were incubated at 37° C with agitation. A 100 µl aliquot was transfered into 10 mLof MHB at predetermined time (0 min, 30 min, 1, 2, 4, 8, 12, and 24 hours) for serial dilution. An appropriate volume (25 µl) was spread onto MH agar and incubated at 37° C for 24 hours to determine the numbers of colony in cfu/mL.

## RESULTS AND DISCUSSION

Susceptibility, MIC and MBC of fermented *P.edule* seeds againts *E.coli* O157:H7*, L. monocytogenes, P. mirabilis, and S.aureus* are showed in Table 1. Meanwhile the time kill curves for *E. coli* O157:H7 and *S. aureus* are showed in Figure 1 and 2.

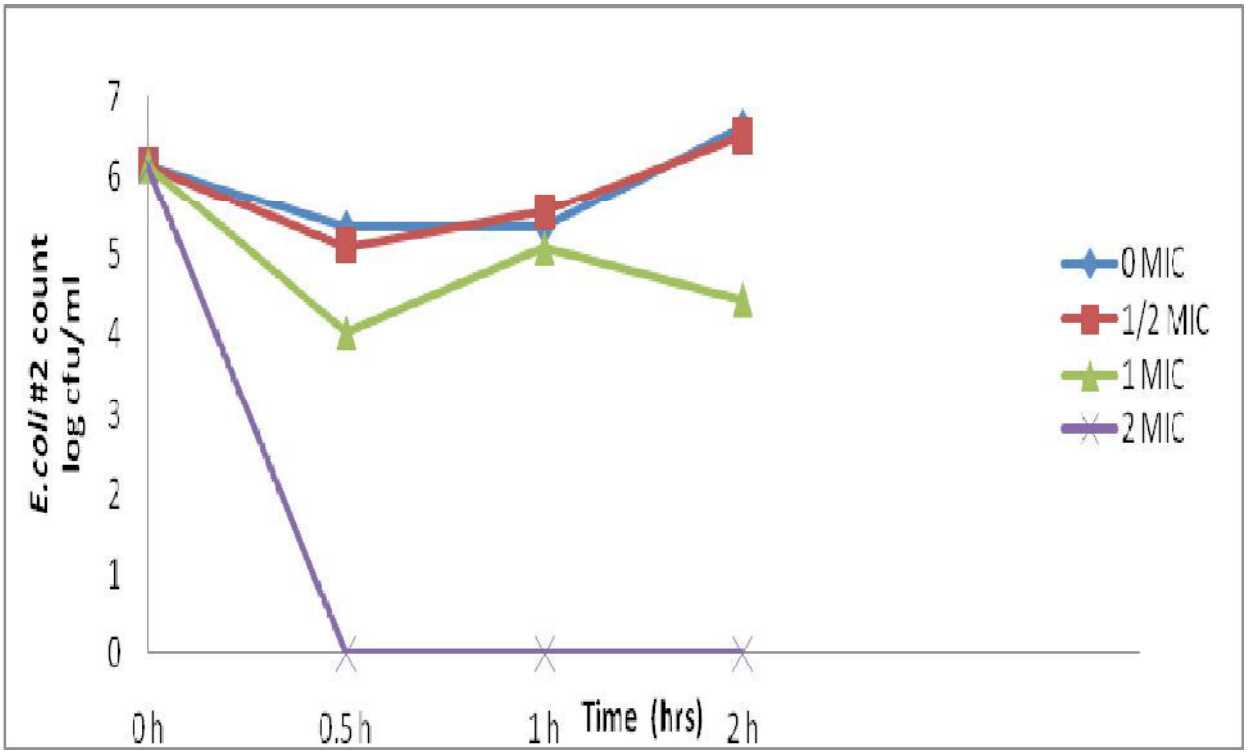


**Figure 1:** Time-kill plots for *Escherichia coli* following exposure to fermented *Pangium edule* extract. *MIC= 25mg/ml

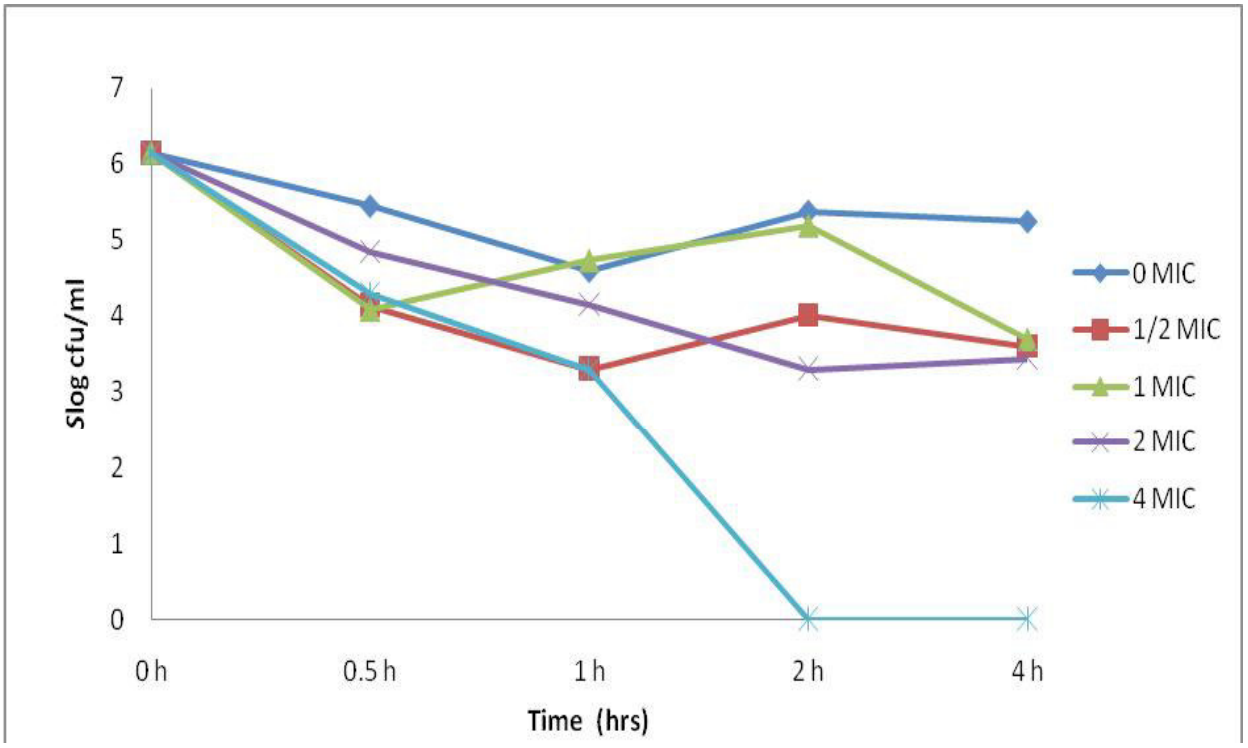


**Figure 2:** Time-kill plots for *Sthaphylococcus aureus* following exposure to fermented *Pangium edule* extract. *MIC= 12.5mg/ml

In this study, the antibacterial activity of *P.edule* seeds were assessed quantitatively by broth dilution method and time- kill assay. The seed extract shows inhibitory effect against all the bacteria tested except for *L. monocytogenes.* Further analysis was done to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the other three bacteria. MIC and MBC of fermented*P. edule*seed extractwere summarized in Table 1. The inhibitory concentration (MIC) for fermented seed extractranged from 12.5 to 25.0 mg/mL and the bactericidal concentration (MBC) ranges from 25.0-50.0 mg/mL. *E. coli* O157:H7 susceptible to the extract with 25.0 mg/mL and 50.0 mg/mL for MIC and MBC respectively. Meanwhile, *L. monocytogenes* do not show susceptibility towards the fermented of *P. edule* seed and it is contrast with the finding by Fook andKheng(2009), which shows the inhibitory zone for all types of extraction include by ethanol extraction for *L.monocytogenes*. The seed extracts showed inhibitory effects against *P. mirabilis* with 9 mm inhibition zone. This facultative anaerobic pathogen has minimum inhibitory concentration (MIC) with 12.5 mg/mLand 25.0 mg/mL for minimum bactericidal concentration (MBC).Last but not least, the Gram-positive bacterium, *S. aureus*also has the inhibition zone of 9 mm with MIC and MBC 12.5 mg/mL and 25. 0 mg/mL respectively.

Table 1: Susceptibility of fermented ***P. edule*** seeds extract

| Bacterial strain | Diameter (mm) | MIC (mg/ml) | MBC (mg/ml) |
|---|---|---|---|
| *Escherichia coli O157:H7* | 8 | 25 | 50 |
| Listeria monocytogenes ATCC 15313 | - | - | - |
| Proteus mirabilis ATCC 21100 | 9 | 12.5 | >100 |
| *Staphylococcus aureus* ATCC 29737 | 9 | 12.5 | 25 |

The crude extracts of fermented *P. edule* showed inhibitory effects against Gram-negative and Gram-positive bacteria and it was supported with the study done by Rauha *et al*. (2000), who found that the crude extracts usually contains substantial quantity of phenolic compound such as phenolic acid derivatives, flavanoids, tannins, essential oil, gallic acid and terpenoid that are effectively react as antibacterial agents. Gallic acid is the most effective compound against foodborne bacteria (Rodriguez-Vaquero *et al*., 2007). The results also showed that Gram-positive bacteria (*S. aureus*) is more susceptible compared to Gram-negative bacteria (*E. coli*) with larger inhibition zone and it might be because the Gram-negative bacteria possess an outer layer surrounding the cell wall, that can restrict the diffusion of active compound through its lipopolysaccharide covering (Fook and Kheng, 2009).

Further determination of the inhibitory concentration on fermented *P. edule* seeds extract by time-kill assay against Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*S. aureus*) are depicted in Figure 1 and 2. The initial load of *E. coli* and*S.aureus* is 6.15 log cfu/mL, all levels of MICs except 0×MIC of fermented seeds extract were able to reduce more than one log cfu of the tested strains after 24 hours incubation. A sharp reduction of the cell density was observed when the *E. coli* strain was exposed to fermented seed extract at a concentration ≥ 1×MIC for 30 minutes to 2 hours. At concentration 2×MIC, which equal to 50mg/mL, the strain can be killed during 30 minutes incubation. The lethal effects of fermented seeds extract for *E. coli* strain was generally permanent at concentration 50 mg/mL. Apart from that, *S. aureus* also showed the reduction in their density level when exposed to ≥1×MIC. The log cfu was reduced drastically when the strain exposed to concentration of 4×MIC. *S. aureus* was completely killed at this concentration within 2 hours incubation. Even though the MIC and MBC for Gram-positive are lower than Gram-negative bacteria, the time consumed to kill Gram-positive bacteria is longer compared to Gram-negative bacteria.

Higher resistance of Gram-negative bacteria to the external agent is attributed by the presence of lipopolysaccharides, outer membrane, cell wall, and inner membrane which make them inherently resistant to antibiotics, detergent and hydrophilic dyes. But, the Gram-negative bacteria possess higher amount of lipopolysaccharide and make it virulent and easy to destroy even it need high concentration of seed extract to kill it. Apart from that, the Gram-positive bacteria contain an outer layer peptidoglycan which is ineffective permeability barrier. So, the differences in sensitivity of Gram-negative bacteria compared to Gram-positive bacteria for the antimicrobial agent might be because of the differences in their cell wall compositions (Negi *et al*., 2005).

The reduction of these bacteria density during time kill assay might be due to diffusion of bioactive compounds that present in the seedextract into the cell and cause leakage of membrane cell and eventually lead to cell death (Fook and Kheng, 2009). The phenolic compounds are able to alter the membranes permeability in the microorganisms and lead to the leakage of protons, phosphates and potassium (Lambert, 2001). Besides that, the alkaloids compound that found in *P. edule* seeds could act as antimicrobial agent. Alkaloids are part of the systemic chemical defense response in plant. The crude alkaloid extract may contain non-phenolic compounds such as quindine, indole, terpenoid, quinolizidine, dopamine and tropane, which were shown as good antimicrobial agents (Roberts and Wink, 1998).

## CONCLUSION

The antibacterial activity of fermented *P. edule* seed extract might be contributed by pre-active compounds in the seed that are activated during fermentation process. Further studies are needed to determine the compounds which are contribute to antibacterial activities in *P. edule* and develop the products that use the *P. edule* seed as safe natural food additive if it processed properly.

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# SHELF- LIFE STABILITY OF MILK CHOCOLATE CONTAINING INULIN

**Norhayati Hussain[1*], Yaya Rukayadi[1] and Mohd Khan Ayob[2]**
[1]*Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia.*
[2]*Department of Food Science, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), 43600 Bandar Baru Bangi, Selangor, Malaysia.*
*****Corresponding author: aryatihussain@upm.edu.my*

**ABSTRACT**

This study was carried out to evaluate the effect of inulin (sucrose replacer) in newly developed milk chocolate (MC1) after storage at two types of temperature and humidity: 18$^0$C, 60%RH (normal condition, MC1a) and 25$^o$C, 80%RH (harsh condition, MC1b) on their shelf stability as compared with control milk chocolate (MCc) containing sucrose as a sweetener. The MC1 had significantly ($p<0.05$) higher WI (whiteness index) value than the control, MCc during storage at 18$^0$C, 60%RH for 12 months. Although no sugar bloom observed on surfaces of MC1a and MC1b, the MC1b samples were rejected after the appearance of fungal growth right before reaching 3 months old of storage. In addition, sensory panelist had earlier detected significant different ($p<0.05$) in the taste and odor attributes especially at 2 months old MC1 and MCc. However, all sensory attributes (hardness, taste, odor and appearance/ color) of MC1 were not significantly ($p>0.05$) different from that of MCc although it was stored for 12 month at 18$^o$C, 60%RH. After 12 month storage, all the initial amount of inulin added was still present in MC1 as determined using HPLC. Our study confirmed that milk chocolate containing inulin has a shelf life of 12 months without degradation of inulin content in the chocolate (chemically stable), no fungal growth (microbiologically safe), undetectable appearance of non uniform colors (physically stable) and well accepted in sensorial properties similar to the control samples stored at 18$^o$C, 60%RH.

***Keywords***: Inulin; milk chocolate; shelf life; stability and sensory evaluation.

## INTRODUCTION

Inulin is frequently used as an additive in functional food especially as substitute for lipid, supplement for sugar and increase dietary fibre content to enhance bifidogenic effect (Terezija *et al*. 2004). Generally, chocolate confectionary has a unique taste, flavor, texture and known to be a rich source of antioxidants, fats and sucrose. However, when the fat content is too low (below 27%, w/w) chocolate loses its smoothness and also melting in the mouth is retarded (Ewa *et al*. 2005). Therefore, we have selected inulin to be incorporated into milk chocolates as a sugar replacer (instead of as fat replacer) and as added dietary fibre in order to meet the increasing demand from consumers for health-boosting foods without altering the taste and compromising quality. Fat blooming is one of the major quality problems for the chocolate industry that can be induced by exposure to high ambient temperatures and causes a non-uniform pattern of colors over the surface of a chocolate. Meanwhile, sugar bloom is a result of water condensation that causes internal sugar to melt thus forming sugar crystals on the surface of the chocolate. The phenomenon can be measured by a computer vision system (Briones and Aguilera, 2005).

Such problems require us to carefully formulate and process the prebiotic milk chocolate in order to provide maintenance of inulin activity during long term storage. Ordinary chocolate normally has a shelf life of 12 months (long-life products) and such food product should be recognized as having a microbiological, chemical and organolaptic shelf lives because all foods deteriorate, albeit at different rates (Man, 2002). Chocolates when stored under correct temperature and humidity is a very stable product. A 'normal' storage temperatures and relative humidity was suggested at 18$^0$C, 60%RH respectively; while storage at 25$^0$C, 80%RH was considered as a harsh condition (Man and Jones, 2000).

Therefore, our aim in this study was to determine and to compare the shelf life stability between prebiotic (with added inulin) milk and control (without inulin) chocolates at different temperatures by means of chemical (HPLC), physical (color evaluation), microbiological analyses and most importantly the sensory evaluation. The incorporation of inulin and isomalt in sucrose-free prebiotic milk chocolate and their influence on product's shelf life has not yet been previously reported.

## MATERIALS AND METHODS

### Materials

Newly developed prebiotic milk chocolate (MC1) by Malaysian Cocoa Board was prepared using cocoa solids, cocoa butter, milk components, emulsifier, a flavor component and sweeteners (inulin and small amount of isomalt) (International Application Patent No: PCT/ MY 2007/000023). However, control milk chocolate (MCc) which has sucrose as a sweetener was also prepared for comparison purposes. Cocoa liquor purchased from Selbourn Food Services at Pelabuhan Klang, Malaysia, full cream and skimmed milk from Promac Enterprises Sdn. Bhd., cocoa butter from Malaysia Cocoa Manufacturing Sdn. Bhd., isomalt from Nutrisweet & Food Specialties Sdn. Bhd., and prebiotic inulin extracted from chicory root (Sensus, The Netherlands). These chocolates, in chocolate boxes lined with bubble plastic, were stored at $18^0$C, 60%RH (in a chiller cabinet) and $25^0$C, 80%RH (in a controlled humidity chamber) for 12 months.

### Color measurement and microbiological analysis

The color of the surface of the chocolate was measured using a HunterLab Miniscan$^{TM}$ XE colorimeter model 45/0 LAV (Hunter Associates Inc., Reston, VA) after calibration with white and black glass standards. Three spots were examined on the major axis of each chocolate bar. The colorimeter yielded L*, a* and b* values for each spot which were converted to whiteness index (WI), where $WI = 100- \{(100 – L^*)^2 + (a^{*2}) + (b^{*2})\}^{0.5}$ (2). In this study, we used the WI value to indicate the appearance of sugar or fat bloom (white or crystallized spot) which could be detected by our naked eye.

Development of fungal growth was also observed with the naked eye especially on chocolate surface with white or mycelia spot in relation to storage at high humidity. We would stop the experiment if any storage condition showed the existence of fungal growth on the surface of the chocolate.

### Inulin determination

MC1 before and after storage with longer shelf life was analysed for inulin content using HPLC to compare whether the content of inulin would decreased after a prolonged storage. Homogenized sample (1g) was accurately weighed into a 200 ml beaker, treated with ca. 100 ml of boiling water at pH 6-8 and kept at 85$^o$C with continuous magnetic stirring on a hot plate for 15 min. After cooled to room temperature, the volume was made up to 100ml and the solution was filtered through a 0.20 µm membrane filters before injection (Zuleta and Sambucetti, 2001).

The HPLC instrumentation consisted of a Waters 1525 Binary HPLC Pump, Waters 717 plus Autosampler (injector with a 50 µl sample volume), an Aminex HPX-42A (Bio-Rad) anion exchange column and Waters 2414 Refractive Index detector. HPLC conditions were utilizing of deionized water at 85$^o$C as the mobile phase at a flux rate of 0.6 ml/ min. Calibration curves were plotted with 0.005 – 1 g/ 100 ml of inulin as standard. All determinations were made in triplicate.

### Sensory analysis

Evaluation of sensory attributes for each sample stored in different storage conditions included appearance or color, odour, hardness and taste. Each of these attributes was evaluated according to the following scale (1-5): 5, better than standard (acceptable); 4, same as standard (acceptable); 3, slight difference, nothing undesirable (tolerated); 2, inferior to standard (rejected); 1, much inferior (rejected). The score depends on its comparison with fresh sample of newly prepared chocolate at the time of tasting (5). One attribute having a reject score means that the whole product must have a reject score thus in this study we had used an acceptance levels of average 3 and above. Evaluation of the sensory properties was carried out by 7 trained panelists.

**Statistical Analysis**

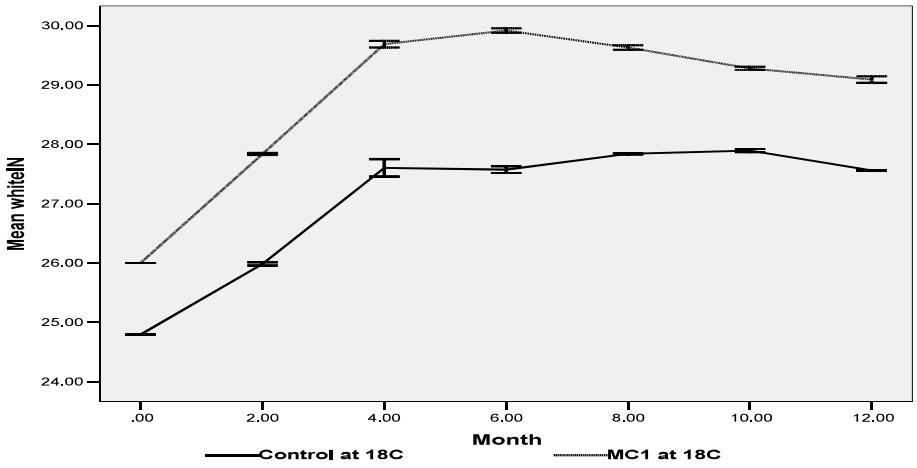
All data obtained from three replications of analysis were analysed using SPSS Inc. software (version 14.0). A 2-factor analysis of variance using the General Linear Model procedure was used to determine significant difference between samples and each month of storage with a significance level of $p< 0.05$.

## RESULTS

**Color measurement and microbiological analysis**

Chocolate containing inulin (MC1) had significantly ($p<0.05$) higher WI value than the control, MCc during storage at $18^0$C, 60%RH for 12 months. Meanwhile, both chocolate was able to withstand the storage conditions without obvious development of sugar blooms (whitish spots) during the 12 month storage (Figure 1A). In contrast, MC1 showed significantly ($p<0.05$) lower WI value than MCc stored at $25^o$C, 80%RH (Figure 1B). Similar increasing pattern was observed for 2 months old chocolates at both storage conditions. Consequently, we had to stop the analysis right before the product reach 3 months old due to fungal growth observed on the surface of both type of chocolate and identified as *Aspergillus sp.* (Figure 2A and 2B).

1A.




1B.

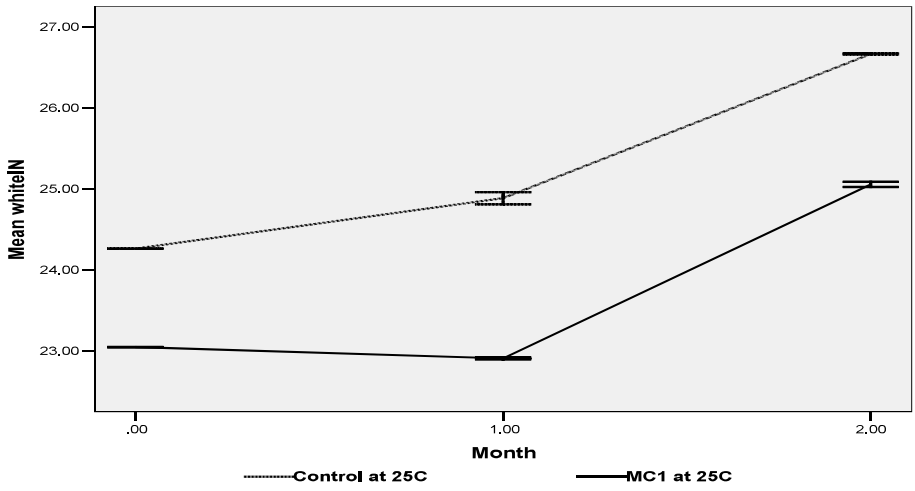
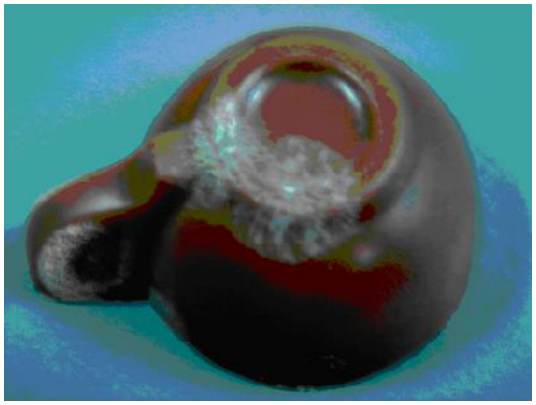



Figure 1A & 1B. Mean of Whiteness Index ± sd for control, MCc and MC1 chocolates stored at $18^0$C, 60%RH and $25^0$C, 80%RH.

2A. 

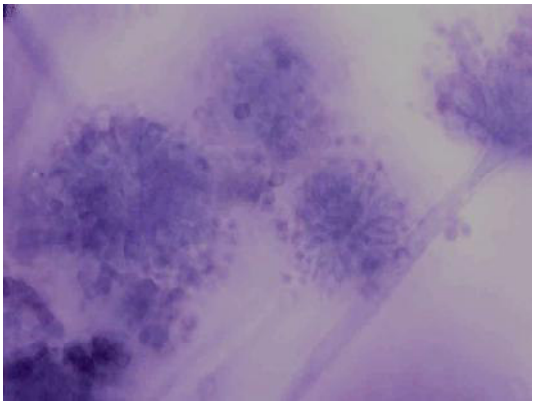
2B. 

Figure 2A & 2B. Mould growth on control, MCc and MC1 kept at 25°C (80%RH) for 2 months showing *Aspergillus sp.* colony at 200x magnification

**Inulin determination**

Figure 3 shows comparison of inulin content in MC1 during before and after 12 months storage at 18°C. There was no significant different ($p>0.05$) in inulin content before and after long storage (12 months) at 18°C, 60%RH as observed in the MC1.

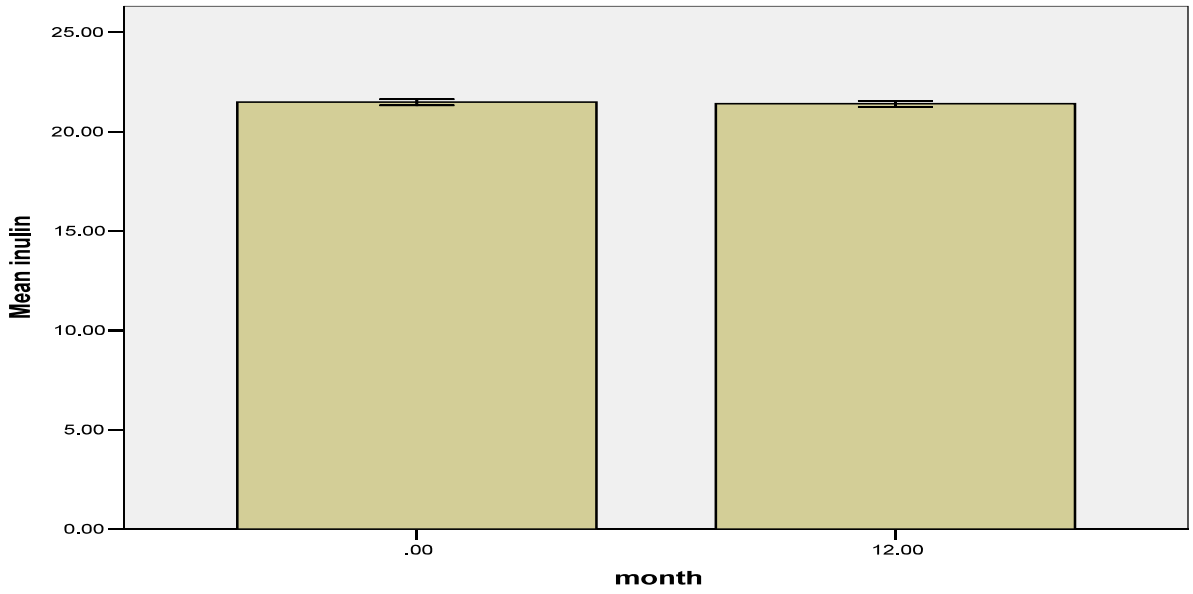



Figure 3. Inulin content ± sd in MC1 during, before and after a long (12 months) storage at 18°C, 60%RH

**Sensory analysis**

Table 1 indicates that the acceptance of MCc and MC1 stored at 18°C, 60%RH was not significantly different ($p>0.05$). Overall scores given by the panelist were more than 3 for each attribute. However, panelist had indicated that both type of chocolate had unacceptable taste with the score given less than 3 after 2 month storage at 25°C, 80%RH (Table 2). There significant different ($p<0.05$) in the taste and odor attributes of MC1 and MCc, especially at 2 month storage.

Table 1. Mean of four different attributes for each chocolate sample stored at 18°C, 60%RH for 12 months.

| | Control, MCc | | | | MC1 | | | |
|---|---|---|---|---|---|---|---|---|
| **Month** | **Hard** | **Taste** | **Odour** | **Appear @color** | **Hard** | **Taste** | **Odour** | **Appear @color** |
| 0 | 3.14 | 3.71 | 4.00 | 3.14 | 3.00 | 3.29 | 3.43 | 3.71 |
| 2 | 3.86 | 3.14 | 3.43 | 3.00 | 3.29 | 3.29 | 3.00 | 3.00 |
| 4 | 3.57 | 3.71 | 4.00 | 3.14 | 3.14 | 3.14 | 3.29 | 3.14 |
| 6 | 3.71 | 3.00 | 3.43 | 3.14 | 3.57 | 3.14 | 3.00 | 3.14 |
| 8 | 3.29 | 3.71 | 4.00 | 3.71 | 3.29 | 3.57 | 3.14 | 3.00 |
| 10 | 3.86 | 3.43 | 3.29 | 3.71 | 3.71 | 3.43 | 3.43 | 3.14 |
| 12 | 3.86 | 3.00 | 3.29 | 3.86 | 3.71 | 3.00 | 3.57 | 3.86 |

Note: Scores for all attributes are not significantly different at $p > 0.05$

Table 2. Mean of four different attributes for each chocolate sample stored at 25°C, 80%RH for 2 months.

| | Control, MCc | | | | MC1 | | | |
|---|---|---|---|---|---|---|---|---|
| **Month** | **Hard** | **Taste** | **Odour** | **Appear @color** | **Hard** | **Taste** | **Odour** | **Appear @color** |
| 0 | $3.43^{a}$ | $4.00^{b}$ | $3.71^{a,b}$ | $3.00^{a}$ | $3.57^{a}$ | $4.14^{b}$ | $3.43^{a,b}$ | $3.29^{a}$ |
| 1 | $3.75^{a}$ | $4.00^{b}$ | $4.25^{b}$ | $3.00^{a}$ | $3.38^{a}$ | $3.63^{b}$ | $3.63^{b}$ | $3.00^{a}$ |
| 2 | $4.00^{a}$ | $2.86^{a}$ | $2.29^{a}$ | $3.14^{a}$ | $4.14^{a}$ | $2.14^{a}$ | $3.14^{a}$ | $3.00^{a}$ |

$^{a,b}$ Different alphabet in the same column is significantly different at $p<0.05$

## DISCUSSION

No sugar or fat bloom was spotted on the surface of the chocolates although samples were stored at high temperature and humidity. In addition, appearance or color attributes for chocolates stored at 25°C was not scored as unacceptable by the panelist but the WI values were higher at 2 months old compared to storage at 18°C. This may be due to faster initial condensation occurred at higher environmental humidity (80%RH). This might be true as our findings showed high humidity storage condition together with high temperature did enhance fungal growth on the chocolates immediately before they reached 3 months old. Temperature and relative humidity are important extrinsic factors in determining whether a food will spoil or not thus at higher relative humidity, microbial growth is initiated more rapidly (Prescott *et al*., 1993). Consequently, both samples in this study were rejected for being microbiologically unsafe (shelf life ended) when stored at 25°C, 80%RH.

Interestingly, sensory panelist had earlier detected significant different ($p < 0.05$) in the taste (scored less than 3) for both 2 months old MCc and MC1 chocolates (25°C, 80%RH) compared to chocolates stored at 18°C, 60%RH (scored more than 3). Given the assurance of product safety, sensory evaluation is undoubtedly the most appropriate technique for evaluating changes during storage trials (Kilcast, 2000). All sensory attributes of MC1 were not significantly different from that of control chocolates, MCc although it was stored for 12 months at 18°C, 60%RH. Until the 14th month, the attributes for those chocolates were

still acceptable by the panelist (data not shown). The shelf life of a chocolate is the period during which it remains acceptable in terms of appearance, aroma, flavour and texture (Man and Jones, 2000).

After 12 months of storage at 18°C (60%RH), all the initial inulin was still present in MC1 ($p>0.05$) when determined using an easy, rapid and cheap alternative HPLC technique as described by Zuleta & Sambucetti (Zuleta and Sambucetti, 2001). Bohm *et al*. (2005) did report that inulin from chicory or Jerusalem artichoke started to significantly degrade for up to 60 min at temperatures between 135°C and 195°C thus the selected storage condition above should not affecting the inulin performance in chocolates.

Our study confirmed that milk chocolate containing inulin has a shelf life of 12 months without degradation of inulin content in the chocolate (chemically stable), no fungal growth (microbiologically safe), undetectable appearance of non uniform colors (physically stable) and well accepted by sensory panelist similar to the control chocolate at 18°C, 60%RH. Thus, this will help in increasing the health benefits of chocolate and related products.

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# SURVIVAL OF FREEZE-DRIED *Lactobacillus rhamnosus* R21 IN THE PRESENCE SKIM MILK AS PROTECTANT DURING STORAGE

**Ni Nyoman Puspawati[1], Lilis Nuraida[2], Dede Robiatul Adawiyah[2]**
*Faculty of Agriculture Technology, Udayana University*
*[2] SEAFAST Center and Department of Food and Agriculture,*
*Bogor Agricultural University*
*Correspondence author: nnpuspa@yahoo.co.id*

**ABSTRACT**

Lactic acid bacteria are the most important bacteria having potential as probiotic. The objectives of the present study were to evaluate influence of skim milk as protectant on survival of freeze-dried *Lactobacillus rhamnosus* R21 which is isolated from breast milk during storage and to calculate the shelf-life of freeze dried *Lactobacillus* culture. To predict the shelf life of freeze dried culture, further experiment was conducted by storage the freeze dried of *Lactobacillus rhamnosus* R21 at RH 75 and RH 90 and shelf life was predicted by sorption isotherm method. Evaluation was done on under act water content, viability, water activity, acidification activity. The result showed that water content and water activity increased during storage from 2.17% db - 21.59% db and 0.099 – 0.801 respectively. Viability and acidification activity of freeze-dried culture decreased from 11.49 log cfu/g – 0 cfu/g. The predicted shelf life of the freeze dried *Lactobacillus rhamnosus* R21 culture if initial water content is 2.17% db, packaged in aluminium foil laminated by PE (polyethylene) and temperature 30°C are 5.86 years at RH 75 and 5.10 years at RH 80.

***Keywords:*** *Lactobacillus rhamnosus* R21*, breast milk, freeze drying, shelf life*

## INTRODUCTION

Lactic acid bacteria as probiotic microorganisms play an important role in improving and maintaining health, thus encouraging the use of lactic acid bacteria for the development of functional foods and pharmaceutical. Lactic acid bacteria have been widely studied and applied in many fermentation processes in the food industry as starter cultures. *Lactobacillus* species are also known to function as probiotics used in yogurt products and food products associated with health. Probiotics are defined as live microorganisms that are able to achieve a certain amount of the digestive tract and beneficial to health. Benefits of lactic acid bacteria as probiotics in improving health can occur when a culture consumed alive and able to survive in the digestive tract. *Lactobacillus rhamnosus* R21 that can be isolated from breast milk are known to have probiotic properties as particularly resistant to bile salts, resistant to low pH, can inhibit the growth of pathogenic bacteria such as *E. coli, Salmonella*, *B. cereus, S. aureus*. Testing in vivo, *Lactobacillus rhamnosus* R21 also has properties to increase the amount of lactic acid bacteria and can inhibit the growth of pathogenic bacteria such as *E. coli* in the cecum (Susanti et al., 2008).

Preserving culture of *lactobacilli* for industrial purposes can be done by various methods such as freezing, spray drying and freeze drying. Bacterial culture preservation process can cause damage to the structure and physiological cell that can lead to decreased viability of the bacteria. Damage to the cell biological systems due to freeze-drying process is characterized by a change in the physical properties of the membrane lipid changes or due to changes in protein structure that is sensitive (Leslie et al., 1995). Cell damage caused by lactic acid bacteria freeze drying process can be minimized by the addition of protective material (cryogenic) given before the freezing and drying (Tamime, 1981). For industrial use, starter cultures to be used must have a high viability during freeze-drying process and a long shelf life. Previous studies have examined the use of different materials to protective cultures of lactic acid bacteria during freeze-drying process (Puspawati, 2008).

The purpose of this study was to determine the effect of protective material to skim milk cultures *Lactobacillus rhamnosus* R21 resistance during freeze-drying process and determine the shelf life of dried cultures.

## MATERIALS AND METHODS

### Bacterial strains

Strains of bacteria used in this study were *Lactobacillus rhamnosus* R21 cultures derived from breast milk (ASI). Stock cultures stored at cryoval tube containing 20% glycerol and frozen at-80°C. Subculture and mass multiplication of lactic acid bacteria cells carried in the media de Man Rogosa Sharpe Broth (MRSB).

### Media

Media used in this study MRSB, MRSA, $KH_2PO_4$, skim milk, NaCl and BaCl2. The tools used in this study are standard tools for microbiological analysis, freeze drier, deep freezer and centrifuge.

### Stages of Research

In this research, manufacture dried culture with freeze drying process. Making dried culture refers Harmayani *et al.* (2001). Culturing dry storage process is then performed to determine the shelf life of dried probiotic cultures. Determination of shelf life storage of dried cultures performed with *L. rhamnosus* R21 on RH environment 75 and 80. Parameters observed during storage includes: total lactic acid bacteria, moisture content and total molds and yeasts during storage capability *L. culture rhamnosus* R21 dry to acidify milk. Analyses were performed on days 0, 1, 3, 5, and 7. From the analysis determined during storage shelf (θ) dried cultures using the model equations Labuza (Labuza, 1982).

$$\theta = \frac{\ln(Me-Mi)/(Me-Mc)}{k/x \,.\, A/Ws \,.\, Po/b} \quad \text{(equation 1)}$$

wherein: Me is the equilibrium moisture content, Mi = initial moisture content, Mc = critical moisture content, k/x = permeability of packaging, A/Ws = the ratio between the area of packaging by weight of the material, Po = saturated water vapor pressure on the state, b = the value of the slope of the curve (slope).

### Making Culture Dry with drying Frozen (Nuraida *et al.* 1995; Harmayani *et al*., 2001; Champagne *et al*., 2001 modified)

Before the freeze drying process performed cell biomass production of lactic acid bacteria (Harmayani *et al*., 2001 modified). Biomass lactic acid bacteria made using MRSB media. In a medium that has been inoculated with sterile cultures of lactic acid bacteria that have been freshened up by 10% and then incubated at 37°C for 18 hours. Further work culture is harvested and centrifuged at a speed of 10000 rpm for 10 minutes. Supernatants were separated with the filtrate thus obtained wet biomass.

The process of freeze drying, the addition of protective material (cryogenic) skim milk with a concentration of 10% (w/v) on a wet biomass of lactic acid bacteria. Comparison between wet biomass with a protective material (cryogenic) is 1: 10. To allow diffusion of protective material (cryogenic) then the solution is stored for 1 hr at 23°C. Subsequent cultures were frozen at-80°C for 12 hours and then dried (drying) the hair Labconco freeze brand on the condition-50o C, 0.01 MPa for 2 days.

### Storage of Lactic Acid Bacteria Culture Dry

Storage process dried cultures of lactic acid bacteria after freeze-drying process is done by storing the dried culture that has set the humidity desiccators using saturated salt solution. Storage conditions used are at room temperature with humidity levels of 75% and 90%. Storage process made up of products (dried cultures of lactic acid bacteria) suffered damage characterized by decreased cell viability. Tests dried cultures performed every 2 days (day-0, 1, 3, 5 and 7) include: viability of lactic acid bacteria, total fungi and yeasts, water content, water activity ($a_w$) and dried cultures acidification activity includes total acid tertitrasi and the pH of the acidified skim milk.

**Estimation of Age Store Lactic Acid Bacteria Culture Dry (Labuza, 1982)**

Estimation of shelf life of dried cultures of lactic acid bacteria selected performed using isotherms sorption curve approach. Critical parameter that is used to predict the shelf life is the viability of lactic acid bacteria; moisture content and water activity (aw) dried cultures of lactic acid bacteria during storage. Shelf based approach isotherms sorption curve can be calculated using the equation Labuza (equation 1).

## RESULTS AND DISCUSSION

**Effect of storage of the dried culture of Lactobacillus rhamnosus resistance R21**

**a. Effect of water content during storage on the viability of Lactobacillus rhamnosus R21 dried cultures**

Storage process can increase the moisture content dried cultures. Increased moisture content during storage dried cultures can reduce the amount of *L. rhamnosus* R21 (Figure 1a). Increased moisture dried cultures stored due to the product freeze drying results have properties very hidgroskopis so it is very easy to absorb water or moisture from the environment. Increased moisture content during storage is also correlated to the activity of dried culture acidification *L. rhamnosus* R21 as indicated by the decrease in total tertitrasi acid and pH values increased in acidified skim milk (Fig. 1b and 1c).

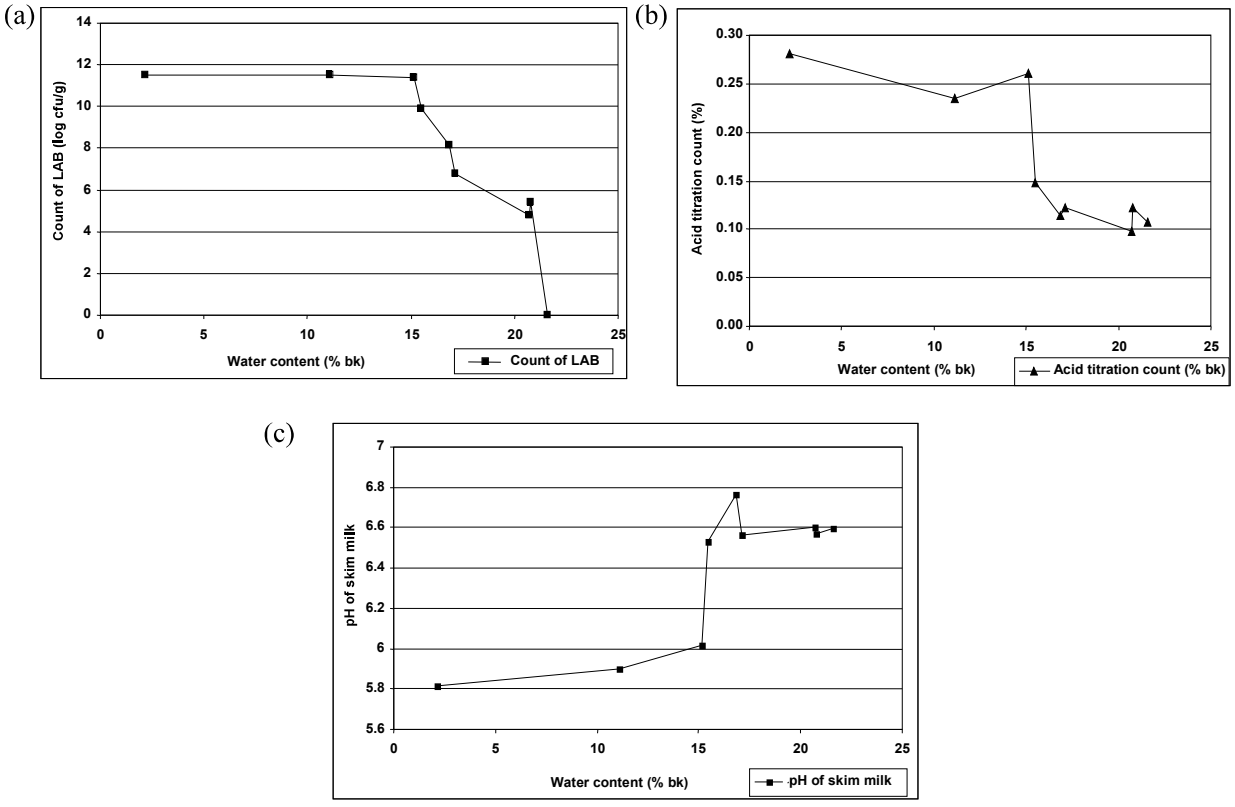


Figure 1. Results of storage L. rhamnosus R21 for 7 days, (a) The relationship of count of LAB with water content, (b) The relationship acid titration count with water content, (c) the relationship of the pH of skim milk with water content

Increased levels of water stimulate the bacteria to grow, but there was not enough water to support the growth of lactic acid bacteria. This is because before it had time to metabolize lactic acid bacteria to grow, bacteria need to fix ourselves first and this process requires considerable energy so that growth becomes stunted and even tend to die. Decrease the amount of lactic acid bacteria causing acidification activity decreased to skim milk as indicated by the decrease in the total number tertitrasi acid and increased

pH values (Figure 2a and 2b). The longer the time required to lower the pH of the medium, the lower the acidification activity of bacteria.

**b. Mold and Yeast count**

The count of mold and yeast in dried cultures of *L.rhamnosus* R21 with a protective material skim milk during storage were observed to determine the mold and yeast contamination in dried cultures. The results showed that overall there was no contamination of molds and yeasts in dried cultures during storage (mold and yeast count is 0 colonies/ml). This is because the tube shelter material to be dried first rinsed with alcohol, other than that the material had no direct contact with the glass container because of the material that will be placed in erlenmeyer freeze drying a sterilized beforehand and are less likely to be contamination. During storage, the salt jar saturated and dry container culture container to be stored has been sterilized. The application of sanitary conditions during drying and storage to minimize contamination by mold and yeast.

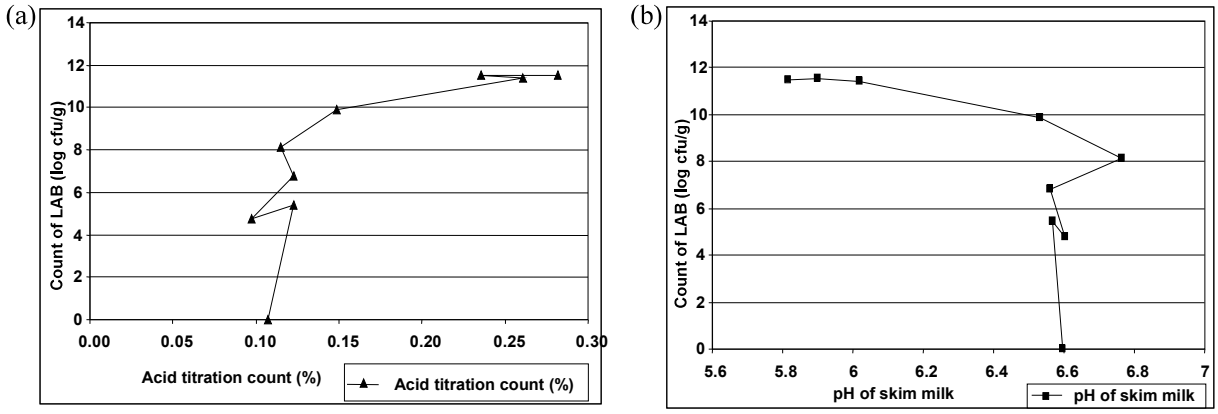


Figure 2. Acidification activity of culture of *L.rhamnosus* R21, (a) the relationship the number of LAB with acid titration count (%), (b) The relationship of the number of LAB with pH of acidified skim milk

**c. Estimation of shelf life of dried cultures *L. rhamnosus* R21 by Labuza model (1982)**

Estimation of shelf life of dried cultures using Labuza model (1982), which utilize the sorption characteristics isotherm food approaches the critical moisture content. The data needed to determine the shelf life of the product is the initial moisture content (Mi), the critical water content products (Mc), the equilibrium moisture content (Me), packing permeability (k/x), extensive packaging (A), weight of products to be packaged (W), saturated water vapor pressure at storage conditions (Po) and the slope of the curve (slope) of the sorption curve isotherms (b). In addition to the water content of the data, other data needed to determine the shelf life is secondary data obtained from the study of literature. External conditions (temperature and RH) assumed to be constant during storage, the ratio of water vapor permeability and extensive packaging (k/x) unchanged and isotherms considered linear sorption on the interval between initial moisture and humidity critical (Labuza 1982). Slope isotherms sorption curve is the rate of increase in the water content of $a_w$ beginning to reach critical water levels to be predicted (Labuza 1982). Slope value can be determined as the ratio between the difference in initial moisture content and moisture content critical to the difference between the values of initial water activity with the activity of water at the critical moisture content is reached.

The results showed that the initial moisture content (Mi) dried cultures *L. rhamnosus* R21 bk is 2.17% or 0.0217 g $H_2O$/g solids. Value of critical moisture content (Mc) dried cultures *L. rhamnosus* R21 in this study is determined by the calculation of the total bacterial culturing dry *L. rhamnosus* R21. Critical moisture content dried culture obtained based on the time it takes to maintain the viability of the culture critically dry before experiencing a decrease in the amount of 2.6 x $10^{11}$ log cfu/g where the water level is

at 14.47% bk or 0.1447 g $H_2O$/g solids (Figure 3a). Determination of the critical viability of 2.6 x $10^{11}$ log cfu/g is determined by the amount of lactic acid bacteria before it has decreased drastically during storage indicating not change the stability of the bacterial cells because of the increased water content.

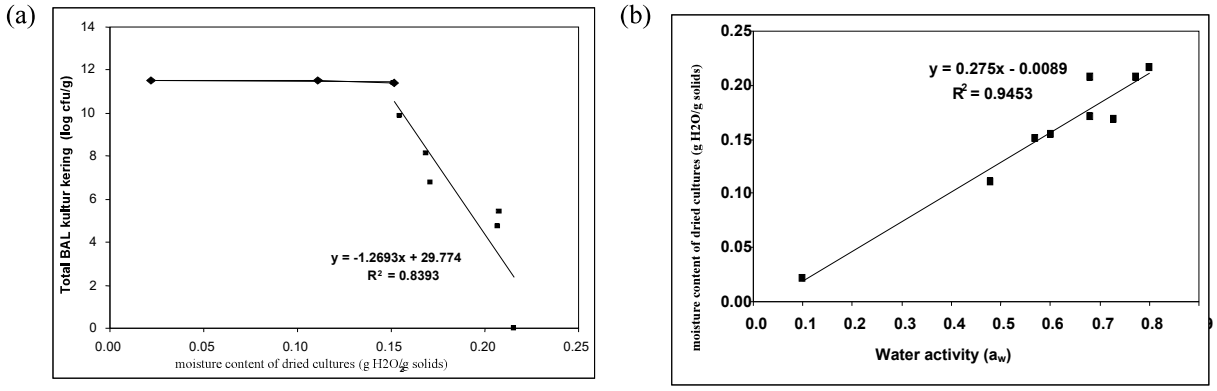


Figure 3. (a) Relations of lactic acid bacteria with the water content during storage for determination of critical moisture content (Mc), (b) sorption curve isotherms dried cultures *L. rhamnosus* R21 for the determination of the equilibrium moisture content (Me).

Equilibrium moisture content (Me) is determined by a linear equation of sorption curve isotherms dried culture on the relationship between water content with water activity of dried cultures were measured using aw meter. Isotherms sorption curve slope values (b) were determined in the linear region of the curve of the relationship between water content with water activity (Arpah, 2001). Slope value of dried cultures *L. rhamnosus* R21 is 0.2789 (from the calculation of the ratio between the difference in initial moisture content and moisture content critical to the difference between the value of initial water activity with the activity of water at the critical moisture content is reached). Of linear equations in Figure 4b obtained equilibrium moisture content (Me) on storage at 75% RH bk 19.75% or 0.1975 g $H_2O$/g solids, while storage at 80% RH obtained the equilibrium moisture content of 21.11% bk or 0.2111 g $H_2O$/g solids. Saturated water vapor pressure (Po) of storage conditions on the shelf life determination is the saturated vapor pressure at 75% RH and a temperature of 30°C in the amount of 31.824 mmHg (Labuza *et al*. 1984).

Packaging used in determining the shelf life of dried cultures are usually aluminum foil packaging, but can also use aluminum foil packaging are plastic coated. The use of plastics for packaging is quite interesting because of its favorable properties such as easy to set up, has a high adaptability of the product, such as non-corrosive metal containers, as well as easy handling (Syarief et al., 1989). According to Latif (1999) packaging permeability (k/x) for PE (polyethylene) = 0.169 g $H_2O$ / $m^2$.mmHg/day; PVC (polyvinylchloride) = 0.544 g $H_2O/m^2$.mmHg/day, while aluminum foil laminated with plastics PE = 0.013 g $H_2O/m^2$.mmHg/day (Histifarina, 2002). Calculation of shelf life of the packaged material assuming weighing 20 g wet weight (19.98 g dry weight), widely used packaging by 11 x 7 $cm^2$ then the ratio of surface area with heavy packs are packaged culture is at 0.0004 $m^2$/g .

Based on the equation parameters shelf has been described above, the shelf life of dried cultures can be determined. If fluctuations in humidity at room temperature between 75% to 80% with initial moisture content dried cultures *L. rhamnosus* R21 at 2.17% bk (according to research), the shelf life of dried culture with a laminated aluminum foil packaging with plastic PE can reach 5.10 to 5.86 years on a slope value of 1 and 5.10 – 5.88 years on the value of slope 2. Results shelf life of dried cultures calculation using the equation Labuza (1982), with a wide range of packaging on the storage RH 75 and 80 to 30°C storage temperature can be seen in Table 1.

**Table 1 Estimation of shelf life of dried cultures *L. rhamnosus* R21 at RH 75 and 80; Temperature 30°C**

| Type of packaging | Permeability packaging (k/x) | Shelf life (years) | |
|---|---|---|---|
| | | RH 75 | RH 80 |
| Aluminium foil laminated with PE | 0,013 | 5,86 | 5,10 |
| *Metallized plastic* | 0,018 | 4,23 | 3,68 |
| PET | 0,020 | 3,81 | 3,31 |
| PE | 0,169 | 0,45 | 0,39 |
| PVC | 0,544 | 0,14 | 0,12 |

Type of packaging with different permeability showed different results. The results obtained show that packing with high permeability have a shorter shelf life compared with the packaging which has a low permeability. On application by using the model Labuza (1982), the permeability of the packaging is crucial shelf dried cultures *L. rhamnosus* R21. Permeability relatively small package capable of inhibiting the transformation of water vapor into a storage environment with good packing so that the level of dryness in the product can be maintained long term. Based on calculations derived shelf life, packaging should be used to store dried cultures *L. rhamnosus* R21 is aluminum foil laminated with plastic PE with relatively low permeability compared to packaging PE (polyethylene) or PVC (polyvinylchloride).

## CONCLUSION

The process of freeze drying (freeze drying) decrease the number of lactic acid bacteria, but can still produce dried probiotic cultures with high viability. From the calculated shelf life estimation using equation Labuza (1982), found that the shelf life of dried cultures *L. rhamnosus* R21 stored at RH 75, temperature of 30°C with aluminum foil packaging materials are laminated with PE for 5.86 years and 5.10 years for RH 80.

From the results of this study suggest that further testing needs to be done on the properties of other probiotic dry cultures *L. rhamnosus* R21 that has been through the process of freeze-drying as the ability to reduce the number of pathogenic bacteria, and should also confirm the results of estimation of the shelf life of dried cultures by measuring and storing the actual conditions.

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# EXTRACTION OF NATURAL COLORANT FROM RED CABBAGE (*Brassica oleracea var. capitata l. f. rubra (l.) thell*) BY USING TARTARIC ACID

**Gusti Ayu Ekawati[1], Putu Timur Ina[2], Marlinsa Mallang[3]**
*[1,2]Department of Food Science And Technology, Faculty of Agricultural Technology, Udayana University, Kampus Bukit Jimbaran, Badung-Bali*
*[3]Department of Food Science And Technology, Faculty of Agricultural Technology, Udayana University, Kampus Bukit Jimbaran, Badung-Bali*
*Email: anangadd@yahoo.com*

**ABSTRACT**

The study was carried out to study the influence of tartaric acid concentration on natural colorant of red cabbage and the highest concentration of tartaric acid in produce natural colorant of red cabbage. This research used Completely Randomized Design. The treatmens were six different addition of tartaric acid concentration namely: 0.15%, 0.30%, 0.45%, 0.60%, 0.75% and 0.90% with three replicates. The results showed that the concentration of tartaric acid influenced the total anthocyanin and water content of natural color of red cabbage. The most effective concentration of tartaric acid to produce natural color was 0.45%. It had characteristic such as total anthocyanin was 0.0107% and water content was 99.4527%.

***Keywords****: red cabbage, extraction, tartaric acid, anthocyanin*

## INTRODUCTION

The use of purple cabbage at present is still limited only for food decorations and salads. Purple cabbages also known as red cabbage and are often found at traditional markets and supermarkets. Cabbages belong to the family *Brassiceae*or better known as *Cruciferae* (Anonymous, 2009). Purple cabbage is one of the vegetables that contain anthocyanin, which can be seen from the purple color of the vegetable. Vegetables and fruits that contain purple color has a remarkable benefit for human health. This is due to the high content of anthocyanin (Astawan and Kasih, 2008). According to Anon. (1992), the purple cabbage contained 1.4 g protein, 0.2 g fat, 5.3 g carbohydrates, 46 mg calcium, 31 mg phosphorus, 0.5 mg iron, 80 IS vitamin A, 0.06 mg vitaminB1, 50 mg vitamin C, and 92.4 mg water, but this is not information about amount of anthocyanin yet.

Anthocyanin purple cabbage is a natural dye that does not harm to the body compared to the synthetic dye. Natural dyes are natural pigments obtained from plants. In Indonesia, people used traditional food coloring derived from natural ingredients such as turmeric for yellow color and suji leaves for green color. Natural dyes are safe for consumption than the synthetic dyes. Anthocyanin a natural pigment that has red purple color, and blue colors that are commonly found in the plants (Tensiska and Dita, 2007). The concentration of pigments play a important role in determining the color formed. According to Winarno (2002), in non thickness concentration anthocyanin produce blue color, in thick concentration produce red color and in usual concentration produce purple color. Amount of anthocyanin content in purple red cabbage is not information yet.

The natural dye extract is produced by maceration extraction. Extraction is a process of separation of solid or liquid material using solvent. The maceration extraction conducted by sub merging/soaking purple cabbage in a tartaric acid solution. Tartaric acid is a natural substance that can be found in many plants such as grapes and is also found in wine. According to Elaine (2005) tartaric acid is a non colorless or white crystal, crystalline powder, found in the tissues of vegetables and fruit juices which can be consumed commercially. Tartaric acid is used as a solvent in producing natural dyes (anthocyanin) from purple cabbage. The selection of tartaric acid as a solvent is based on the experiment conducted by Tensiska and Dita (2007). They compared tartaric acid, citric acid and acetic acid, to produce a large amount of natural dye (anthocyanin) after being checked by using a spectrophotometer with absorbance of 510 nm and 700 nm. Acid can cause lots of vacuola cell walls to break/divide so more anthocyanin pigment can be extracted.

## MATERIALS AND METHODS

The materials used are purple cabbage (*Brassiceaeoleraceaevar. CapitataL.f. rubra (L.) Thell)* purchased at the traditional market at Denpasar Badung. Chemicals used are tartaric acid, KCL, citric acid, Na-citrate, 0.2 M HCl, distilled water, buffer solution pH 1.0 and pH 4.5 buffer solution

The method used is a Completely Randomized Design (CRD) with six treatments of different tartaric acid concentration and three repeated (Hanafi, 1993).

### Implementation of Research

Purple cabbage was cut, washed, blended then weighed for 5 g. Twenty five ml of tartaric acid was added with different concentration (0.15%, 0.30%, 0.45%, 0.60%, 0.75% and 0.90%) and macerated extracted for 24 hours. The mixture solution were filtered with filter paper to obtain anthocyanin pigment. The flow chart of the process of producing natural dyes can be seen in Figure1.

### Parameters Observed

Parameters observed are total anthocyanin (Giusti and Worlstand, 2001) and water content (Sudarmadji*etal*., 1997).

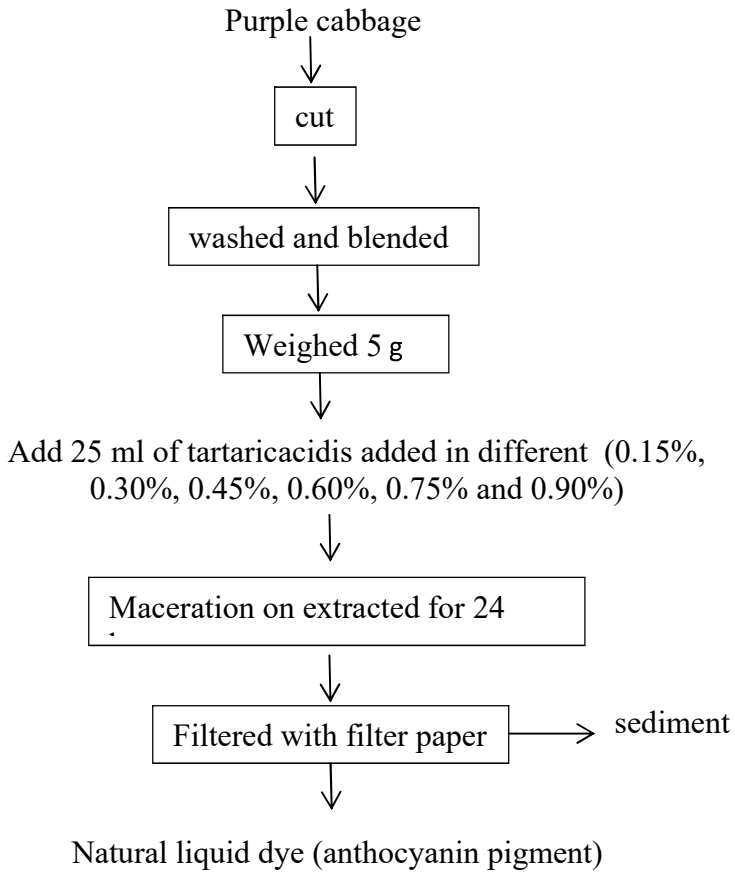


Figure 1.The flow chart of the process of producing natural liquid dyes of purple cabbage (*Brasisicaoleraceavar. CapitataL.f. Rubra(L.) thell*)

## RESULTS AND DISCUSSION

### Concentration of Total Anthocyanin Produced by Extraction of Natural Dyes of Purple

Based on the variance analysis, the concentration of tartaric acid had significant difference effect ($P < 0.05$) on total anthocyanin natural dyes of purple cabbage. The average value of total anthocyanin of purple cabbage can be seen in Table 1. Table 1 shows that the highest average value obtained from the total anthocyanin produced was $K_3$ with 0.0107%. The value was not significance compare to the value of $K_2$ and $K_1$, while the lowest average value obtained at the total anthocyanin is $K_6$ with 0.0097% of total anthocyanin that showed a significance different from all treatments. According to Winarno (2002), total anthocyanin is influenced by acid concentration and pH. According to Riata (2010), low pH (pH3) causes anthocyanin to have a strong/thick color, that shows purple to red color, andt pH around 5 causes anthocyanin colorless.

Table 1.The average concentration of total anthocyanin in natural dye extract of purple cabbage(%)

| Acid Concentration | Mean of total anthocyanin |
|---|---|
| $K_1$ (0.15%) | 0.0105 a |
| $K_2$ (0.30%) | 0.0106 a |
| $K_3$ (0.45%) | 0.0107 a |
| $K_4$ (0.60%) | 0.0102 b |
| $K_5$ (0.75%) | 0.0099 c |
| $K_6$ (0.90%) | 0.0097 d |

Description: Means not followed by the same letter in the same column represent a significant difference ($P < 0.05$)

### Water Levels on Natural Dye Extract of Purple Cabbage

Analysis of variance showed that the concentration of tartaric acid had significance ($P < 0.05$) on water content of purple cabbage extract. The average value of the water content of natural dye of purple cabbage can be seen in Table 2.

Table 2. The average value of the water content of natural dye extract of purple cabbage (%)

| Acid Concentration | Mean (%) |
|---|---|
| $K_1$ (0.15%) | 99.3510 c |
| $K_2$ (0.30%) | 99.4204 b |
| $K_3$ (0.45%) | 99.4527 a |
| $K_4$ (0.60%) | 99.1884 d |
| $K_5$ (0.75%) | 99.1278 e |
| $K_6$ (0.90%) | 98.9397 f |

Description: Means not followed by the same letter in the same column represent a no significant difference ($P > 0.05$)

Table 2 shows that the average value of the highest water content is obtained o $K_3$ with water content of 99.4527%, and was significant difference to all treatments. The average value of the lowest water levels for the treatment of acid concentration is $K_6$ with 98.9397%. This is because $K_6$ is more acidic than the other treatments. The more acid in the extraction solution will be obtained more total anthocyanin. The due to content of water will be low.

## CONCLUSIONS

Conclusions of the research are:

1. The concentration of tartaric acid affects total anthocyanin and water content.
2. The highest total anthocyanin was produced by using tartaric acid concentration of 0.45% with total anthocyanin is 0.0107% and moisture content is 99.4527%.

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# THE USAGE OF BEANS FLOUR AS PROTEIN SOURCE IN WET NOODLES PROCESSING

**Anak Agung Made Dewi Anggreni*, I Wayan Arnata, dan I Agus Gede Amanda Paratama**
*Study Program of Agricultural Industrial Technology, Faculty of Agricultural Technology, Udayana University, Kampus Bukit Jimbaran, Badung, Bali, Indonesia*
**Correspondence author: dewianggreni06@yahoo.co.id*

**ABSTRACT**

The purpose of this research were 1) to determine the influence of substitution of wheat flour with beans flour at different concentration on the wet noodle's characteristics and 2) to find out the appropriate kind and concentration of bean flour to produce wet noodle with the best characteristic. This research used randomized block design with factorial pattern. The first factor was types of bean that were soybean, mungbean, and kidney bean. The second factor was concentration of bean flour that consisted of 4 levels namely, 0%, 15%, 30%, and 45%. All treatments had 2 replication, in order to obtain 24 trial units. All data was analyzed using ANOVA follow by duncans multiple range test when significant different among treatment were found. The result shows that interaction between treatment had very significant effect (P<0,05) on ash content, crude fiber content, protein content, color, taste, and overall acceptance of wet noodle, however no significant effect on moisture content, texture, and flavor. The best treatment was to substitute the wheat flour with kidney bean at 30 % level with the following characteristics 57.97% moisture, 1.42% ash, 13.29% protein, 36.62% crude fiber, the color score was 5.8 (moderate like), the texture score was 4.73 (elastic till very elastic), the flavor score was 4.8 (neutral till rather like), the taste score was 5.07 (rather like till like), and overall acceptance score was 5.27.

***Keywords***: bean flour, concentration, wet noodle

## INTRODUCTION

Noodle was one kind of food contained lot of carbohidrate but low in protein. It was very important therefore to increase its protein content. One of methods was using bean flour as source of protein. The purpose of this research were: 1) to determine the influence of substitution of wheat flour with beans flour at different levels on the wet noodle's characteristic, 2) to find out the appropriate type and level of bean flour to produce wet noodle with the best characteristic.

## MATERIAL AND METHOD

### Material

The materials used for making noodle are : wheat flour, salt, vegetable oil, soy bean, mung bean, and kidney bean were bought at Sanglah Market, Denpasar then beans were grounded at Graha Pasek, Sesetan, Denpasar. *Sodium tripolyphospat* (STPP), and drinking water.

### Method

A randomized block design with factorial pattern was used in this experiment. The first factor was type of bean that consisted of three types namely, soybean, kidney bean, and mungbean. The second factor was levels of bean flour used namely, 0%, 15%, 30%, and 45%. Each treatment has 2 replicates, in order to obtain 24 trial units. All data was analyzed using ANOVA follow by duncans test when significant different among treatment were found (Gomez dan Gomez, 1995).

### Noodle Processing

Wet noodle processing was done as follow: 1) a mixture wheat flour and bean flour (depend on the treatment; to make total weight 100 g) was mixed with row egg (20 g), STPP (0,5 g), salt (2 g) and water (30 ml), then stirred by tea spoon. 2) mixed by hand until became dull dough then let it stand for 15 minute. 3) the dough was pressed by using dough sheet roller (until dough sheet thickness of 1.5 mm). 4) dough sheet was spreaded by 5 g wheat flour, then was place to the roll press to get noodle with the length of 20

cm and the width of 2 mm. 5) acquired noodle was boiled in the oil – contained water for 5 minute at temperature of 100 °C while slowly stired, drained, and then warmed.

**Analysis**

Chemical Analysis for water, ash, protein, and crude fiber method following the method explained by Sudarmadji *et al*. (1997). Organoleptic test integrated color, flavor, taste dan overall acceptance by hedonic test (Soekarto, 1985) while texture by score test (Soekarto, 1985).

## RESULTS AND DISCUSSION

**Water Content**

Analysis of varian showed that types and levels of bean flour had high significant effect (P< 0.01) on water content while treatments interaction had non-significant effect (P>0,05) on the water content of wet noodles (Table 1).

Table 2. The average of water content of wet noodle (%)

| Inclusion rate | Bean Flour Level ( %) | | | | Average |
|---|---|---|---|---|---|
| | 0 | 15 | 30 | 45 | |
| Soybean | 53.9 | 63.0 | 66.4 | 66.2 | 62.4 a |
| Kidney bean | 53.9 | 56.4 | 58.0 | 61.8 | 57.5 b |
| Mung bean | 53.9 | 53.4 | 57.2 | 58.3 | 55.7 b |
| Average | 53.9 a | 57.6 a | 60.5 b | 62.1 c | |

Table 1 shows that the increasing of bean flour concentration has increased the water content of wet noodle. The reason was that wheat flour had lower water content than all type of the bean flour used. Water content of soybean was 13.8% (AAK, 1998), kidney bean was 10%, and mung bean was 11% (Anggreni *et al*., 2011) (unpublished).

**Ash Content**

Analysis of varian showed that types and levels of bean flour had high significant effect (P< 0.01) on the ash content of wet noodles (Table 2).

Table 2. The average of ash content (%)

| Inclusion rate | Bean flour level % | | | |
|---|---|---|---|---|
| | 0 | 15 | 30 | 45 |
| Soybean | 0.6 e | 0.8de | 0.9 de | 1.1 cd |
| Kidney bean | 0.6 e | 0.8 de | 1.4 bc | 1.7 b |
| Mung bean | 0.6 e | 2.3 a | 2.4 a | 2.7 a |

Table 2 shows that the inclusion of different types of bean flour produced wet noodle with different ash content due to the fact that ash content of each bean flour used was higher than wheat flour.

**Protein Content**

Analysis of varian showed that types and levels of bean flours had high significant effect (P< 0.01) on the protein content of wet noodles (Table 3).

Table 3. Protein content of wet noodle (%)

| Inclusion Rate | Bean flour levels % | | | |
|---|---|---|---|---|
| | 0 | 15 | 30 | 45 |
| Soybean | 9.3 e | 18.1 b | 23.0 a | 23.9 a |
| Kidney bean | 9.3 e | 12.3 d | 13.3 cd | 14.4c |
| Mung bean | 9.3 e | 13.1 cd | 17.1 B | 18.2 b |

Table 3 demonstrates that the inclusion of different types of bean flour produced wet noodle with different protein content because protein content of each bean flour was higher then wheat flour. Protein content of soybean was 41% (AAK., 1998), kidney bean was 22.9% (Anon., 2010[d]), dan mung bean was 23.86% (Anon., 2010[b]). Protein content of wheat flour was 10 – 11 % (Adrianto and Idranto, 2004).

**Crude Fiber Content**

Table 4 shows that type and level of bean flour inclusion had high significant effect (P< 0.01) and there was had significant interaction effect (P<0,05) on the crude fiber content of wet noodles.

Table 4. Crude fiber content of wet noodle (%)

| Inclusion Rate | Bean flour level % | | | |
|---|---|---|---|---|
| | 0 | 15 | 30 | 45 |
| Soybean | 30.8 e | 34.1 d | 34.9 cd | 35.8 bc |
| Kidney bean | 30.8 e | 35.8 bc | 36.6 b | 37.8 a |
| Mung bean | 30.8 e | 35.2 c | 35.8 Bc | 38.2 a |

Table 4 shows that increasing of bean flour levels has increased the crude fiber content of wet noodle. Based on the previous study, wheat flour had crude fiber 0.4% lower than bean flour. As we know, beside as protein source bean also contain high dietary fiber.

**Organoleptic Characteristics**

Analysis of varian showed that interaction between type and levels of bean flour had high significant effect (P< 0.01) on color, taste and overall acceptance but had a non-significant effect (P>0,05) on texture and flavor of wet noodles (Table 5).

Table 5. Color, texture, taste, flavor, and over all acceptance of wet noodle

| Treatment | Color | Texture | Taste | Flavor | Overall Acceptance |
|---|---|---|---|---|---|
| Soybean at 0 % level | 5,13 abc | 4,67 a | 5,27 a | 4,40 a | 5,07 ab |
| Soybean at 15 % level | 5,60 ab | 4,67 a | 4,80 ab | 4,07 a | 5,13 ab |
| Soybean at 30 % level | 4,87 abc | 4,20 a | 3,87 bcde | 3,87 a | 4,40 abcd |
| Soybean at 45 % level | 3,87 d | 3,67 a | 3,33 e | 3,53 a | 4,00 d |
| Kidney bean at 0% level | 5,13 abc | 4,67 a | 5,27 a | 4,40 a | 5,07 ab |
| Kidney bean at 15% level | 4,20 cd | 4,20 a | 3,60 cde | 3,53 a | 3,73 d |
| Kidney bean at 30% level | 5,80 a | 4,73 a | 5,07 a | 4,80 a | 5,27 a |
| Kidney bean at 45% level | 5,13 abc | 4,33 a | 4,47 abcd | 4,20 a | 5,00 ab |
| Mung bean at 0% level | 5,13 abc | 4,67 a | 5,27 a | 4,40 a | 5,07 ab |
| Mung bean at 15% level | 5,00 abc | 4,80 a | 4,60 abc | 3,93 a | 4,87 abc |
| Mung bean at 30% level | 4,67 bcd | 4,87 a | 3,87 bcde | 3,33 a | 4,27 bcd |
| Mung bean at 45% level | 3,80 d | 3,80 a | 3,53 de | 3,33 a | 4,07 cd |

Table 5 shows that the highest average color was on the treatment of kidney bean at 30% levels of 5.8 (moderate like – like) and the lowest was on the treatment of soybean at 45% levels of 3.8 (moderate dislike –netral. The average of texture of wet noodles was between 3.67 (neutral – elastic) – 4.87 (elastic – very elastic). The average of wet noodle's flavor was between 3.33 ( moderate dislike – neutral) till 4.80 (neutral – moderate like). The average of taste was between 3.33 ( moderate dislike – neutral) till 5.27 (moderate like - like). The highest average of overall acceptance on the treatment of kidney bean at 30% levels was 5.27 (moderate like – like) and the lowest on the treatment of soybean at 45% levels was 3.73 (moderate dislike – neutral).

## CONCLUSION

1. Interaction between types and levels of bean flour had high significant different on ash content, protein content, crude fiber, color, taste, and over all acceptance of wet noodles but had non significant effect on water content, texture and flavor of wet noodle.
2. The best inclusion level was kidney bean at 30% level.

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# IDENTIFICATION OF GEL FORMING COMPONENT (GFC) OF *KACAPIRING* LEAF (*Gardenia jasminoides* Ellis)

**Widnyana Yoga[1*], Nuri Andarwulan[2], and Endang Prangdimurti[2]**
*[1]Faculty of Agriculture Technology Udayana University, Kampus Bukit Jimbaran, Bali. [2]Bogor Agricultural University, Kampus IPB Dramaga Bogor Indonesia *Corresponding author: ibkyogi@yahoo.com*

**ABSTRACT**

Leaf of *kacapiring* (*Gardenia jasminoides* Ellis) is a part of plant which has hydrocolloid component and can form gel. The aims of this research were (1) to evaluate physical and chemical properties of leaves and gel, (2) to extract, isolate, fractionate and identify of gel forming component Physical characterization of gel was tested with sensory analysis. Chemical components of leaf and gel were quantified by *proximat analysis* and mineral was analyzed by *atomic absorption spectrophotometer* (AAS). Isolation of GFC was done by extraction using 0.028M with *ethylendiamintetraacetate* (EDTA). GFC was fractionated by ultrafiltration membrane. Monomer of GFC was identified by *paper chromatography*, and estimation of pectic substances was done by *spectrophotometer*. Sensory data was analysed by ANOVA and others by descriptive analysis. The result showed that *kacapiring* leaves contained water, ash, protein, fat, and carbohydrate. The mineral content in leaf and gel consisted of Ca (19974.70±49.31 and 5429.71±68.98 $mgKg^{-1}$ db), Mg (4263.15±38.66 and 2800.63±110.96 $mgKg^{-1}$db), dietary fibre (24.98±0.72 and 90.61±1.02 % db respectively. Isolate of 0.25% GFC was fractionated by ultrafiltration membrane and 96.78 % of retentate was separated in 5 µm membrane filter. Analysis by paper chromatography showed that GFC isolate contained galacturonic acid and glucose. Pectic substance analysis by GFC isolate was contains 56.53±0.61% db of galacturonic acid. This meant that GFC isolate was pectin polymer. From this result, it can be concluded that *kacapiring* leaves were very important and potential to be developed as functional foods.

***Keywords****: gel forming component, Gardenia jasminoides* Ellis*, identification.*

## INTRODUCTION

*Kacapiring* (*Gardenia jasminoides* Ellis) is a multi-purpose plant, because every part of this plant has a function. The root of *kacapiring* is used for toothache and fever. The flower can be processed to be essential oil or cosmetic ingredient. The stem can be used as raw incense for aroma therapy (PPT 2007). The fruit is for food coloring, *antitumor, antihiperlipid, antihepatik, diuretics, laxatives, koleratik* (Zhou *et al*., 2007), while *kacapiring* leaves are used as medicine heartburn, ulcers and diabetes (Dalimartha 2005). *Kacapiring* leaves are dark green, contain chlorophyll which is the main natural pigments in leaves. Identification of phytochemical of *kacapiring* leaves showed that the leaves contain flavonoids, saponins, tannins, gallic acid, steroids or terpenoids (Fatmawati 2001). This phytochemical compounds serve as natural antioxidants, so it is potential to be developed.

The extracts of the leaves with water has the ability to form gel. Gel is a hydrocolloid containing mostly water with characteristic as solids, especially the nature of elasticity and rigids (Fardiaz 1989). Gel contains bioactive components, namely dietary fibre. Dietary fibre has a very important role in preventing obesity, atherosclerosis, coronary heart disease, colon cancer and diabetes (Nawirska & Kwasniewska 2005). Dietary fibre also have some mechanism in counteracting toxins (detoxification), antibacterial and antioxidant effects in the gastrointestinal tract (Muchtadi 2000).

Functional properties of the gel-forming component (GFC) of *kacapiring* leaves should be studied pundamentally, to be applied in commercial scale. Functional properties of GFC are influenced by molecular weight, concentration, presence of minerals, environmental conditions and constituent sugar units (Southgate 1991 referred to Artha 2001). Therefore, this study was carried out (1) to evaluate physical and chemical properties of leaves and gel, (2) to extract, isolate, fractionate and identify of gel forming component **(**GFC) of *kacapiring* leaf.

## MATERIALS AND METHODS

The raw materials used were fresh *kacapiring* leaves, were taken from the campus of IPB Dramaga-Bogor. Picking was done in the afternoon at 5 pm, which leaves at the position number 3, 4 and 5 from

the top. The tool used were analytical balance, refrigerators, freeze dryer, pH meter, waterbath, heater, furnace, nitrogen gas, paper chromatography, oven, magnetic stirrer, ultrafiltration reservoir Amicon and membrane filter (Millipore). The instruments used were atomic absorption spectrophotometer (AAS) and spectrophotometer UV-Vis.

The materials used in this study were ethanol 95% (Merck), EDTA (Merck), HCl (Merck), NaOH (Merck), KOH (Merck), $H_2SO_4$ (Merck), Petroleum Ether (JT Baker), $HNO_3$ (Merck), 1000 ppm of standard Mg, Cu, Fe and Ca (Merck), Glucose (Merck), Galactose (Merck), Lactose (Merck), Raffinose (Merck) and Galacturonic acid (Wako), Acetone (Merck), Thermamyl enzyme A9972 (Sigma), Pepsin (JT Baker), Pancreatin (JT Baker), $CaCO_3$ (Merck), Fehling A and Fehling B, 2-propanol (JT Baker), Ethyl acetate (Merck), Diphenylamine (Merck), Aniline (Merck), Orthophosphate acid (Merck), o-hidroksidifenil (Wako), Natrium tetraborate (Merck), Carbohydrase enzyme complex V2010 (Novozyme).

**Characterization of chemical physical properties of the leaves and gel**

The first step was to identify the varieties of plants and to measure the dimension of the leaves. The chemical components of the fresh leaves were analyzed by proximate and mineral content (AOAC 1998). Fresh leaves were dried with a freeze dryer, crushed and sieved 30 mesh to obtain powder.

Gel was obtained by extracting of the fresh leaves using bottled water (BW) and aquades (AQ) with the ratio of leaves: water 1:5, 1:10, and 1:15. The best physical character of the gel was determined by criteria not frothy and compact with sensory test, (Soekarto 1985) was involving 26 panelists. The best of gel was analyzed the chemical composition of mineral content by AAS (AOAC 1998), dietary fibre (Asp *et al*. 1983), and pectic substance with a spectrophotometer (McCready & McComb 1952, modified).

**Isolation, Fractionation and Identification of Gel Forming Component (GFC)**

Gel forming component (GFC) was isolated by the best character of gel, using a 20% of EDTA solution 0.028 M, and heated at a temperature of 90$^o$C for 15 minutes. The filtrate was acidified with HCl 0.1 N until pH 3 and added by ethanol 96% to get GFC isolates (Farida 2002). GFC isolates was dried and crushed to be a powder for fractionation and identification.

The fractionation of GFC isolate 0.25% was done ultrafiltration membranes. Identification of GFC isolates monomer was conducted qualitatively by Fehling test and chromatography paper using glucose, galacturonic acid, lactose, raffinose, fructose and galactose standard with color indicator solution consisting of a mixture of aniline, diphenylamine and phosphoric acid (Gammar *et al*. 1997).

**Statistical analysis**

Sensory data were analyzed using one-way ANOVA. Duncan's was used to assess differences between means. A significant difference was considered at the level of $p< 0.05$. The other data were expressed as mean $\pm$ deviation standars.

## RESULTS AND DISCUSSION

**The Characteristics of *Kacapiring* Leaves**

The result of the identification of plant varieties indicated that *kacapiring* plant in a group of Rubiaceae by scientific name *Gardenia Jasminoides* Ellis. *Kacapiring* leaves have length between 5-20 cm with an average between 4-5 cm. Lemmens and Soetjipto (1999), stated that *kacapiring* leaves were from 5-15 cm long and 2-7 cm wide. The chemical analysis constituents can be seen in Table 1.

Table 1 Chemical composition of *kacapiring* leaves

| Parameter | Unit | Content | |
|---|---|---|---|
| | | wb | db |
| Water | % | 67.29±0.09 | - |
| Fat | % | 2.40±0.01 | 7.35±0.03 |
| Protein | % | 4.85±0.06 | 14.83±0.19 |
| Carbohydrates (*by difference*) | % | 23.67±0.13 | 72.41±0.12 |
| Dietary Fiber | % | 8.17±0.23 | 24.98±0.72 |
| Ash | % | 1.76±0.04 | 5.39±0.14 |
| Ca | ($mgKg^{-1}$) | 6532.51±16.12 | 19.974.70±49.31 |
| Mg | ($mgKg^{-1}$) | 1394.21±12.64 | 4263.15±38.66 |
| Fe | ($mgKg^{-1}$) | signed | signed |
| Cu | ($mgKg^{-1}$) | signed | signed |

signed = undetected, wb = wet basis, db = dry basis

Water, ash and protein content of *kacapiring* leaves, lower than *cincau* leaves (*Cyclea barbata* L.Miers), that was 75.33% wb, 8.47% db and 23.51% db (Farida 2002). Fat and carbohydrate content higher than the *cincau* leaves 0.93% db and 67.09% db, mineral content of Cu and Fe was not detected, whereas the dominant mineral was Ca 19,974.70 $mgKg^{1}$db and Mg 4263.15 $mgKg^{1}$db.

**The Gel Characteristics** of ***Kacapiring*** **Leaves**

Ananta (2000), reported that water was the best solvent to extract the *Cincau* leaves in forming gel. The data subjectively can be seen in Table 2. The best treatment of AQ 1:15, so was continued for characterize the physical and chemical gel. Fresh gel had a moisture content of 98.75 ±0.09% wb. Mineral content test showed that the mineral Ca (5429.71 $mgKg^{-1}$) was higher than Mg (2800.63 $mgKg^{-1}$). Tang *et al.* (1995) stated that the gel formed by the addition of $Ca^{2+}$ ions was stronger than ion $Mg^{2+}$ due to differences in the size of the cation, so that the mineral Ca had stronger role in the mechanism of gelation than Mg.

Table 2 The results of sensory test

| Treatment | The average value of sensory test | | | | |
|---|---|---|---|---|---|
| | Hedonic quality test | | | | Hedonic test |
| Leaves: Solvent | Foamy | Color | Aroma | Consistency | General acceptance |
| **AQ** | | | | | |
| 1:5 | $1.9^{d}$ | $6.1^{ab}$ | $4.0^{b}$ | $5.7^{a}$ | $3.1^{b}$ |
| 1:10 | $3.1^{b}$ | $6.3^{a}$ | $5.5^{a}$ | $4.2^{b}$ | $4.8^{a}$ |
| 1:15 | $6.0^{a}$ | $6.0^{b}$ | $5.6^{a}$ | $4.5^{b}$ | $5.3^{a}$ |
| **BW** | | | | | |
| 1:5 | $1.1^{e}$ | $5.1^{c}$ | $3.7^{b}$ | $5.4^{a}$ | $2.8^{b}$ |
| 1:10 | $2.2^{c}$ | $6.2^{ab}$ | $4.1^{b}$ | $5.9^{a}$ | $4.6^{a}$ |
| 1:15 | $3.0^{b}$ | $5.9^{b}$ | $3.5^{b}$ | $5.6^{a}$ | $4.9^{a}$ |

Means with difference letters were significanly different at the level $p<0.05$.AQ =aquades. BW = Bottled water. Foamy (1 = very foamy, and 7 = very lease foamy), aroma (1 = very lease typical leaf, and 7 = very typical leaf), color (1 = very lease green, and 7 = very green), viscosity (1 = not lease thick, and 7 = very thick), general admission (1 = quite dislike, and 7 = quite like)

Table 3 Composition of dietary fibre and pectic substances

| Specimen | Total dietary fibre | | Soluble fibre | | Insoluble fibre | |
|---|---|---|---|---|---|---|
| | %db | %wb | %db | %wb | %db | %wb |
| **Leaves** | 24.98±0.72 | 8.17±0.24 | 18.52±0.57 | 6.06±0.19 | 6.46±0.17 | 2.11±0.05 |
| **Gel** | 90.61±1.02 | 1.13±0.01 | 58.94±1.01 | 0.74±0.01 | 31.67±0.03 | 0.39±0.00 |
| | **Total GFC isolate** | | **Pectic substance** | | | |
| | %db | %wb | %db | %wb | - | |
| **GFC isolate** | 89.52±0.44 | 1.11±0.01 | 56.53±0.61 | 0.62±0.00 | | |

The composition of the the biggest chemical gel after water was dietary fibre. Dietary fibre is needed to protect the human digestive order. The observation result of the dietary fibre in the leaves showed that the soluble dietary fibre was greater than the insoluble dietary fibre. The content of soluble dietary fibre in the gel was 58.20 %db, approaching the content of the GFC isolates pectic substance was 56.53 %db.

**Isolation of the Gel Forming Component**

GFC isolates in the gel was obtained by addition the compound *ethylenediaminetetracetate* (EDTA). The compound was a metal ion binding agent and it would increase the activation energy (Nostrandis 1976). The addition of EDTA was intended to form a complex between the mineral in the gel with EDTA (Nabrzyski 1997). GFC isolates *Kacapiring* leaf was obtained as much as 1.19% wb, with water content 6.64 ±0.19% wb. This GFC isolates was not much different from GFC isolates of other plants which was about 1 to 5%wb (Walter 1991), but it was still lower than GFC isolates of *Cincau* leaves was 1.78 to 3.78% wb (Artha 2001).

**Fractionation and Identification of Gel Forming Component**

Fractionation GFC isolates to estimate the molecular weight. Table 4 shows the results of fractionation with filter membrane 5 µm, 3 µm, 1.2 µm and 0.6 µm. Fractionation results were retentated only in the membrane fraction of 5 µm (F5). This indicates that the fraction has a molecular weight of 1000-2000 kDa (51.78%) and the passing fraction was lower than 10 kDa (3.26%).

Table 4. The results of fractionation of 100 ml of GFC isolates 0.25% with 5 µm membrane (MWCO 1000-2000 kDa)

| Component | Weight (g) | Volume (ml) | Dry weight materials (g) | Dry material of GFC Isolates (%) |
|---|---|---|---|---|
| KPG | 0.25 | 100.0 | 0.24+0.004 | 100.0 |
| Retentated fraction 5 µm | | | | |
| Above the membrane | | 22.38 ± 0.48 | 0.12 ± 0.01 | 51.78 |
| In the membrane | | 3.37 ± 0.48 | 0.11 ± 0.01 | 45.00 |
| Passing fraction 5 µm | | 74.25 ± 0.50 | 0.0074± 0.00 | 3.26 |

The identification of GFC isolates monomer was initiated by hydrolysis with *complex carbohydrase* enzymes at 5.02 of pH and incubation at 25 °C of room temperature for 60 minutes to obtain hydrolyzate. The results of the analysis hydrolizate using solution Fehling A and B produced brick red precipitate. This shows that the GFC hydrolyzate contains reducing sugar. The hydrolyzate was identified by chromatography paper technique using several standard sugars can be seen in Figure 1.

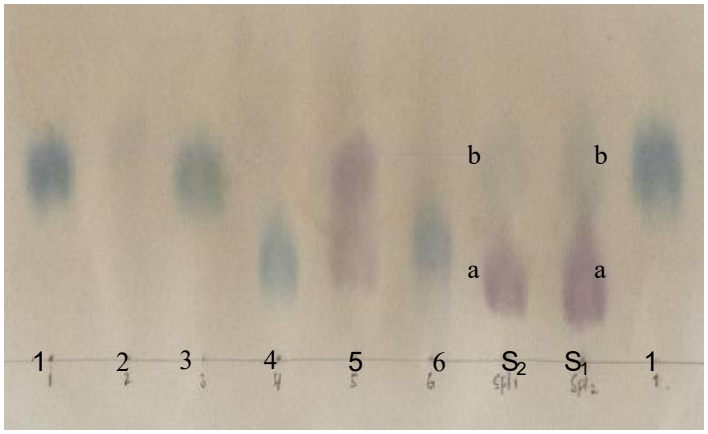


Figure 1 The separation of standard and the sample using chromatography paper. 1% standard and 2% sample concentrations. Eluent (2-propanol, ethyl acetate and distilled water 7:1:2). Dye solution (diphenylamine, aniline, and phosphoric acid). Spot description: 1 = glucose, 2 =fructose, 3 = lactose, 4 =galactose, 5 = galacturonic acid, and 6 = raffinose, S1 = replicates sample 1, S2 = replicates sample 2, a = spot 1, b = spot 2.

The results of separation using chromatography paper showed that the GFC (code S) had two clearly separated spots (code a and b), in accordance with the color and the standard retention factor (Table 5). The two spots were about the same as glucose standard (code 1, blue with Rf 27.09) and galacturonic acid standard (code 5, purple color, with a value of Rf 12.35). Therefore, the GFC of *kacapiring* leaves composed galacturonic acid monomer, as a unit of monomer of the pectin and glucose polymer as the monomer unit of the cellulose or hemicellulose polymer.

Some natural hydrocolloid, consisting of xylosa, fructose and glycerol in squash were as monomer unit. The *Cincau* leaves were composed by galacturonic acid and galactose (Artha 2001), alginate contained guloronat and manuronat as well as in gum xanthan containing mannose, glucose and glucoronat.

Table 5 Rf standard value and GFC isolates of *kacapiring* leaves which were analyzed using chromatography paper

| Code | Sample | Color | Rf Value | Comparer Rf |
|---|---|---|---|---|
| **1** | Glucose **std** | **blue** | **27.09±0.00** | 35.0 (Hana 2007) |
| 2 | Fructose std | light green | 31.08±0.14 | 38.0 (Hana 2007) |
| 3 | Lactose std | greenist blue | 17.13±0.07 | - |
| 4 | Galactose std | light blue | 14.47±0.21 | 12.0 (Harborne 1987) |
| **5** | Galacturonic acid std | **purple** | **12.35±0.07** | 15.0 (Harborne 1987) |
| 6 | Raffinose std | light blue | 15.94±0.00 | 11.0 (Hana 2007) |
| **S** | Spot KPG 1 | **purple (a)** | **11.55±0.07** | |
| **S** | Spot KPG 2 | **blue (b)** | **27.89±0.00** | |

Harborn 1987 (solvent elution were used butanol: acetonitril: water 4:1:5)

## CONCLUSION

*Kacapiring* leaves have components to form gel. The best subjective character gel was obtained at the treatment of AQ 1:15 (1 part of leaf and 15 parts of distilled water). The leaves and gel contained mineral Ca (19.974.70 ±49.31 and 5429.71 ±68.98 mgKg$^{-1}$db), Mg (4263.15±38.66 and 2800.63 ±110.96 mgKg-1db). The main component of dietary fibre in the gel was 90.61 ±1.02% db, with the distribution of dietary fiber which was soluble higher than insoluble fibre (58.94 ±1.01 and 31.67 ±0.03% db) and GFC isolates contained pectic substance of 56.53 ±0.61% db as galacturonic acid and it have molecular

weight between 1000 and 2000 kDa. The monomers by GFC isolates is composed by galacturonic acids and glucose.

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# THE APPLICATION OF *Rhizopus oligosporus* MK 1 TO REDUCE AFB1 CONTAMINATION IN TEMPEH FERMENTATION

**Putu Ari Sandhi Wipradnyadewi**
*Department of Food Science and Technology, Faculty of Agriculture Technology, Udayana University*
*Email: ptarisandhiw@yahoo.com*

**ABSTRACT**

This research was to apply *Rhizopus oligosporus* MK 1 for reducing AFB1 contamination in tempeh fermentation. The AFB1 analysis method was conducted by HPLC. The result showed that raw material (*Aspergillus flavus* contaminated and uncontaminated soybean) contained AFB1 about 149,38 ppb and 53,82 ppb, while cooked soybean or before fermentation has concentration was 99,60 ppb and 36,84 ppb. Fermentation used MK 1 isolate obvious could produced tempeh with low AFB1 concentration was 71,63 ppb and 21,56 ppb.

***Keywords:*** *Rhizopus oligosporus* MK 1, AFB1, tempeh.

## INTRODUCTION

AFB1 is a secondary metabolite being a potent toxin, carcinogenic, teratogen, and mutagen produced by *Aspergillus flavus* and *A. parasiticus.* Peanut and their product are usually contaminated by AFB1. Tempeh is a tradisional fermented food of Indonesia made from soybean fermented by *Rhizopus oligosporus.* This research was concerning on the ability of strain *Rhizopus* isolated from several tempeh inoculum in reducing AFB1 and during tempeh fermentation.

Isolated *Rhizopus oligosporus* MK 1 (isolated from Muklar merk of tempeh ) showed highest mycelia growth rate at logarithmic phase. *Rhizopus oligosporus* MK 1 also decrease AFB1 in synthetic broth, so it was choosed in tempeh fermentation to yield tempeh with low concentration of AFB1.

## MATERIALS AND METHODS

**Materials and equipment**. This research used *Rhizopus oligosporus* MK 1 isolated from Muklar merk of tempeh, *Aspergillus flavus* FNCC 0546 from Laboratory of Microbiology at Gadjah Mada University $10^6$ spora/ml contaminated and uncontaminated soybean during 7 days , standard AFB1 of liquid from Departement of Research Veteryner Bogor West Java, ether, hexane, dicholoromethane ($CH_2Cl_2$), benzene, asetat acid, sodium sulfat anhydrous, silica gel,chloroform, methanol, nitrogen gas, deionized water, High Performance Liquid Chromatography (HPLC), clean-up column, scales, erlenmeyer, beaker and petridish.

**Methods**. This research used strain *Rhizopus* isolated from several tempeh inoculum in reducing AFB1 and during tempeh fermentation. Isolated *Rhizopus oligosporus* MK 1 (isolated from Muklar merk of tempeh ) showed highest mycelia growth rate at logarithmic phase. *Rhizopus oligosporus* MK 1 also decrease AFB1 in synthetic broth, so it was choosed in tempeh fermentation to yield tempeh with low concentration of AFB1. The AFB1 analysis method was conducted by HPLC.

Modificated Tempeh Making :

1. Basic commodity preparation, soybean.
2. *Aspergillus flavus* FNCC 0546 $10^6$ spora/ml contaminated and uncontaminated soybean during 7 days.
3. After 7 days, then cleaned with water and submerged with water for 24 hours again. After that soybean were peeled and cleaned with water.
4. Soybean were boiled or steamed until the texture of rather soft until 30 minute. Then it was cooled and sterilized.

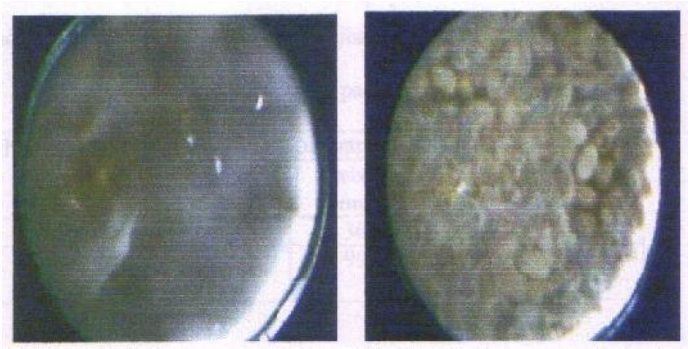
5. After that, they were inoculated with *Rhizopus oligosporus* MK 1 and fermented during 2 days.
6. Parameter observed in this research were AFB1 with HPLC method.

## RESULTS AND DISCUSSION

The chosen isolate (*Rhizopus oligosporus* MK 1) was tested its ability to reduce AFB1 contamination in CY-broth media. In this research, this isolate was tested again in order to detect its ability to reduce AFB1 when applied in tempeh fermentation. *Rhizopus oligosporus* MK 1 isolate obvious had ability to fermented *Aspergillus flavus* contaminated and uncontaminated soybean for making tempeh (Figure 1 and 2).

From up From buttom

Figure 1. *Rhizopus oligosporus* MK 1 fermented tempeh with *A.flavus* contaminated soybean



From up From buttom

Figure 2. *Rhizopus oligosporus* MK 1 fermented tempeh with *A.flavus* uncontaminated soybean

Two days fermentation aged tempeh result used *Rhizopus oligosporus* MK 1 isolated produced tempeh that formed conidia, seen from up and buttom. Raw material (*Aspergillus flavus* contaminated soybean) contained AFB1 about 149,38 ppb while cooked soybean or before fermentation has concentration was 99,60 ppb. It mean that the AFB1 decreased about 33,32%. Boiling in only temperature 100 $^{o}$C gave 28% depreciation of aflatoksin in corn (Samarajeewa *et al*. 1990). Fermentation process used *Rhizopus oligosporus* MK 1 was obvious could produced tempeh with low AFB1 concentration (ends concentration was 71,63 ppb). It mean that the AFB1 decreased about 28%.

Raw material (*Aspergillus flavus* uncontaminated soybean) contained AFB1 about 53,82 ppb while cooked soybean or before fermentation has concentration was 36,84 ppb. It mean that the AFB1 decreased about 31,55%. Physical and chemical treatment efficiency in detoxification of aflatoxins between only 40-80 %, but if combinated process so the result may be very effective. Fermentation process used *Rhizopus*

*oligosporus* MK 1 was obvious could produced tempeh with low AFB1 concentration (ends concentration was 21,56 ppb). It mean that the AFB1 decreased about 41,47%. Detoxification of aflatoxins in food with biological treatment could decreased aflatoxins about 67-100% depend on the beginning of aflatoxins level and process condition (Bata and Lasztity 1999).

The result showed that raw material (*Aspergillus flavus* contaminated and uncontaminated soybean) contained AFB1 about 149,38 ppb and 53,82 ppb, while cooked soybean or before fermentation has concentration was 99,60 ppb and 36,84 ppb. Fermentation used MK 1 isolate obvious could produced tempeh with low AFB1 concentration was 71,63 ppb and 21,56 ppb.

## ACKNOWLEDGEMENT

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# STUDY PROTOCOL: EFFECT OF IRON FORTIFICATION OF FERMENTED MILK WITH LOCAL PROBIOTIC *Lactobacillus plantarum* DAD 13 AND PREBIOTIC FRUCTO OLIGOSACCHARIDE ON THE IRON CONTENT AND TOTAL LACTIC ACID BACTERIA DURING STORAGE

**Siti Helmyati[1], Endri Yuliati[1], Narendra Yoga Hendarta[2], Siti Fadhilatun Nashriyah[1], Kurnia Widyastuti[1], and Euis Nurdiyawati[1]**
[1]School of Health Nutrition, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia
[2]Dept. of Health Analyst, Health Polytechnics of Yogyakarta, Yogyakarta, Indonesia
Corresponding author: siti_helmyati@yahoo.com

**ABSTRACT**

Iron is an essential trace element in animal and human nutrition. Its deficiency induces anemia which a□icts about 9.4% of children age 5-14 years in Indonesia. Iron fortification of milk or dairy products might be an effective way to prevent iron deficiency. Fermented milk is commonly consumed and preferred by children, requiring limited infrastructure and technology. Moreover, the probiotic and prebiotic may help maintain a healthy digestive tract and increase the iron absorption in the gut. However, iron fortification can cause several biophysicochemical modifications. All of these modifications depend on many factors, one of them is the physicochemistry of iron fortificant. Thus, it is needed a research to determine the effect of iron fortification on the iron content and total lactic acid bacteria of fermented milk after their enrichments with iron. This study is an experimental study. The fermented milk will be made from probiotic *Lactobacillus plantarum* Dad 13 and prebiotic fructo-oligosaccharides (FOS). Fermented milk will be divided into 3 portions; first portion is not fortified with iron and regarded as control. The rest 2 portions are fortified with $FeSO_4$ and NaFeEDTA respectively at a level of 50 ppm iron. Fermented milk samples will be analyzed chemically and microbiologically when fresh and after 1, 2, 3, 4 weeks of cold storage. Iron content is analyzed by AAS method (Atomic Absorption Spectophotometry). MRS (de Man, Rogosa and Sharpe) agar medium will be used to enumeration total lactic acid bacteria.

***Keywords***: fermented milk, iron fortification, probiotic, lactic acid bacteria, anemia

## INTRODUCTION

Iron is an essential trace element in animal and human nutrition. Its deficiency induces anemia which afflicts about 9.4% of children age 5-14 years in Indonesia (Basic Life Survey, 2007). Iron supplementation and fortification are the optimal approach to reducing the high prevalence of iron deficiency in developing countries. But apparently, iron supplementation can cause negative effects. This effects is increase pathogenic enterobacteria, decrease good bacteria such as lactic acid bacteria (LAB) (Zimmermann et al., 2010), increased the frequency of diarrhea (Lee et al., 2008; Gera and Sachdev, 2002), increased the number of coliforms in the gastrointestinal tract of pigs (Radji, 2009). This condition can cause intestinal microbiota imbalance.

Bacteria need iron for their growth (Marx, 2002). In other hand iron can increase the virulence of pathogenic bacteria (Kortman *et. al*, 2012). Many strains of bacteria are competing to take the unabsorbed iron in the intestine, due to the formation of bacterial colonization depends on the ability of bacteria to acquire iron and other nutrients for growth (Andrews et al., 2003). For major gram-negative intestinal bacteria, such as Salmonella, Shigella, or *E. coli*, the addition of iron or the presence of excessive iron plays a role in virulence and colonization of pathogenic strains (Naikare et al., 2006). So using Fe chelator to prevent the bacteria acquirement of iron is needed.

Although it has negative effects, iron supplementation and fortification is important to be done since iron deficiency anemia is still become a nutritional problem in Indonesia (Supariasa et al., 2002). Therefore, we need a way to maintain the balance of intestinal microbiota.

Iron fortification need less cost than supplementation. However, there are many technical difficulties in fortifying food with iron because bioavailable forms of iron are chemically reactive and often produce undesirable effects when added into the food. Iron fortification can cause several biophysicochemical modifications which alter the appearance or taste of the food vehicle. All of these modifications depend on many factors, one of them is the physicochemistry of iron fortificant. The success of iron fortification

depends as much on the fortification compound as on the food vehicle. The food vehicle and fortification compound must be chosen in tandem because most iron compounds cause discoloration or rancidity.

EDTA is a substance that able to bind metal, so it is used to bind iron in the form of NaFeEDTA (Ethylene diamine tetraacetic Iron Sodium Acid). NaFeEDTA is high bioavailability iron compounds and recommended as food fortificant such as flour, fish sauce, soy sauce and sugar (Allen et at., 2006 *cit* Yang et al., 2011). In NaFeEDTA, iron is bound strongly to EDTA so that Fe can not react with the substance in the food. As consequences, it does not cause food deterioration. According to Hurrel et al. (2000), NaFeEDTA provide many benefits; iron absorption 2-3 times better than $FeSO_4$ (Hurrell, 2000), not oxidize fat or precipitate peptides, and stable at least for 12 months (Thuy et al., 2005). In addition, because bound strongly, iron can not be utilized by the pathogen bacteria.

Iron fortification of milk or dairy products might be an promise way to prevent iron deficiency. Fermented milk is commonly consumed and preferred by children, consumed regularly, required limited infrastructure and technology. Moreover, fermentation product demand in the market is high, and the public aware of the importance of maintaining gastrointestinal health increased. The probiotic and prebiotic may help maintain a healthy digestive tract and increase the iron absorption in the gut. Milk is fermented functional foods that have health effects that have been clinically proven that for intestinal health (Lourens-Hattingh & Viljoen, 2001). Prebiotic can increase mineral absorbtion (Rastall, 2010).

We will develop synbiotics fermented milk fortified with iron in attempt to minimize the negative effect of iron supplementation and fortification. We use probiotic *Lactobacillus plantarum* Dad 13 is local isolates of probiotic isolated from curd buffalo milk (traditional fermented foods of West Sumatra) and prebiotics fructo-oligosaccharides (FOS). Probiotic *Lactobacillus plantarum* Dad 13 is local isolates of probiotic isolated from curd buffalo milk (traditional fermented foods of West Sumatra) provide humoral immune respond effects, antimicrobial properties, and hypocholesterolemic effects (Lestari, 2003). Thus it is needed a research to determine the effect of iron fortification on the iron content and total lactic acid bacteria of fermented milk after their enrichments with iron.

## OBJECTIVES

The aim of this study is to develop the fermented milk, then assessed for the lactic acid bacteria (LAB), iron content and pH of fermented milk using various type of iron fortificant (no added iron, $FeSO_4$ and NaFeEDTA). The research questions are:

1. Is there any differences in lactic acid bacteria between fermented milk with no added iron, $FeSO_4$ and NaFeEDTA during storage?
2. Is there any differences in iron content between fermented milk with no added iron, $FeSO_4$ and NaFeEDTA during storage?
3. Is there any differences in pH between fermented milk with no added iron, $FeSO_4$ and NaFeEDTA during storage?

## METHODS

### Place and time of study

The making of fermented milk will be conducted in Nutrition Laboratory, Faculty of Medicine, UGM; LAB enumeration in Microbiology Laboratory, The Center of Food and Nutrition Study, UGM while iron and pH analysis in Chemistry Laboratory, UGM. This study will be conducted on Oct – Dec 2012.

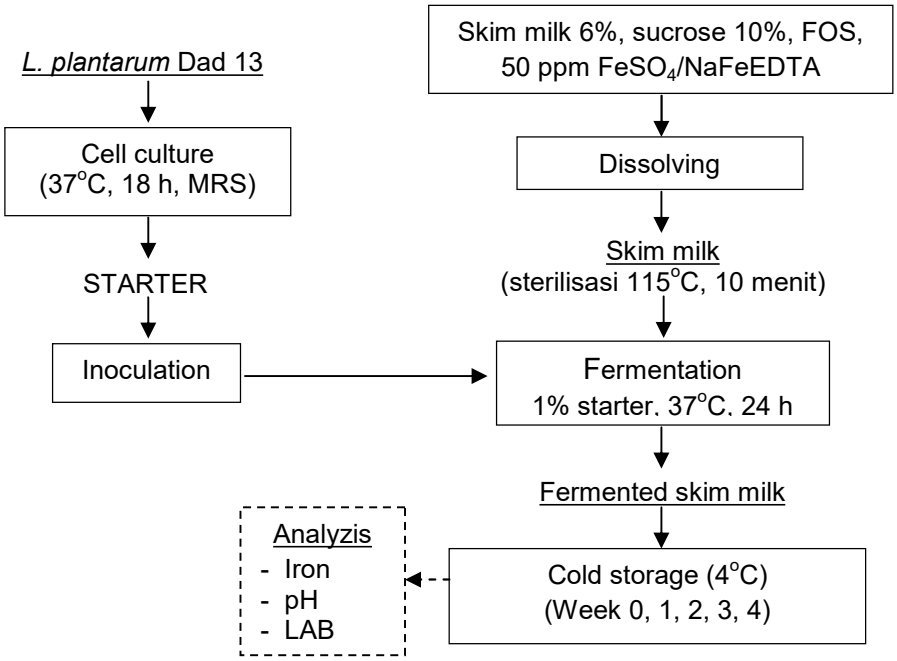
### Starter preparation

We use pure culture of *Lactobacillus plantarum* Dad 13, taken from Food and Nutrition Culture Collection, The Center of Food and Nutrition Study, UGM. Starter cultures are inoculated in 10 mL MRS

broth then incubated at 37 ° C for 18 hours and ready to be used.

**Fermented milk**

The fermented milk is made from probiotic *Lactobacillus plantarum* Dad 13 and prebiotic fructo-oligosaccharides (FOS). Fermented milk will be divided into 3 portions; first portion is not fortified with iron (control). The rest 2 portions are fortified with $FeSO_4$ (Merck) and NaFeEDTA (Akzo Nobel) respectively at a level of 50 ppm iron. pH, iron content and LAB will be analyzed on 0 (after 24 h fermentation), 1, 2, 3 and 4 weeks storage at 4°C. Each sample is made with 2 replications. The fermented milk making procedure is below:




Preparation of fermented milk based on Sari (2011). The using of 6% skim milk fermentation showed the best physical properties of fermented milk because they did not cause sedimentation, cell viability and pH relatively stable during fermentation and cold storage.

**Chemical analysis**

The iron is analyzed by wet digestion method, the determined by Flame Atomic Absorption Spectrophotometry (ASS). pH is measured by pHmeter, using distilled water, pH 4 buffer, pH 7 buffer, and pH meters.

**Microbiological analysis**

Total LAB analysis is carried out using pour plate technique with MRS medium agar. Serial dilutions are made for each sample and 1 ml of the appropriate dillution is transferred aseptically into a sterile petri dish (2 replicates per dilution). Plates of different samples are all incubated at 37°C for 48 hours then stored at 4°C. Plates containing 20 to 200 colonies are enumerated, and the counts are expressed as log10 cfu/g of the product.

**Data Analysis**

The samples are analyzed for LAB, iron and pH using analysis of variance for multiple comparisons (ANOVA). Means values are compared using the Bonferroni test with $p \leq 0.05$.

## DISCUSSION

This study addresses an important problem, because iron deficiency anemia still become major health problem in Indonesia. We need to look for the way to combat iron deficiency without raise any adverse effects. From this study, we hope to find the appropriate formulation in iron fortificants for fermented milk. Moreover, the using of local probiotic for fermented milk can be encouraged to be scaled up by industry. Finally, the results of our study can contribute to more effective way of iron deficiency controlling program.

**Trial Status**

Active. Trial started 17st September 2012.

**Abbreviations**

NaFeEDTA, Natrium Ferric Ethylene Diamin Tetraacetate; LAB, Lactic Acid Bacteria; FOS, Fructo-oligosaccharides; AAS ,Atomic Absorption Spectophotometry;. MRS ,de Man, Rogosa and Sharpe;$FeSO_4$ Ferro Sulfat; ANOVA, Analysis Of Varians;

**Competing interests**

The author(s) declare that they have no competing interests

**Authors' contributions**

SH and EY were involved in the initial conceptualization and study design. SH, EY and NYH developed the data collection methods and piloted these with the help of SFN, KW and EN. SH , EY and NYH wrote the paper. All authors read and approved the final manuscript. SH is responsible for this manuscript.

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# EXTRACTION OF BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY OF THE LOCAL WHITE, RED, AND BLACK RICE BRAN EXTRACTS BY VARIOUS SOLVENTS

**I Wayan Rai Widarta[1], KA Nocianitri[1], and LP Intan Pramita Sari[2)]**
*[1)]Faculty of Agriculture Technology*
*[2)] Graduated of Agriculture Technology Faculty Udayana University*

**ABSTRACT**

The aim of this study was to determine the bioactive compounds and antioxidant activities in local white, red and black rice bran. Three types of solvents were employed, namely methanol 99.9%, ethanol 96% and Aqua DM. Each bran extract was obtained by shaking for 24 hrs in a single solvent, respectively. The extract was vacuum dried prior to use. The extracts were analyzed for the total phenolic content, total anthocyanin content and antioxidant activities by DPPH radical. The results indicated that the levels of phenols, anthocyanin, antioxidant activities varied with the solvent and the ethanol extract contained the highest level of total anthocyanin (33.19 mg/100 g sample) in black rice bran extract. The highest level of total phenolic was found in methanol black rice bran extract (7.52 mg/100 g sample) but there are not significant differences with ethanol black rice bran extract (7.48 mg/100 g sample). The highest antioxidant activity was found in methanol black rice bran extract, namely 88.84%.

***Keywords**:* extraction, bioactive compound, antioxidant activity, local rice bran, solvent

## INTRODUCTION

Rice (*Oryza sativa* L) is one of the cereals were also a source of staple food for the people, especially in Indonesia. Bali as one of the provinces in Indonesia also participate in improving the self-sufficiency of rice in the country. It can be seen from the high production of rice in Bali reached 869,160 tons in 2010 and Tabanan regency is known as the "rice granary" in Bali, which contribute more than 25 percent of grain production in Bali. Tabanan as rice production center in Bali, has an area of harvested rice paddy fields and around 41,643 ha of the 152,190 ha harvested area in Bali. Tabanan produce 235 thousand tons of grain per season harvest (Central Bureau of Statistics / BPS Bali Province, 2012).

Tabanan has some local varieties such as brown rice seeded (sandalwood), white rice (mansur), and black rice. Rice production in Tabanan is marketed throughout the region in Bali, be it home, restaurants, and hotels. However, behind the value to rice are not offset by the value of the waste generated from the grain milling process.

Rice bran is a rich source of phytochemicals that may be working both synergistically and in parallel with each other to promote health and fight disease. Rice bran is much richer in phytonutrients and antioxidants than are corn, wheat, or oat bran. Variation has been detected in the amounts and types of phytochemicals in rice bran with respect to cultivars, growing conditions, bran separation, and kernel thickness. Rice bran fractionation of various cultivars has revealed that both the inner and outer portion of the bran layer contains phenols with anticancer properties; the outer portion contains the highest concentration. Another recent study revealed that significant phytochemical diversity was detected among a set of rice varieties (Ryan, 2011).

The extractability of phenolic compounds and their antioxidant activities in the crude extract depends on many factors including polarity and pH of solvents, extraction time and temperature, as well as chemical structure of phenolic compounds (Awika et al., 2004). Therefore, it is necessary to find the proper solvent in order to obtain the extract with high amount of phenolic compounds and high antioxidant activities on white, red, and black rice bran extracts. The purpose of this study is to obtain the most appropriate solvent used in extracting bioactive components contained in the bran of white rice, brown rice and black rice, resulting in a high antioxidant activity.

## MATERIALS AND METHODS

### Materials

White, red, and black rice bran used in this study was obtained from Tabanan regency, Bali province, Indonesia. The hulled rice bran were milled and passed through the 60 mesh. Chemicals reagent was purchased from Bratachem such as Folin–Ciocalteu, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ethanol 96%, NaOH, Aqua DM (*aquademineralized)*, methanol (99,9%), buffer potasium chlorida 0.025 M and buffer sodium acetate 0.4 M.

### Methods

Three types of solvents were employed, namely methanol 99.9%, ethanol 96% and Aqua DM. Each bran extract was obtained by shaking for 24 hrs in a single solvent, respectively. The mixed extract was filtered by whatman no 1 filter paper. The extract was vacuum dried prior to use. The extracts were analyzed for the total phenolic content (Garcia et al., 2007), total anthocyanin content (Giusti dan Wrolstad, 2001) and antioxidant activities by DPPH radical (Sompong *et al*., 2011).

### Statistical analysis

All measurements were duplicated samples. The results were statistically analysed by analysis of variance (ANOVA) and Duncan's multiple range test (DMRT). Statistical significance was accepted at a level of $P < 0.05$.

## RESULT AND DISCUSSION

### Yield of extract

The overall results of the yield of the crude extracts obtained from different rice bran and various solvents were shown in Figure 1. Statistical analysis showed that different rice bran and various solvents significantly affected yield of the crude extract ($p<0.05$). While interaction of this factors did not significantly influenced yield of crude extracts ($p >0.05$). Conducted research shows that values of yield of crude extracts vary from 6.10% - 13.54%.

Figure 1 shows that the yield produced by solvent aqua DM by 6.26%, 4.61% for ethanol, whereas methanol at 4.01%. The average value obtained by solvent aqua DM highest yield of 6.26%. This is because the aqua DM relatively polar solvent. Based on the level of polarity, aqua DM had a higher level of polarity than methanol and ethanol. Compounds in rice bran were extracted with a solvent local aqua DM have the appropriate polarity, that can produce the highest yield.

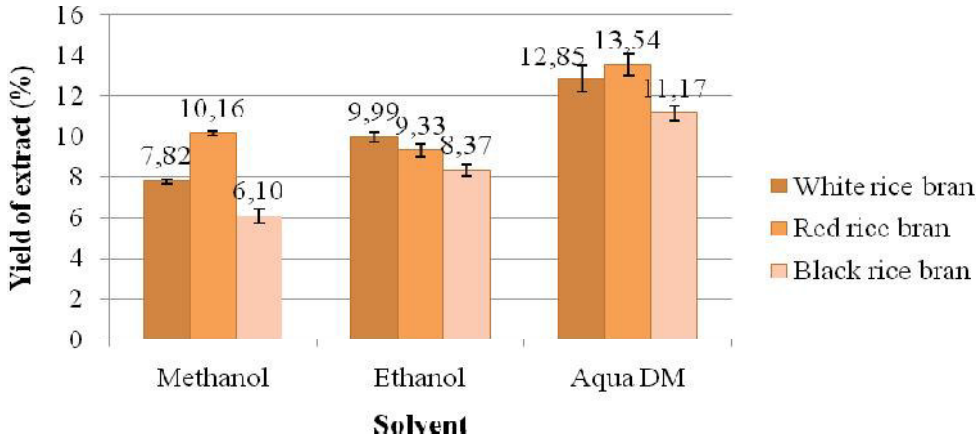
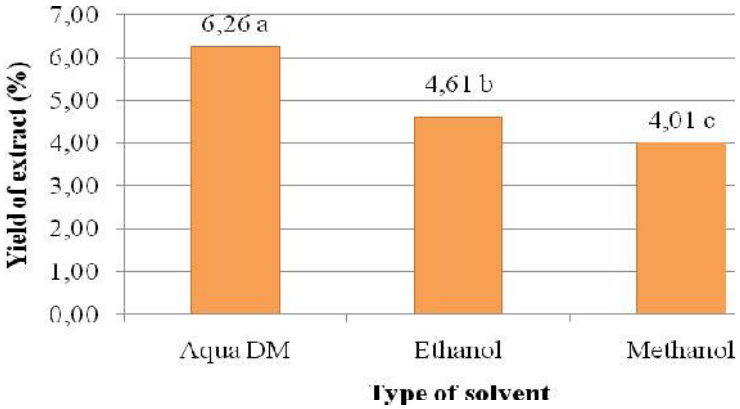
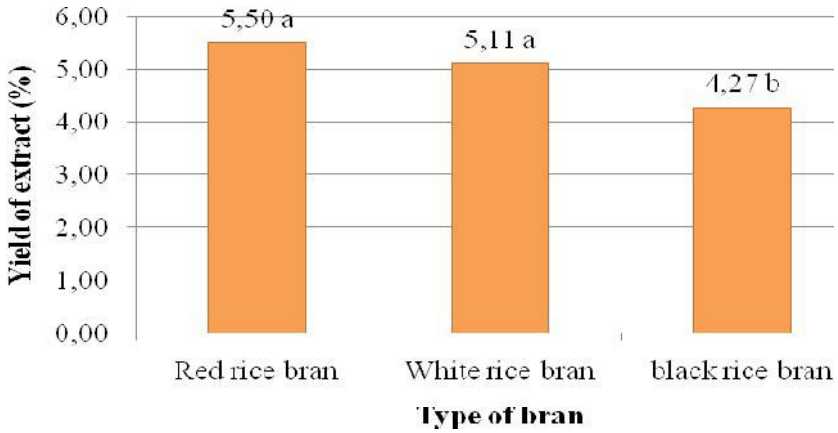



Figure 1. Yield of crude extracts from different rice bran and various solvent




* The same notation indicates not significantly different (P>0.05)
Figure 2. The relationship between the type of solvent with the extract yield (%)

According Lestiani and Lanny (2008), the polar solvent determine the type and number of compounds that can be extracted from the material. The solvent will extract compounds that have the same or similar polarity to the polar solvent used. Types of compounds that allegedly participated in the bran extracted the vitamin B1 (thiamin), vitamin B2 (riboflavin), vitamin B3 (niacin), carbohydrates, fiber and minerals that dissolve in water. The nature of the compound soluble in water, so that the resulting yield on solvent aqua DM higher than methanol and ethanol.

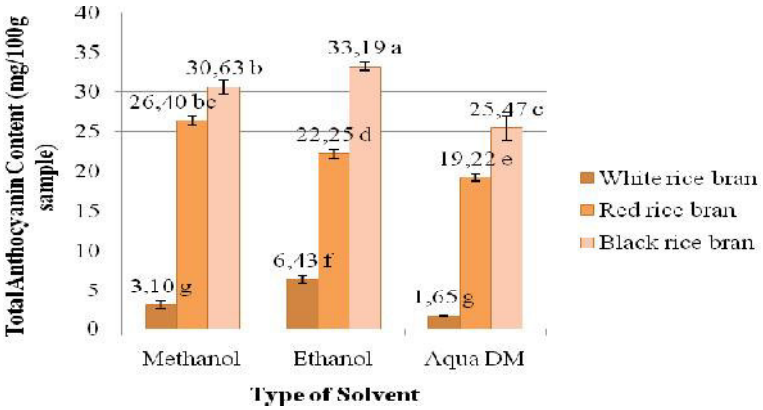



* The same notation indicates not significantly different (P>0,05)
Figure 3. The relationship between the type of bran with extract yield (%)

Judging from the type of bran in the same solvent, the highest yield of extracts obtained from rice bran of brown rice and white rice while the lowest yield of the extract obtained from black rice bran. It can be caused by the bran of brown rice and white rice have more non-phenolic compound content with polarity similar to the solvent used. According Adzkiya (2011) non-phenolic compounds that are soluble in organic solvents such as methanol, ethanol, and water are sugars, organic acids and proteins.

**Total Anthocyanins**

The results of the analysis showed that treatment of various types of solvents and bran as well as their interaction was highly significant (P <0.05) to total anthocyanin local rice bran extract. The average value of total anthocyanin extracts in the treatment of local rice bran solvent type can be seen in Figure 4.




* The same notation indicates not significantly different (P>0.05)

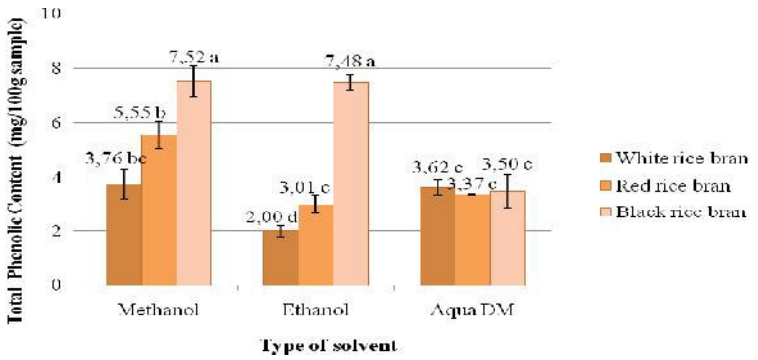
Figure 4. The relationship between the type of solvent and the bran on levels of total anthocyanin (mg/100 g rice bran)

Figure 4 shows that the combined treatment of black rice bran were extracted with ethanol has the highest total anthocyanin at 33.19 mg/100g bran, whereas combination treatment of white rice bran extracted with solvents aqua DM has the lowest total anthocyanin 1.65 mg/100g the bran and not significantly different from white rice bran combination treatment were extracted with methanol is 3.10 mg/100g bran. Highest total anthocyanin levels generated in this study, together with high levels of anthocyanins in black glutinous rice, which is extracted under acidic conditions ranged from 31.3 mg/100g - 34.2 mg/100 g sample (Tananuwong and Tewaruth 2010). Meanwhile, Chanphrom (2007) reported that levels of total anthocyanins in pigmented rice bran was 28.61 ± 10.22 mg/100 g.

Combination treatment of black rice bran were extracted with ethanol producing content highest total anthocyanin can be caused by a high content of anthocyanins and anthocyanin in black rice has a very similar polarity solvent ethanol. Black rice bran has a more intense color so that the content of anthocyanins in black rice bran more than white rice bran and brown rice. Sompong et al. (2011) states that black rice contains more anthocyanin than red rice while white rice has a lower flavonoid content compared to red rice (Adzkiya 2011). Ethanol is a polar solvent that can dissolve well anthocyanin based on the principle of "like dissolve like" (Amelia et al., 2013). Abou-Arab et al. (2011) reported that ethanol is more effective than the water used for the extraction of anthocyanins from flowers Rosella.

**Total Phenolic**

Results of analysis of variance showed that the interaction between the type of solvent and the type of bran was highly significant (P <0.01) of total phenols local rice bran extract. Value - average total phenols local rice bran extract on the treatment of the type of solvent can be seen in Figure 5.




* The same notation indicates not significantly different (P> 0.05)

Figure 5. The relationship between the type of solvent and the bran of the total phenolic content (mg/100 g of bran)

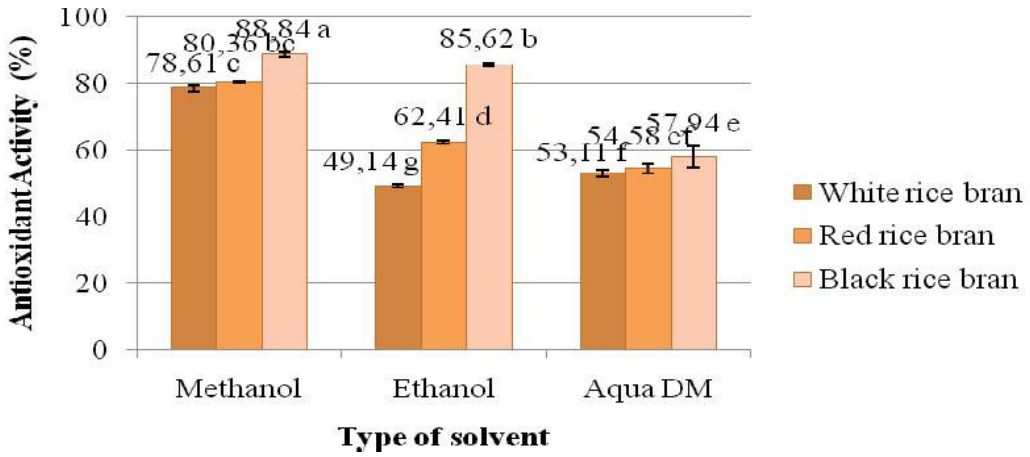
Figure 6 shows that the combined treatment of black rice bran were extracted with methanol has the highest content of total phenols 7.52 mg/100g bran were not significantly different from the treatment of black rice bran were extracted with ethanol is equal to 7.48 mg/100g bran, while the combination of white rice bran were extracted with ethanol has the lowest content of total phenols, namely 2.00 mg/100g bran. This suggests that ethanol has the same relative ability to extract the content of methanol in the phenolic compounds contained in black rice bran. According to Amelia et al. (2013) Ethanol is a good solvent for the extraction of polyphenol compounds.

The results also showed levels of total phenols in white rice bran lower than black rice bran and brown rice bran. Similar research results obtained by Adzkiya et al. (2011) reported that the total phenolic content of white rice (IR 64) is very small when compared to the levels of total phenols in brown rice. According to Tian et al. (2004), Zhou et al. (2004) in Walter and Marchesan (2011) grain with a darker color perikarp like black rice and brown rice contains a higher polyphenol.

**Antioxidant Activity of Rice Bran Extract**

The results of the analysis indicate that the interaction between the various types of solvent and type of bran was highly significant (P <0.05), the antioxidant activity of rice bran extracts local. The average value of the antioxidant activity of local rice bran extract on the treatment type of solvent can be seen in Figure 6.

Figure 6 shows that the combined treatment of black rice bran were extracted with methanol has the highest antioxidant activity of 88.84%, while the combination of white rice bran were extracted with ethanol has the lowest antioxidant activity of 49.14%. It can be caused by a combination of black rice bran were extracted with methanol has the highest total phenols as shown in Figure 5. Total phenols were obtained had positive correlation with the antioxidant activity. Something similar has been reported by Walter and Marchesan (2011) that the higher total phenol, the antioxidant activity will be higher as well.




* The same notation indicates not significantly different (P> 0.05)

Figure 6. The relationship between the type of solvent and the bran on antioxidant activity (%)

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# THE USAGE OF PURPLE SWEET POTATO (*Ipomoea batatas*) IN RATION FERMENTED BY *Aspergillus niger* FOR IMPROVING FEED CONVERSION AND BLOOD LIPID PROFILE OF BALI DUCK

**Tjokorda Gede Belawa Yadnya**
*Faculty of Animal Husbandry, Udayana University*
*Email: belawayadnya_fapet@yahoo.com*

**ABSTRACT**

This study aims to examine the usage of purple sweet potato (*Ipomoea batatas*) in ration fermented by *Apergillus niger* for improving feed conversion and blood lipid profile of bali duck on growth phase. This study use completely randomized design (CRD) with three treatments are: ration without purple sweet potato (treatment A), ration containing 10% purple sweet potato without fermentation (treatment B), and ration containing 10% fermented purple sweet potato by *Aspergillus niger* (treatment C). Each treatment consist of four replications and each replication consist of five ducks with homogeneous age and weight. Variables observed are: feed efficiency usage (feed consumption, body weight gain, feed conversion ratio), and blood lipid profile (total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) , and triacylglycerol). Based on the result of the experiment, it can be concluded that the provision of rations containing purple sweet potato (*Ipomoea batatas)* fermented by *Aspergillus niger* can improve the feed conversion ratio and blood lipid profile of bali duck on growth phase

***Keywords****: bali ducks, fermented purple sweet potato, feed conversion, blood lipid profile, growth phase*

# COMPARING EXTRACTION METHODS TO ISOLATE ANDROGRAPHOLIDE FROM THE BITTER HERB (*Andrographis paniculata* Burm.f Ness)

**Ni Kadek Warditiani[1*] and Suwidjiyo Pramono[2]**
[1]*Pharmacy Department, Faculty of Mathematic and Natural Sciences, Udayana University, Kampus Bukit Jimbaran, Bali.*
[2]*Pharmaceutical Department, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta.*
[*]*Corresponding author: kadek.warditiani@gmail.com*

**ABSTRACT**

Andrographolide diterpene lactone compound is the major component that contained in the bitter herb (*Andrographis paniculata* Burm.f Ness). Andrographolide has many pharmacological activities such as anti-diabetic, anti-bacterial, anti-oxidant, anti-hyperlipidemia, anti-cancer, etc. The purpose of this study was to determine the extraction method that is able to obtain more andrograpolid compound which will be isolated with recrystallization method. The extraction methods in this study were maceration and soxhletation, The result showed that maceration produced a lot higher yield of extract if compared to soxhletation method. The purity of the andrographolide isolates obtained was 88.29 ± 1.94% measured using HPLC.

***Keywords***: andrographolide, bitter herb, isolate, extraction.

# POSTER PRESENTATIONS: BIODIVERSITY AND ENVIRONMENT

# ALLELES VARIATION OF COCONUT ACCESSIONS (*Cocos nucifera* L., Arecaceae) BASED ON MICROSATELLITE DNA

**Eniek Kriswiyanti[1*], I Gede Rai Maya Temaja[2], Made Sudana[2] and G.N. Alit Susanta Wirya[2]**
[1]*Dept Biology of Faculty Mathematic and Natural Science*
[2]*Faculty of Agriculture, Udayana University.*
Corresponding author: eniek_kriswiyanti@yahoo.co.id

**ABSTRACT**

Coconut plays a more prominent role in the social, economic and cultural for Balinese people, especially for ritual purpose. The aimed of this research to determined alleles variation of sixteen coconut accessions (*Cocos nucifera* L., Arecaceae). Alleles variation in 16 coconut accessions from Br. Babung, Gunaksa and Pikat villages, Dawan, Klungkung regency was determined using 4 microsatellite markers. A total of 28 alleles were detected by microsatellite with an average 7 alleles per locus, there are 7 alleles of microsatellite primer CNZ05, 8 alleles of CNZ09, 7 alleles of CNZ21, and 6 alleles of microsatellite primer CNZ51. Height heterozygosity (0.8752) on locus CNZ09

***Keyword:*** *Alleles, coconut accessions, microsatellite markers, heterozygosity.*

## INTRODUCTION

There are two main types of coconut: 'tall', the naturally cross pollinating group with more economic value and 'dwarf', the naturally self pollinating group with reduced size and growth habit. It is believed that the 'dwarf' originated from earliest 'tall' coconuts in atolls of the far east and maintained most of its original genome because of its autogamous behaviour. Thus, dwarf coconuts are of similar stature and fruit features irrespective of the geographical location. However, 'tall' genome has undergone many changes because of bottle-neck effects of selection, though it had retained the tall stature and fruit characteristics irrespective of its dispersion from far east to Indo-Atlantic regions across Africa (Bourdeix *et al*., 2005, Dasanayaka *et al,* 2009).

The coconut palm (*Cocos nucifera* L., Arecaceae) is the most widely cultivated crop in Philippines, Indonesia, India, Sri Lanka and China, where coconut palm plays an important role in economy. It provides food supply and industrial products, such as coconut oil, copra, liquid endosperm and desiccated coconut. Almost every part of the coconut tree can be used in either making commercial products or meeting the food requirements of rural communities (Teulet et al., 2000).

Investigation of coconut genetic diversity provides sufficient scientific data for germplasm management. Diversity analysis in coconut palm has been done by morphological traits, biochemical and molecular markers. Morphological and biochemical markers have shortages as follows: Long juvenile phase, high cost, long-term of field evaluation, environment factors and limited number of available phenotypic markers (Manimekalai *et al*., 2006). However, since molecular markers are detectable at all stages of development and can cover the entire genome, they, which detect variation at DNA level, overcome most limitations of morphological and biochemical markers, (Lebrun *et al*, 1998; Perera *et al*.,1996, 2000, 2001; Rivera *et al*., 1999; Teulat *et al*., 2000; Dasanayake *et al*., 2003; Upadhyay *et al*., 2004; Manimekalai *et al*., 2006, 2007, 2010). Among various available molecular marker techniques, simple sequence repeat (SSR) or microsatellite markers provide good signal in evaluating genetic diversity and genetic relationships in plants. The increased number of SSR markers greatly improves the previously established genetic relationships among coconut varieties/populations (Liu *et al*., 2011).

## MATERIAL AND METHODS

*Plant material used*: Leaf samples were obtained from 16 coconut accessions from Br. Babung Gunaksa and Br Pikat Dawan Klungkung residence. Among the 16 coconut accessions, 13 were of the 'tall' category (*nyuh ancak, barak/red, bingin, gadang/green, kebo, kopyor/polo/srogsogan, manjangan,*

*mulung, penyu, rangda, salak, sangket, sudamala, nyuh* = Balinese coconut) and 3 were of the 'dwarf' category (green, white and yellow dwarf).

*DNA extraction and detection of microsatellite polymorphisms*:

DNA was extracted from fresh coconut leaves using a CTAB based protocol modified from Doyle and Doyle (1987). The primer sequences and associated information are given in Table 1.

Polymerase Chain Reactions (PCR) assay and gel analysis

DNA was amplified in 13 µL reactions containing 2 µL sample, 3.5 µL $H_2O$, 6.5 µL Mastermix/ hotstart (Qiagen), 1 µL primer. The PCR programmed for 30 cycles of 60 seconds each at 94°C, annealing temperature 39-52°C, extension temperature 72°C. The first cycle was preceded by a 3 min denaturation at 94°C and the last cycle ended with 5 min extension at 72° C. Reaction products were separated on 6% polyacrylamide (denatured) and visualized with silver nitrate staining (Tegelstorm, 1984).The alleles were scored based on the size of each PCR amplified fragment by electrophoresing all samples in a single gel. Allele size was determined by semilog plotting of distance migration of amplicon on PAGE (Hutchinson, 2001). Diversity values based on allele frequencies were calculated for each microsatellite locus using Nei's methods (1987).

Table 1: Detail of microsatellites Primer used

| Primer name | Forward primer (5'-3') | Reverse primer (3'-5') |
|---|---|---|
| CNZ05 | CTTATCCAAATCGTCACAGAG | AGGAGAAGCCAGGAAAGATTT |
| CNZ09 | ATCTACCAGTGTGGTCCTCTC | ACCAGGAAAAAGAGCGGAGAA |
| CNZ21 | ATGTTTTAGCTTCACCATGAA | TCAAGTTCAAGAAGACCTTTG |
| CNZ51 | CTTTAGGGAAAAAGGACTGAG | ATCCATGAGCTGAGCTTGAAC |

## RESULTS AND DISCUSSION

The sixteen accessions coconut with four specific microsatellite primer pairs produced a total of 28 alleles ranging from 6 to 8 alleles per locus (Fig.1 and Table 2).

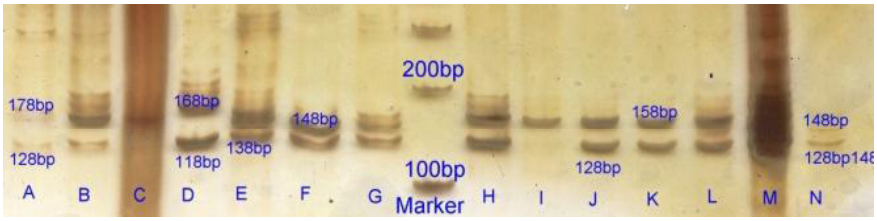


Fig.1 Allelograph from locus CNZ05
A.Green dwarf, B.Yellow dwarf, D.White dwarf, the tall type: C.Green tall, E.Red tall, F. *Kopyor/Polo/Srogsogan*, G.*Salak*, H. Brown, I.*Rangda*, J.*Kebo*, K.*Mulung*,L.*Bingin*, M.*Penyu*, N.*Sangket*

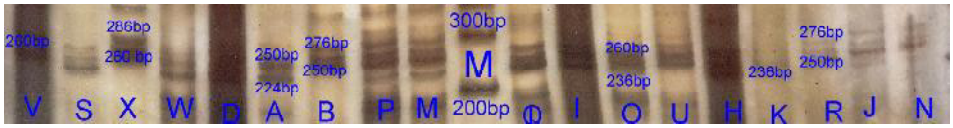


Fig.2 Allelograph from locus CNZ21
V: yellow dwarf; X: white dwarf and W:green dwarf; A: *Ancak*. B.*Barak*/red, P: *Salak*, M:*Mulung*, Q: *Sangket*, O: *Rangda*, H: Green/*Gadang*, K: *Kopyor*,
R: *Sudamala*, J: *Kebo*, N: *Penyu*: "tall category"

Table 2. Detail of microsatellite loci, alleles detected in coconut accessions

| Microsatellite | No of alleles | Allele size (bp) | Heterozygosity |
|---|---|---|---|
| CNZ05 | 7 | 118, **128**, 138, 148, **158**, 168, 178 | 0.838645 |
| CNZ09 | 8 | 115,120, **125**, 130, **135**, 140, 145, 155 | 0.87525 |
| CNZ21 | 7 | 224, 236, **250**, **260**, 270, **276**, 286 | 0.785092 |
| CNZ51 | 6 | 160, 170, 180, **190**, **200**, 210 | 0.80658 |

In the present study, the mean number of allele**s** per locus (7.0) was similar to that found in other studies of coconut palm tree populations using SSR markers. Rajesh *et al*. (2008) **t**he genetic diversity in 26 coconut accessions from the Andaman and Nicobar Islands was determined using 14 microsatellite markers. A total of 103 alleles were detected by the microsatellite markers with an average of 7.35 alleles per locus**.** Dasanayaka *et al*. (2009) sixteen primer pairs identified 79 alleles, averaging 4.9 alleles per locus ranging from 3 to 10 simple sequence repeat polymorphisms among the 43 coconut accessions assessed. All 16 loci were polymorphic and a total of 76 alleles were observed in tall category, ranging from 3 to 10 with an average of 4.7 alleles per locus. A total of 29 alleles were observed in dwarf category ranging from 1 to 3 with an average of 1.8 alleles per locus, Ribeiro *et al*. (2010) found a total of 68 alles, ranging from 2 to 13 alleles per locus, with an average of 5.23, and heterozygosity were 0.459 and 0.443, Kumar, *et al*. (2011) found a total 28 polymorphic alleles produce of 8 primers were used of 14 accessions, Liu *et al.* (2011) used 26 simple sequence repeat (SSR) markers, were detected a total of 188 alleles with an average of 7.23 alleles per locus.

On the table 3 the locus CNZ05: the highest frequency of alleles is size 128 bp, (0.281), rare frequency 118 bp was found only on coconut accession '*nyuh Rangda*" (0.063). The Locus CNZ09 height frequency on allele size 125 bp and 135 bp both with frequency 0.218, rare frequency 155 bp only on coconut accession '*nyuh Ancak*" frequency 0.031. Locus CNZ21 height frequency on size allele 250 bp with frequency 0.344, rare frequency 286 bp only on coconut accession '*nyuh white dwarf*" with frequency 0.063. Locus CNZ51 height frequency on size allele 190 bp with frequency 0.344, rare frequency on 118 bp, on coconut accession '*nyuh ancak and gadang/green tall* " frequency 0.063

## ACKNOWLEDGEMENT

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# GENETIC DIVERSITY OF SOROH CELAGI (PASEK CATUR SANAK CLAN) BASED ON Y-CHROMOSOMAL MICROSATELITES DNA

**I Ketut Junitha, Made Pharmawati and Wayan Rosiana**
*Laboratorium of Genetics, Department of Biology, Faculty of Mathematic and Natural Sciences, Udayana University, Bukit Jimbaran Campus, Bali. 80361*
*HP: 087862384570, E-mail: junithaketut@yahoo.com*

**ABSTRACT**

The Balinese people were grouped into family clans or commonly known as *soroh*. The clans are grouped based on patrilineal genealogy system and they believe that they were descended from one ancestor. Each clan has one main temple known as *kawitan* temple. It is compulsory for Balinese to know and pray in their *kawitan* temple of their *soroh*. The Celagi clan together with Kayu Selem, Kayuan and Terunyan are part of Bali Mula origin group which is believed descended from one ancestor, Empu Kamereka. Four Y-chromosomal microsatellites markers (DYS19, DYS390, DYS393 and DYS395) were used in this study to observe genetic diversity of Celagi clan. The result showed that nine alleles were identified, two alleles each on DYS19, DYS390, DYS393 loci, and three alleles on DYS395 locus. Base on alleles frequencies it was found that the genetic diversity of Celagi clan was low (D=0,148 ± 0,054). Five haplotypes were found in this study, and was dominated by haplotype 1 with frequency of 0,839. Haplotype 1 consisted of allele combination from DYS19, DYS390, DYS393 and DYS395 loci with sizes of 200, 207, 133, 131 bp. This haplotype is wide spread in Bali.

***Keywords***: Clan, locus, microsatellite, Y-chromosom, haplotype

## INTRODUCTION

Bali society was differentiated into several genealogist groups known as clan or *Soroh*. Each member of clan believed that he derived from one ancestor. Each clan has one main temple, namely *Kawitan* temple. *Kawitan* (kawitan=origin), means that members of a Kawitan temple are believed to come from one same ancestor (Bagus,1971). Everyone in Bali society family tries to know his soroh to respect its ancestor. Pasek Clan in Bali is a genealogy group of people who have been living in Bali before the entry of Majapahit people. Pasek clan is divided into two mayor groups that are Pasek Bali Mula or Catur Sanak and Pasek Sanak Sapta Rsi. *Soroh* Kayu Selem, Celagi, Kayuan and Terunyan represent the part of Pasek Catur Sanak. They believe to represent clan from one ancestor that is Empu Kamareka. Empu Kamareka as foster child of Semeru is member of Bali Mula clan. Same thing is also expressed by Riana (2011). Bali Mula people have been living in Bali before ancestor of Pasek Sanak Sapta Rsi come.

DNA microsatelite markers represent part of DNA that do not encode protein so that they do not relate to level of human being quality. This marker has high mutation rate therefore suites for research of genetics variation between society groups (Bowcock et al, 1982). Autosomal DNA microsatelite markers have been used for forensic analysis to determine consanguinity relationship (Junitha and Alit, 2011). Microsatelite Chromosom-Y markers were used to determine the genetics variation between caste in India (Mitchell et al, 2006). Research using DNA microsatelites chromosom-Y markers in Bali societies of Aga and Tri Wangsa found the existence of typical alel of 208pb at DYS19 locus in group Hyang Kumpi Mulianis dadia. This alel was not found in other Bali Mula dadia in Bali Mula society in Sembiran village and in other Balinese societies (Junitha, 2004; Junitha and Suryobroto, 2010). This previous research used four DNA microsatelites chromosome - Y ( DYS19, DYS390, DYS393 and of DYS395) and found nine kinds of haplotipes in society of Terunyan and 12 kinds in Soroh Kayu Selem. In both clans same haplotipe was not found ( Junitha and Sudirga, 2007; Junitha et al., 2009).

This research conducted using DNA microsatelites chromosom-Y markers ( DYS19, DYS390, DYS393 and of DYS395) on Celagi clan. This research aims to determine type of haplotipe and to upgrade DNA database of Balinese clan especially of Catur Sanak clan.

## MATERIALS AND METHODS

This research was started by visiting main Kawitan Temple of Soroh Celagi in Pedahan village, regional of Tianyar Tengah, district of Kubu, regency of Karangasem. From the temple priest and head of Kawitan Temple organization, we got information about the people come to pray in the Kawitan Temple from around of Bali. Buccal sweb cell samples were collected using sterile cotton bud from 42 males that agreed with informed consent. There were 23 people from Pedahan, one person from Tianyar and Munti district of Kubu, Karangasem regency. One person from Pikat village district of Dawan Klungkung, two people from Tihingan village and 1 person from Koripan village, district of Banjarangkan, regency of Klungkung. One person from Suter and 12 people from Songan village, district of Kintamani, Bangli Regency. The buccal cells were suspended in 500 µl buffer (10mM NaCl, 100mM Tris-HCl, 100 mM EDTA and 4M urea) in 1,5 ml ependorf tube. The DNA from buccal samples were extracted used Qiagen Kit. The DNA pellet were resuspended in 50 µl Tris-EDTA buffer and stored in – 20$^{o}$C. Amplifications of DNA were done in ApliedBiosystem PCR machine using four primer of microsatelites chromosom-Y (DYS19, DYS390, DYS393 and DYS395). The PCR solution in total volume of 13 µl consisted of 3,5 sterile water, 2 µl DNA sample, 1 µl primer mix and 6,5 µl HotstarPCR Taqplus master mix (Qiagen). PCR process were run a follow: 94$^{o}$C preheating temperature for 5 minute, 94 $^{o}$C DNA denaturation for 45 second, 52 $^{o}$C annealing temperature, 72$^{o}$C elongation temperature and 72$^{o}$C extended process and 4 $^{o}$C for final process, Amplificatioes were run for 30 cycles.

PCR products were electrophoresed on 10% PAGE and visualized with silver nitrate staening (Tegelstrom, 1986). The alleles size were determined using DNA band size semi-log plotting (Hutchinson, 2001). Genetic diversities were calculated with formula of $D= (1-\sum pi^2)n/n-1$ (Parra et al, 1999)

## RESULT AND DISCUSSION

The Soroh Celagi has main Kawitan Temple located at Pedahan village, Tianyar Tengah Karangasem regency. According to Empu Reka Adnyana from Songan village, district of Kintamani, Bangli Regency, Empu Kamareka who lived in Tampurhyang at Batur kaldera has a son namely Empu Gnijaya Mahaireng and five grand children namely Empu Kayu Selem, Empu Made Celagi, Empu Terunya, Empu Kayuan and Ni Nyoman Nyelem. The four sons of Empu Gnijaya Mahaireng were suggested to live in different places. Empu Kayu Selem lived in Kayu Selem Gwa Song where the Kawitan Temple of Soroh Kayu Selem is now located. Empu Made Celagi lived in Pedahan, Empu Nyoman Terunyan lived in Terunyan village and Empu Kayuan lived in Siakin village. All of those places now become the place of Kawitan Temples of soroh of Pasek Bali Mula or Pasek Catur Sanak (Empu Reka Adnyana, personal communication, 2011). The same story was also reported the book of Lelintihan of Sang Catur Sanak Bali: Kayu Selem, Celagi, Terunyan, Kaywan Balingkang and Bali Aga citizen (Riana, 2011).

DNA samples were extracted from 42 male volunteers (probandus) from Karangasem, Bangli, Klungkung regencies. The DNA sequence has been amplified in PCR machine using four pairs Y-chromosom primers (DYS19, DYS390, DYS393 and DYS395). The electrogram is presented in Figures 1 a-d. There are some DNA samples that did not produce band in gel electrophoresis e.g line 6 Fig 1c. There are two possibilities that cause this result. First, there is no DNA template because of failure in DNA extraction process, and secondly the annealing temperature was not optimal. The temperature used was lower than optimal temperature.

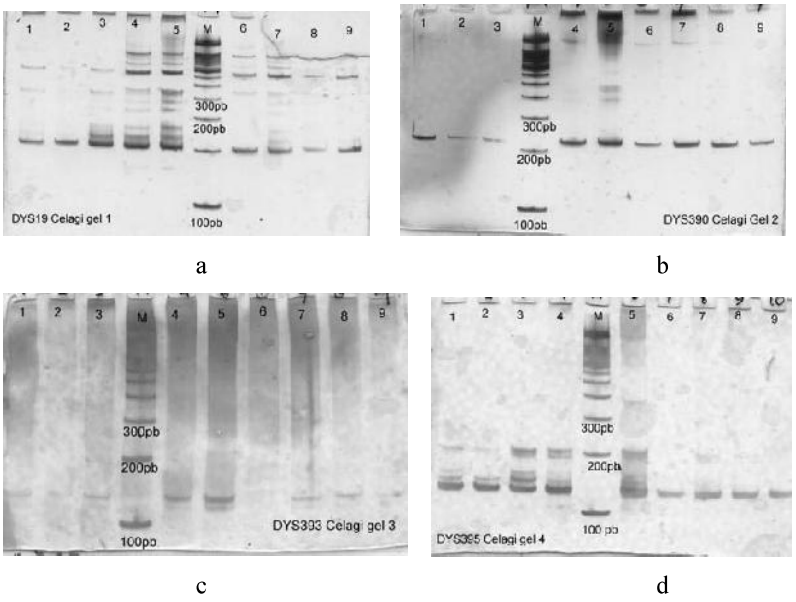


Figure. 1. Electrogram of amplicon on 10% PAGE
Locus DYS19 (a), DYS 390 (b), DYS393 (c) and DYS 395 (d)
DNA 100bp ladder (M) and lines 1-9 are amplification products of DNA samples.

For locus DYS390, one sample was not amplified, but using other loci this sample produced band. In this case, there is possibility that at that DNA sample mutation occurred at annealing site and the primer did not match for sequence annealing site. As a result, amplification of DNA was not running. Thereby sample like that, do not yield allele or referred as null allele (Avise and Dankin, 2004).

The allele sizes were determined based on migration of DNA amplicon on PAGE and plotted on semi-log paper. Allele sizes of DNA amplicon were expressed as set of the amount of basa. Size of alleles in each locus and its frequencies is presented at Table 1. The result of this research showed that all of the loci are polymorphic. There are nine alleles for all loci, two alleles at DYS19 (204 and 200bp), DYS390 (207 and 203bp), DYS393 (133 and 125bp) and three alleles for locus DYS395 (135, 131 and 127 bp). From analysis using four loci, it was found that each locus predominated by one allele which has frequencies more than 0,9, except locus DYS395 locus with the highest frequency only 0,88. The allele 207 bp at locus DYS390 showed the highest frequency (0,97) because this allele represents common allele for the other communities e.g. Sembiran, Terunyan communities and Tri Wangsa society (Junitha and Sudirga, 2007; Junitha and Suryobroto 2010).

Table 1. Locus, alleles size and frequencies of DYS19, DYS390, DYS393 and DYS395

| No | Locus | Allele | Frequencies |
|---|---|---|---|
| 1 | DYS19 | 204 | 0.09 |
| | | 200 | 0.91 |
| 2 | DYS 390 | 207 | 0.97 |
| | | 203 | 0.03 |
| 3 | DYS 393 | 133 | 0.94 |
| | | 125 | 0.06 |
| 4 | DYS 395 | 135 | 0.06 |
| | | 131 | 0.88 |
| | | 127 | 0.06 |

The high frequency of 207 bp allele of DYS390 locus can be related to the existence of gene flow from India to Bali where allele 207 bp owning high frequency at all caste in India (Bhattacharyya et al., 1999; Ramana et al., 2001). Karafet et al. (2005) found that 2,2% Balinese men have India gene. Although all loci have the polymorphic gene, the number of allele in each locus was only 2 or 3 alleles

and predominated by just one of allele. This result indicated that not may mutation occur, and if mutation occurred, the presence of mutation was not for long time. Although the Soroh Kayu Selem has nine alleles, for same kind of loci the frequencies of alleles are more flatten (Junitha et al., 2009). DYS19 locus was predominated by 200bp allele, that is because of the 200bp allele is a common allele of the other Bali societies such as Soroh Kayu Selem and Terunyan as member of Pasek catur Sanak Clan, Sembiran, Tenganan Pegringsingan and also Tri Wangsa societies (Junitha, 2004; Junitha and Sudirga, 2007; Junitha et al., 2009; Junitha and Suryobroto, 2010). This allele is universal allele because it spread over to flatten in the world (Ruiz-Linnares, 1996; Hammer and Horrai, 1995; Hammer et al., 1997).

Alleles of locus DYS393 that found in Soroh Celagi were found in Terunyan and Soroh Kayu Selem, allele 125 bp was found in Terunyan society with frequency of 0.06 but was not found in Soroh Kayu Selem. Allele 133 bp was found in Terunyan as well as Soroh Kayu Selem. While for allele 131 bp at DYS395 locus higher frequency (0.88) in Soroh Celagi, was not found in Terunyan as well as in Soroh Kayu Selem. That allele was also not found in others Balinese people, Javanese and tribe of Batak (Parra et al., 1999; Junitha, 2004; Junitha and Sudirga, 2007; Junitha et al., 2009). Therefore, because this allele was not found in other societies outside Soroh Celagi, this allele can be used as a mark for Soroh Celagi.

Genetic diversities of each locus are very low range from 0.062 ± 0.04 at DYS 390 locus and the highest was only 0.234 ± 0.06 at DYS395 locus. The whole diversity among the samples was 0.148 ± 0.05 (Table 2), therefore the diversity of Soroh Celagi was low. Classification of lower diversity is determined by the presence of which are only two or three alleles in each locus, and predominated by one allele for all loci (Slatkin, 1995). In Balinese culture the marriages system is patrilineal, in that system a woman comes into man or husband community and become his clan member. By this way alleles variation of Y-chromosome only derived from mutation. The genetic diversity of Soroh Celagi is lower (0.148 ± 0.054) compared to Soroh Kayu Selem (0.511 ± 0.03) and Terunyan society (0.280 ± 0.05) as a part of Catur Sanak Clan. The Soroh Celagi gene diversity bigger compare to Tenganan Pegrisingan society where they are very close culturally (Junitha et al., 2009; Junitha and Sudirga, 2007; Junitha, 2004).

Table 2. Genetic diversity

| Locus | Diversity |
|---|---|
| DYS 19 | 0.175 ± 0.059 |
| DYS 390 | 0.063 ± 0.040 |
| DYS 393 | 0.121 ± 0.053 |
| DYS 395 | 0.234 ± 0.067 |
| Average Diversity | 0.148 ± 0.054 |

Based on alleles combination of four loci of DNA microsatellites Y-chromosome markers (DYS19, DYS390, DYS393 and DYS395), haplotype in Soroh Celagi was determined. There are five haplotypes found in this research such as presented at Table 3.

Table 3. The haplotypes, frequencies and distribution

| Haplotype | Alleles combination (bp) | Individu | Frequency | Pedahan | Songan | Other places |
|---|---|---|---|---|---|---|
| 1 | 200,207,133,131 | 26 | 0.84 | 12 | 11 | 3 |
| 2 | 200,207,133,135 | 1 | 0.03 | | 1 | |
| 3 | 200,207, 125,127 | 2 | 0.08 | | | 2 |
| 4 | 204,207,133, 131 | 2 | 0.06 | | 2 | |
| 5 | 204,203,133,131 | 1 | 0.03 | | 1 | |
| | Jumlah | 32 | 1 | 12 | 15 | 5 |

Other places are: Suter, district of Kintamani, Bangli Regency, Koripan, Tihingan district of Banjarangkan and Pikat village, district of Dawan Klungkung regency.

This result showed that haplotype 1 with alleles combination of 200 bp, 207 bp, 133 bp, 131 bp has the highest frequency (0.84) and spread at Pedahan village as place of Kawitan Temple of Soroh Celagi, Songan village as place of origin of Soroh Celagi and others place in Bali. Only haplotype 1 was found in Pedahan village. On the other hand, in Songan village four haplotypes were exist that are haplotype 1, haplotype 2, haplotype 4 and haplotype 5 (Table 3). This because Songan village is place of origin of soroh Celagi and most of member of Soroh Celagi live in Songan village, so that haplotypes will spread over as a result of local alleles mutation. Haplotype 3 which is not exists in Songan village, owned by probandus of member Soroh Celagi from Suter village district of Kintamani regency of Bangli and Tihingan village district of Banjarangkan regency of Klungkung. The haplotypes that found in Soroh Celagi are not found in previous research in Soroh Kayu Selem, Terunyan community, both of the same part of Pasek Catur Sanak clan and also other societies such as Tri Wangsa society and Tenganan Pegringsingan (Junitha, 2004; Junitha and Sudirga, 2007; Junitha et al., 2009). This result gives opportunity to use these data as reference to trace Soroh Celagi using DNA microsatelites Y-chromosome marker, although further research must be developed using more probandus.

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# THE STUDY OF SOCIO-ENGINEERING OF SUBAK SYSTEM DEVELOPMENT WITH Agro-ecotourism ORIENTED

**Sumiyati[1*], Lilik Sutiarso[2], Wayan Windia[3] and Putu Sudira[2]**
*[1]Agricultural Engineering Department, Agricultural Technology Faculty, Udayana University, Bali, Indonesia.*
*[2]Agricultural Engineering Department, Agricultural Technology Faculty, Gadjah Mada University, Yogyakarta, Indonesia.*
*[3]Agribusiness Department, Agricultural Faculty, Udayana University, Bali, Indonesia.*
**Correspondence author: sumiyatiftpunud@gmail.com*

**ABSTRACT**

Subak is a traditional irrigation institution in Bali. The feature of Subak management is based on the principle to the concept of Tri Hita Karana (THK). Since Bali have become one of the world's tourist destinations to date, it needs an attempt of integration between agriculture and tourism. Therefore, subak is not only as objects but take a role as a subjects. It is required a Study of Socio-engineering of the Subak System Development with Agro-ecotourism Oriented.

The study was begun with the identification system. The boundary system in this study is the subak system and the agro-ecotourism system. Aspects studied include the technical and social of society aspects. Data was taken from the field through a survey method. Research was conducted in Subak Lodtunduh in Singakerta Village, Ubud District, Gianyar Regency, Bali Province and Subak Anggabaya, in the Penatih Village, District of East Denpasar, Denpasar, Bali Province.

Agro-ecotourism facilities utilize resources owned by the subak or subak members, the principle does not interfere with existing subak operations. Line tracking, can take advantage of the irrigation dikes or barrier fields, conditioned to ensure the comfort and safety of visitors. So that irrigation facilities has also improved.

With agro-ecotourism development is not expected to decrease the social conditions in the subak systems, however it is expected to increase social conditions of the Subak Anggabaya subak system and Lodtunduh subak system.

***Keywords***: subak, agro-ecotourism, socio-engineering

## INTRODUCTION

Subak is a traditional irrigation institution in Bali and its function is to manage irrigation water that comes from a specific source. One special feature of subak system is its management based on Tri Hita Karana (THK) concept. This concept allows subak to manage the irrigation as well as the agriculture field in harmony, so that subak can be lasted for centuries.

Until now, Bali is still one of the world tourism destinations. As the tourism has been developing, the phenomenon of the youth interest becomes a farmer has been decreasing, in addition the shrinkage of the agriculture field due to land use conversion. Rice paddy fields and farmers are the asset of tourism. The noble agrarian culture values have discovered from agriculture life which are able to be used as a tourism asset. Integrating agricultre and tourism are necessary to be done, so that subak is not only to be an object, but able to play a role as a subject. The transformation compatibility studies are required for subak and agro-eco-tourism to encourage the regional development.

The regulations of local government of Bali province No. 02/PD/DPRD/1972 stated that subak is an indigenous community who has a socio-agrarian-religious characteristic and also a farmer association who manages water irrigation in the rice paddy fields. While, the government regulation No. 23 tahun 1982 on irrigation, subak is defined as an indigenous community who characterizes as a socio-agrarian-religious and also historically grown and developed as an water-use organization at the farm level.

Originally, subak system was only managing irrigation water for its member benefits. Since there have been economic activities, in its developments subak system is also managing organization's finance.

Water distribution is using a particular system in each ownership lot of farmers' subak members. Supply and drainage system are separated in one ownership lot, known as one inlet and one outlet system. Water in drainage canal can be used by others. Water allocation system and its distribution were performed regularly. Irrigation water is delivered to subak members; commonly it flows continually and proportionally.

Water measurement unit has been used known as tektek.

Agro-ecotourism is a trip to a special place to take an advantage of agribusiness as a tourism object for recreation or leisure, to enjoy, admire, value and study the nature, environment and agriculture culture in a particular place/ farmland in order to preserve/conserve the environment and improve the local community prosperity.

Agro-ecotourism is one of the tourism activities which its object is a farm including all activities that associated with it and in its implementation is still considering environmental preservation, in order to reduce negative impact of tourism activities, i.e. the environmental damage or pollution and local culture. In addition, it also provides financial benefits and local community empowerment by creating tourism products that promote local values.

Agriculture is an activity to support the culture. Subak is one of Bali's cultural heritage and also part of Balinese indigenous knowledge. Agriculture is becoming uninteresting, the area of subak is shrinking due to land use conversion, the different interest in using water and subak is not yet a legal institution. Compatible transformation of subak system and tourism is required to support regional development.

Subak is a system; is a commensurate entity with socio-cultural community, achieving its goals based on harmony and unity according to THK concept and to maintain the environmental balance. In the study of "Study of Socio-engineering the Subak System Development with Agro-ecotourism Oriented" offered an innovation that should be beneficial economically, feasible technically, acceptable socially and culturally, also does not contradict with the socio-culture of local values and not lead to pollution.

## MATERIALS AND METHODS

This study was begun with the system identification which consists of subak system and agro-ecotourism system. Field data collection was performed using survey method and on the matters that need further information, in-depth interview was conducted based on interview guidelines which refer to the aim of the study (Mantra, 2008). System identification study consists of two aspects i.e. technical aspect and the social aspect of society. Based on the identification system study, subak system and agro-ecotourism system were arranged. The research was conducted on Subak Lodtunduh which is located in Singakerta Village, Ubud District, Gianyar Regency, Bali and Subak Anggabaya which is located in Penatih Village, Denpasar Timur District, Denpasar, Bali.

## RESULTS AND DISCUSSION

### Technical Aspects

Based on the elements of the technical aspect as the results of the system identification study, subak system development with agro-ecotourism oriented were arranged as follows.

Most of irrigation water requirement (IWR) in Subak Anggabaya and Lodtunduh could be met by the available discharge (Q). There was a significant increase in water demand during the soil tillage, but it could be solved by water credit system between the subak members. Water excess in a subak system was managed by separated supply and drainage system in a particular rice paddy ownership area which is known as one inlet and one outlet system.

The procurement of agro-ecotourism facilities are utilizing subak or subak members' resources and still considering the existing subak operation. For example tracking line; it is possible to use the dike in rice paddy field which is conditioned to ensure the visitor's comfort and safety. For that reason, irrigation facilities are needed to improve.

Agro-ecotourism facilities utilize resources owned by the subak or subak members, the principle does not interfere with existing subak operations. Line tracking, can take advantage of the irrigation dikes

or barrier fields, conditioned to ensure the comfort and safety of visitors. So that irrigation facilities has also improved.

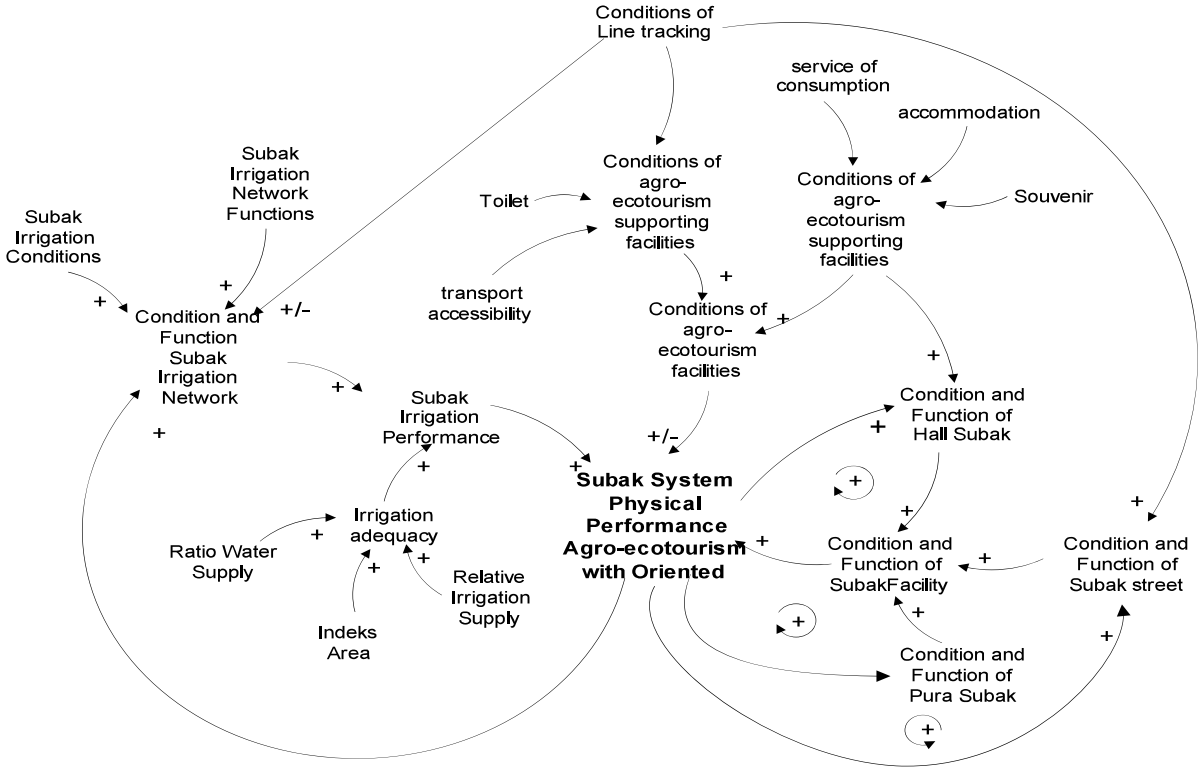


Figure 1. Causal diagram of the technical aspect

**Social Aspects of Society**

Based on the elements of the social aspect as the results of the system identification study, subak system development with agro-ecotourism oriented were arranged as follows.

Subak Anggabaya and Lodtunduh were in a "good category" in term of the routine ritual ceremonies' value and their member's participation in religious ceremonies. The implementations of religious ritual on both subaks are fully supported by the active participation of their members. In addition, its rituals in subak system have shown that subak system is a socio-agrarian-religious irrigation system; to manifest the concept of Tri Hita Karana (THK) which is associated in maintaining the harmonic relationship between The Creator. In order to develop an agro-ecotourism oriented subak, the religious rituals can be a charming attraction.

With agro-ecotourism development is not expected to decrease the social conditions in the subak systems. Expected to increase social conditions of the Subak Anggabaya subak system and Lodtunduh subak system.

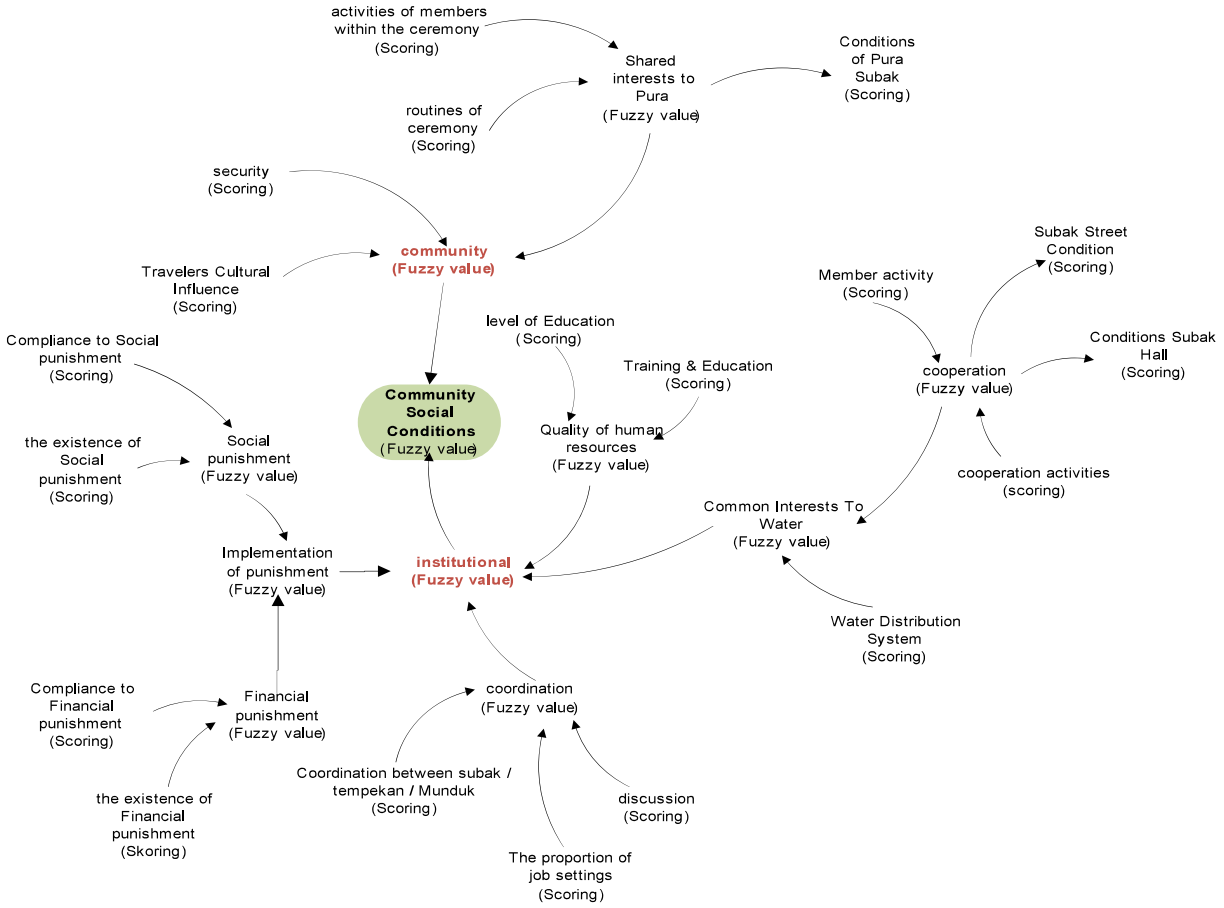


Figure 1. Causal diagram of the social aspect

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# BIOCATALYTIC DESULFURIZATION OF DIBENZOTHIOPHENE BY *Pseudomonas* sp. STRAIN KWN5

**Ida Bagus Wayan Gunam[*1], I Gusti Ayu Lani Triani[1], Nyoman Semadi Antara[1], Agus Selamet Duniaji[2], Yohanes Setiyo[3] and Dewa Adi Supata[1]**

*[1]Bioindustry Laboratory, Department of Agro-Industrial Technology, [2]Department of Food Science and Technology, [3]Department of Agricultural Engineering,*
*Faculty of Agricultural Technology, Udayana University, Denpasar, Bali, Indonesia*
*[*]Corresponding author:* Phone: +62 361 222006; Fax: +62 0361 701801;
E-mail: ibwgunam@yahoo.com

**ABSTRACT**

*Pseudomonas* sp. strain KWN5 was tested for the ability to use dibenzothiophene in *n*-tetradecane as the sole of sulfur source. The strain could grow on mineral salt sulfur-free (MSSF) medium with the *n*-tetradecane oil phase containing 200 ppm dibenzothiophene (DBT) and desulfurize this compound. The DBT-desulfurizing ability of KWN5 is high over a wide temperature range from 27 to 42°C, and the highest at 37°C. This strain could grow well on incubation period for 4 days at 37°C, pH 7, and glucose as the carbon source. In that condition, growing cells of KWN5 could degrade 200 ppm DBT around 75.21% within 96 h, indicating that this strain was very useful for the removal of DBT from oil.

***Keywords**:* Desulfurization, dibenzothiophene, tetradecane, strain KWN5

## INTRODUCTION

Production and world primary energy consumption showed a high increase. Energy demand increases with increasing income level. The energy of the most widely used today is still from fossil energy. Use of fossil energy, especially petroleum widely acknowledged having benefits but also having a negative impact.

The result of incomplete combustion of petroleum and coal produce sulfur oxides ($SO_x$), which can cause environmental pollution such as air pollution and acid rain (Gunam *et al*., 2006). Sources of air pollution in each area are different. Sources of air pollution may come from motor vehicles, household activities, and industry (Laras, 2006).

Petroleum contains sulfur compounds, including aromatic sulfur compounds such as alkyl dibenzothiophene and benzothiophene. Its compounds cannot be removed by conventional hydrodesulfurization (HDS) treatment using metallic catalysts (Furuya *et al*., 2003; Gunam *et al.*, 2006). This proves that the use of HDS require high costs, so many researchers turned its attention to seeking a more efficient alternative technologies (Guerinik and Muttawah, 2003).

One attempt to reduce the sulfur content of aromatic compounds in petroleum is biodesulfurization process. In this process, the microbes use sulfur from petroleum as an energy source for growth. To obtain optimal results in lowering the sulfur content of petroleum required certain types of bacteria that have the ability to degrade these compounds. Efforts to reduce the sulfur content of aromatic compounds in petroleum can be optimized (Jasrizal, 2009).

Results of previous studies (Gunam *et al*., 2009), showed that one strain has the highest ability to degrade aromatic sulfur compounds, known as KWN5 strain (this strain was isolated from soil samples derived from petroleum-contaminated soil near oil fields Kawengan, Bojonegoro, East Java). Based on the above, it is necessary to investigate the optimum conditions (temperature and pH) for the growth of this strain, which can degrade the highest DBT compounds.

## MATERIALS AND METHODS

### Materials

Dibenzothiophene (DBT) was purchased from Aldrich and Tetradecane was supplied by Wako Pure Chemical Co., Osaka, Japan. Mineral Salt Sulfur Free (MSSF) Medium was prepared by previous method

(Gunam *et al*., 2006) and petroleum (light gas oil) was supplied by Pertamina. All other reagents were of analytical grade and commercially available.

A concentrated fraction of aromatic compounds (CA) was prepared by fractionation of commercial light gas oil (Gunam *et al.*, 2006).

**Bacterial strains and culture media**

The KWN5 strain was grown in a mineral salt sulfur-free (MSSF) medium (pH 7) with CA as the sole sulfur source, as reported in our previous paper (Gunam *et al.*, 2011). MSSF-TD was the standard media for the desulfurization assay, consisted of MSSF: *n*-tetradecane = 5:1, with DBT dissolved in *n*-tetradecane. Bacterial growth was determined by measuring $OD_{660}$ of water layer (Gunam *et al*., 2006).

**Seed culture preparation**

For seed culture production, KWN5 was cultivated at 30°C in 500-ml Erlenmeyer flasks containing 200 ml of MSSF medium with CA as the sole source of sulfur for 4 days. The cells were harvested by centrifugation at 3500 rpm for 20 min at 4°C, washed twice with 0.85% saline solution and suspended in the same solution. The optical density at 660 nm ($OD_{660}$) of the cell suspension was adjusted to 5.

**Bio-desulfurization assay**

Six milliliter of MSSF-TD medium containing DBT were inoculated with 0.1 ml of the seed culture ($OD_{660}$ = 5) and incubated at various temperature and initial pH for 4 days with shaking (200 rpm). After incubation, the organic layer of *n*-tetradecane and water layer were separated by centrifugation at 3500 rpm for 20 min at 4°C. Un-inoculated medium served as controls and were treated in the same manner.

**Analytical methods**

Cells growth was measured turbidimetrically at 660 nm. The cell concentration was determined from the linear relationship between the optical density at 660 nm ($OD_{660}$) and dry cells weight (drying at 105°C for 36 h). Measurements of DBT were performed using GC with a flame ionization detector (GC-FID). The concentrations of DBT in growth culture were analyzed by GC-17A (Shimadzu, Kyoto, Japan). Samples for GC analysis were acidified to pH 2.0 with 1 M HCl and extracted from aqueous cell/DBT suspensions by liquid–liquid extraction using ethyl acetate in a 2:1 ratio. A portion of the ethyl acetate layer was removed and centrifuged and 1 µl of the supernatant was injected into a gas chromatograph. The gas chromatograph was equipped with a fused silica capillary column, CBPI-m25-025 (25 m 0.22 mm id, df ¼ 0.25 l), packed with silicone OV-1, SE-30 (Shimadzu, Tokyo, Japan). The flow rate of helium carrier was 1 ml/min. The column temperature was programmed from 140 to 250ºC at 8ºC/min. The injector and detector temperatures were maintained at 280 and 310ºC, respectively.

## RESULTS AND DISCUSSIONS

**Temperature-dependent desulfurization of DBT by growing cells of KWN5**

The temperature is a major environmental factor that affecting physiological activity of most prokaryotes. At optimum temperature, microbes perform biological activities at the maximum rate such as growth and metabolism. The effects of temperature on DBT degradation by growing cells of KWN5 were examined 96 hours cultivation under different temperatures. As shown in Fig. 1, KWN5 grew significantly in the MSSF medium containing 200 ppm DBT in tetradecane as the sole source of sulfur. Moreover, growing cells of KWN5 exhibited high desulfurizing ability toward 200 ppm DBT in TD over a wide temperature range from 27 to 47°C, and this was most efficient from 32 to 37°C. From the results, it was clearly shown that microbial growth of KWN5 strain at 37°C resulted the highest growth and desulfurizing activity. In that condition showed that the sulfur content in model oil decreased from 200 to 55.74 ppm DBT (72.13% degradation) over 4 days incubation. However, the activity suddenly decreased at 42°C. In

contrast, no reduction of sulfur was detected for the un-inoculated samples after treatment under the same conditions. Kirimura et al. (2001) also reported that *B. subtilis* WU-S2B also exhibited DBT-desulfurizing ability over a wide temperature range from 30 to 55°C, and the activity suddenly decreased at 52°C.

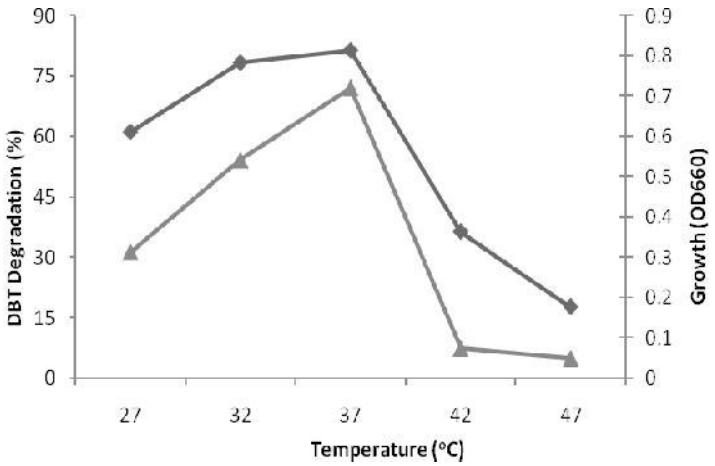


Fig. 1. Effects of temperature on DBT desulfurization by growing cells of KWN5 strain. KWN5 was cultivated in MSSF medium with 200 ppm DBT as sole source of sulfur at various temperatures for 96 hours. Symbols: □, growth ($OD_{660}$), and ▲, DBT degradation.

**Effect of various initial pHs on the growth and desulfurization activity**

The effect of initial pH towards growth and desulfurization activity of KWN5 was demonstrated in Figure 2. Growth of KWN5 was studied over a wide range of pH, ranging from 6.0 until 8.0, and the maximal growth and desulfurization activity were obtained at pH 7.0. The growth rate ($OD_{660}$) and desulfurizing activity of KWN5 strain in pH 7.0 medium were 1.2 and 75.21%, respectively. The activity of KWN5 sharply decreased was observed when the bacteria were grown in the medium with initial pH at lower and higher then pH 7. However, growth of bacteria was not significantly affected by pH.

The result was almost same with other desulfurizing bacteria such as S. subarctica T7b (Gunam *et al*., 2006), Gunam *et al*. (2011). Gunam *et al*. (2006) reported that *S. subarctica* T7b had optimal growth and desulfurization activities when the value of pH was 7. When the pH changes from 6.5 to 7.5, the degradation rate of DBt by KWN5 strain was kept at about 72-75%. Whereas the degradation ability of the suspended cells was only 30-34%. Meanwhile, the suspended cells lost more than 40% activity when the pH was lower than 5.5.

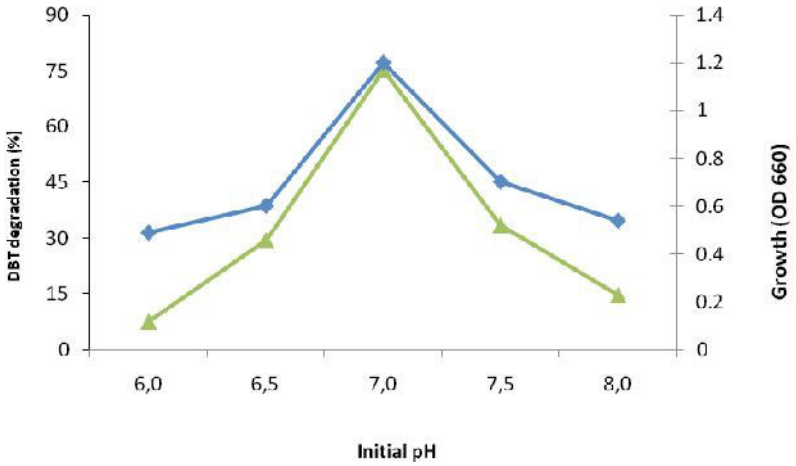


Fig. 2. Effects of initial pH on DBT degradation by growing cells of KWN5 strain. KWN5 was cultivated in MSSF medium with 200 ppm DBT as sole source of sulfur at various pH for 96 hours. Symbols: □, growth ($OD_{660}$), and ▲, DBT degradation.

## CONCLUSIONS

The conclusion that can be drawn from this research were: At 37°C and pH 7 can provide optimal conditions for growth of strain KWN5 with OD values at a wavelength of 660 was 1.2 and 200 ppm can degrade DBT in TD was 75.21%.

*Pseudomonas* sp. Strain KWN5 was great potential in degrading aromatic sulfur compound contained in petroleum, it is necessary to conduct further research especially to choose an effective method in its application in industry.

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# INDUCE POLIPLOIDY OF *Lilium longiflorum* THUNB. USING ORYZALIN TREATMENT

**Gusti Ayu Mirah Dwidaputri[1], Eniek Kriswiyanti[1] and Ida Ayu Astarini[1]**
[1]*Biology Department, Faculty of Mathematics and Natural Sciences, Udayana University, Bali*
*Email: idaastarini@yahoo.com*

**ABSTRACT**

*Lilium longiflorum* Thunb (Liliaceae), known as trumpet lily is a popular and high value ornamental plant around the world. Increasing genetic diversity of this plant will provide more choice for consumer and therefore increase economic value of the plants. One method to create diversity is via poliploidyzation. The objective of this study was to determine the morphometric variation and ploidy level of chromosome in *L. longiflorum* after treatment with Oryzalin 0.005% and 0.01%. Research was conducted at Biotechnology Laboratory, Agroecotechnology Department and Plant Structure Laboratory, Biology Department, Udayana University. Treated root tips were fixed with Farmer solution, followed by hydrolisis with 2N HCl prior to root squash. Squashed roots were then stained with Aceto-orcein 2% to obtain contrasting image of the chromosome. Results show that chromosome type of control plants is the same with Oryzalin 0.01% treatment, which is metacentric and submetacentric, whereas Oryzalin 0.005% treatment shows metacentric, submetacentric and subtelocentric type. Chromosome shape varied among treatments; i.e. stem, V, U and J on control and Oryzalin 0.005% treatment, while Oryzalin 0.01% treatment resulting in stem shape. Chromosomes number on control plants are 24, whereas treatment with Oryzalin 0.005% resulting in 48 chromosome, and treatment with Oryzalin 0.01% resulted in 32 chromosomes. In conclusion, Oryzalin 0.005% effective to doubled chromosome in trumpet lily.

***Keywords:*** chromosome, mutation, trumpet lily

## INTRODUCTION

Lilium (*Lilium longiflorum* Thunb.) family Liliaceae popularly known as Easter Lily or white trumpet lily. Trumpet lily is a perenial shrub with for 3 – 4 year production time. Trumpet lily commonly found in mountaineous areas but can also live in muddy areas (Marlina, 2009).

Lilium is a high value cut flower around the world. Demand for international market increase around 26% per year. It is the fifth most important cut flower in the Netherland with prodcution more than 300 million stem per year (Van Tuyl dan Van Holsteijn, 1996). In the USA, 11.5 million lilium bulb was produced and ready to be sent around the world. In Indonesia, lilium production is relatively low so that domestic market is still rely on imported lilium. Local lily value at around Rp 7.500 per stem. Imported lily value at around Rp. 12.500 per stem (Wuryan, 2009). Low production of lily in Indonesia mainly due to lack of superior cultivars and limited variation available.

Plant breeding is a method to manipulate plant genes and induce genetic diversity. Induce mutation is become an important tool to obtain high value superior cultivars and can be done using chemical and physical mutagen. Oryzalin is a chemical mutagen that able to induce poliploidy. Oryzalin strongly bind tubulin in plant to form a complex tubulin-Oryzalin. This complex do not able to polimerize into microtubulus so it inhibit cell division. Oryzalin has been use to obtain new genotype with variation on flower and leaf size and colour (Notsuka *et al*. 2000). Van Tuyl *et al*. (1992) describe that tetraploid lilium which has bigger flowers and strong stems is favourable to consumer.

The aim of this research is to find out ploidy level and morphometric variation of *Lilium longiflorum* Thunb. after induction using Oryzalin 0.005% and 0.01%. Long term benefit is to provide more variety in Lilium.

## MATERIALS AND METHOD

Research was done at Biotechnology Laboratory, Department of Agroecotechnology; and Plant Structure and Development Laboratory, Department of Biology, Udayana University, January - May 2011. *L. longiflorum* bulb was obtained from Donomulyo Village, Batu, Malang, East Java. Each bulb was planted in *polybag* containing sand and compost (1:2). Freshly grown roots of ± 2 mm was soaked in Oryzalin solution with concentration 0,005% and 0,01% for ± 3 hours. Bulb were then moved to water

solution for 24 hours to remove residu.

Squash method was used to examined chromosome of *Lilium longiflorum* after treatment. Root tips were cut ± 2 mm at 7 am to obtained best stage of the chromosome. Treated root tips were fixed with Farmer solution, followed by hydrolisis with 2N HCl prior to root squash. Squashed roots were then stained with Aceto-orcein 2% for 30 minutes, flame over Bunsen burner to obtain contrasting image of the chromosome (Ahmad *et al*., 1983). Chromosome counting and karyotyping was done on 10 cells with metaphase and pro-metaphase stage using Olympus Light Microscope. Photograph of the chromosome was taken using Digital Camera Kodak M863.

## RESULTS AND DISCUSSION

Results show that Oryzalin treatments gave variation in term of size, type, form and number of chromosome. Control plants has chromosome size ranging 32,5 µm - 73,8 µm. Treatment with Oryzalin 0.005% produced chromosome size 26,3 µm - 80 µm, while Oryzalin 0.01% produced chromosome size 36,3 µm - 68,8 µm.

Control plants have the same type of chromosome with plant treated with Oryzalin 0.01%, i.e. metacentric and submetacentric, whereas Oryzalin 0.005% treatment shows metacentric, submetacentric and subtelocentric type. Chromosome shape varied among treatments; i.e. stem, V, U and J on control and Oryzalin 0.005% treatment, while Oryzalin 0.01% treatment resulting in stem shape. Morphometric variation on chromosome occurred due to Oryzalin induced mutation, causing chromosome structure aberration. Type of aberration that can occur includes deletion, duplication, inversion and translocation (Brown and Caligari 2008).

Chromosomes number on control plants are 24, whereas treatment with Oryzalin 0.005% resulting in 48 chromosome, and treatment with Oryzalin 0.01% resulted in 32 chromosomes (Figure 1). This was in accordance with research results by Takamura (2002) which revealed the most effective ploidy induction on *in vitro* Lilium using Oryzalin was at 0.005%. However, optimum concentration to produce bulblet and highest ploidy level was at 0.003%. Higher concentration inhibits bulblet formation on *L. longiflorum* explants.

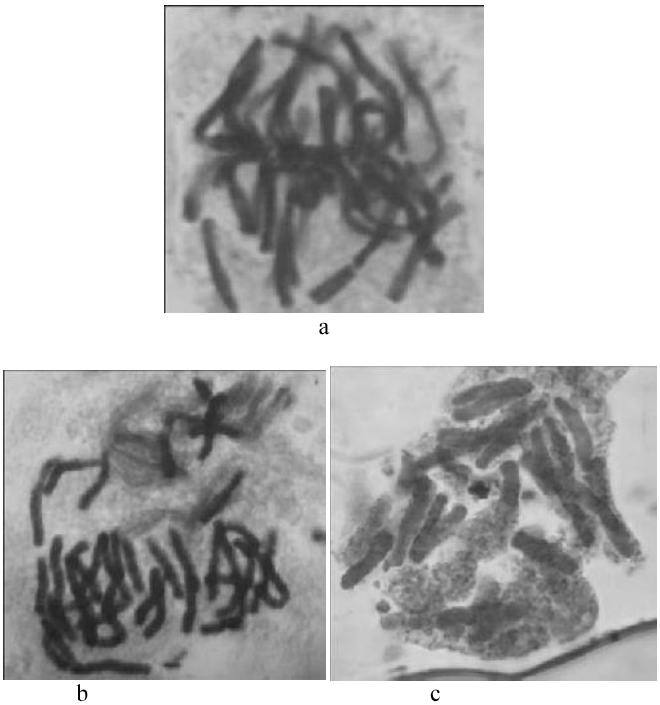


Figure 1. Lilium Chromosome. a. Control, b. Oryzalin 0.005%, c. Oryzalin 0.01%

## CONCLUSION

In conclusion, Oryzalin 0.005% effective to doubled chromosome in *L. longiflorum*, from 24 on untreated plant to 48 on treated plants. There are 3 types of chromosome found, i.e. metacentric and submetacentric (on control plants and Oryzalin 0.01%, 0.005% treatment), and subtelocentric type only on Oryzalin 0.005% treatment.

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# COMPOSTING ACCELERATION OF KITCHEN WASTE USING MICROBIAL INOCULANT

**Ni Made Indri Hapsari Arihantana[1], Ni Luh Yulianti[1]**
*[1]Faculty of Agriculture Technology, Udayana University, Kampus Bukit Jimbaran, Bali.*
*Correspondence author: mdindri@yahoo.com*

**ABSTRACT**

Organic waste such as leftover foods from kitchen, vegetables, leaf, and other spoiled foods can be utilized into composting organic material and also decrease the risk of environmental hazardous. Naturally, decomposition process takes longer time, human power and generate annoying health problems. Referring to these reasons, a new technology is required. The addition of microorganism as a decomposer will accelerate the process if they have a favourable condition. The research was conducted to produce an inoculant from rumen of cows, goats, chicken manure and also to analize kitchen waste (left over foods) by using different inoculants. Firstly moisture content, total microorganism, fungi, and pH of the inoculants were measured. The second step was to determine pH and ash content of kitchen waste. Moisture content of cow, goat rumen, and EM4 was 90% respectively, while chicken manure remained 60%. All inoculants have a neutral pH, total microorganism 1,1 x $10^{11}$ cfu/ml and total fungi, 3,4 x $10^{4}$ cfu/ml, while chicken manure contains 1,1 x$10^{11}$ cfu/ml microorganism and 1,0 x $10^{6}$ cfu/ml fungi. The.average pH of the kitchen waste was 7,0 and ash content was 2,3%. The highest ash content was produced from the kitchen waste treated with inoculants from chicken manure.

***Keywords*****:** *cow rumen, goat rumen, chicken manure, inoculant, kitchen waste*

## INTRODUCTION

Organic waste such as leftover foods from kitchen, vegetables, leaf, and other spoiled foods can be utilized into composting organic material and also decreased the risk of environmental hazardous. Compost is an organic waste which have decomposed by interaction of microbe. Naturally, decomposition process takes more time, human power and generated annoying also health problem. Refers to those reason, the need of technology in composting is required. Additional of microbe decomposer can help composting acceleration process if appropriate and favorable conditions are available.

Kitchen waste which can be composted consists of cellulose. Therefore, if the materials were inoculated with microorganism, the composting of kitchen waste is expected to run faster and produced better quality compost. Mala (1994) reported that inoculation of *Trichoderma harzianum Rifai Aggr*. (cellulolytic microorganism) on rice straw composting, composting able to accelerate to 19 days to reach the ratio of C/N 20 as the criteria of mature compost.

Manure is one of the materials that can be used as inoculants. Manure or rumen contents of cattle (ruminants) is a type of slaughter house waste generated continuously at a large amount, which ranges from 10-20% of animal live weight. It is known that rumen microorganism could accelerate the composting process and also improve the quality of compost. Rumen fluid contains protozoa and bacteria digesting cellulose, hemicellulose, starch, sugar, protein, lactate-eating bacteria and methane-forming bacteria (Hungate, 1966). Each ruminants contain various rumen microorganism, that influences composting and compost quality produced. The aim of this research is to obtain rumen microorganism from cows, goats, and chicken manure to be used as inoculants, and also to analize macro nutrients of kitchen waste.

## MATERIALS AND METHODS

Fresh cow rumen was obtained from Sanggaran slaughter house at Denpasar, fresh goat rumen from goat abattoir in Haji Rifai Wangaya Denpasar. Chicken manure was obtained from a poultry farm in the village of Kuum, Marga, Tabanan regency. Kitchen organic waste was obtained from several residential homes in Nuansa Hijau Utama Ubung, Denpasar. Media for inoculants are Nutrient Agar (oxoid) and 0.1% peptone water. The carrier material used is filtered compost. EM4 is used as a positive control for composting. The research consists of two phases, the first phase is the manufacture of inoculants

and composting. The study lasted for 9 months. The first phase includes: counting the initial population and observing the parameter of total microorganism, total fungi and pH. The second phase includes the implementation of composting that consists of sorting, addition of inoculant, planting litter bags and reversal process. The parameters observed are: an initial analysis of nutrient kitchen waste content and pH value. At this stage, the data were analyzed descriptively

## RESULTS AND DISCUSSION

The first stage measured levels of initial water of cow, goat rumen and chicken manure, by burning the material at 105°C for 24 hours. The measurements used is toluene on water content of rumen as reported by Sofyan, 1983. The water content of the rumen of cows and goats generally has an average moisture content ranging between 70-80% (pkplk-PLB). The results showd moisture content ranged around 90% for both types of rumen. The moisture content of the chicken manure ranged between 30-40%, while moisture content of the chicken manure ranged around 60%. Compared to the inoculants that are sold at market, EM4 has a moisture content of 90% which is quite similar with moisture contents of rumens of cow and goats (Figure 1).

pH of rumen usually ranges from 5.5 to 5.6, the research reported that rumen of cows and goats pH ranges around 6.5, chicken manure ranges around 7 and EM4 has a pH of 5.4. All materials were categorized as neutral pH (Figure 1).

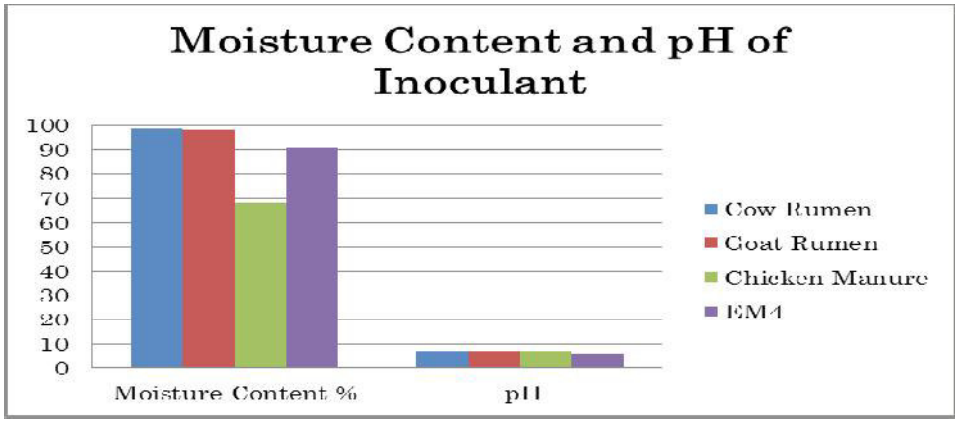


Figure 1. Moisture Content and pH Inoculant

Rumen fluid contains bacteria and protozoa. The concentration of bacteria is around $10^9$/ml rumen contents, while protozoa varies about $10^5$-$10^6$/ml rumen contents (Tillman *et al.*, 1991). Some types of bacteria / microbes present in the rumen contents are (a) bacterial / microbial lipolytic, (b) bacteria / microbes forming acid, (c) bacterial / microbial amylolytic, (d) bacterial / microbial cellulolytic, (e) bacteria / microbes proteolytic (Tillman *et al.*, 1991). In the goat rumen microbial population are divided into three main groups of bacteria, protozoa, and fungi (Tillman *et al.*, 1991). Types of bacteria found in the rumen of goat have the same function with the bacterial species present in the solution of EM4, quite higher in complexity.

The high TPC indicates the high quality of microorganism inoculants produced. In this study, the results of TPC show in Figure 2. The concentration of the bacteria found in the rumen of cows is 9.8 x $10^9$ cfu/ml. TPC on rumen of goats is 2.6 x $10^9$ cfu/ml and the chicken manure is 1.1 x$10^{11}$ cfu/ml. The EM4 inoculant is 10.4 x $10^9$ cfu/ml. Total fungi produced by rumen of cows is 0.5 x $10^4$ cfu/ml, 0.8 x $10^3$ cfu/ml for rumen of goats and chicken manure 3.6 x $10^4$ cfu/ml. The total EM4 mold inoculant produced is 3.1 x$10^3$ cfu/ml.

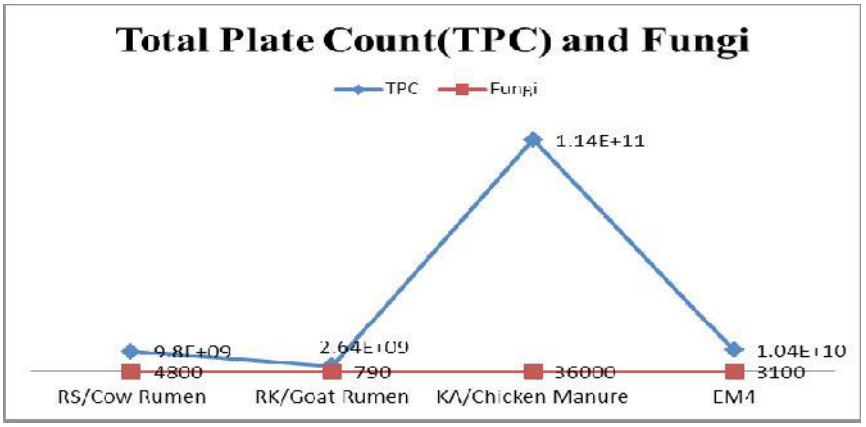


Figure 2. Result of Total Plate Count (TPC) and Fungi of Inoculant

Generally, kitchen wastes consist of a number of bacteria / microbes that are ready to revamp the organic materials contained. After the phase separation of organic waste, inoculant applied into each sample. In this study, the TPC results indicate, the concentration of bacteria found in kitchen garbage treated with rumen of cows inoculant is 1.7 $x10^{10}$ cfu/ml. TPC on rumen of goats inoculant is 1.2 x $10^{10}$ cfu/ml and the chicken manure inoculant is 1.1 $x10^{11}$ cfu/ml. The EM4 inoculant is 4.2 x $10^{10}$ cfu/ml. Total fungi produced by rumen of cows is 7.9 x $10^{3}$ cfu/ml, 1.0 x $10^{5}$ cfu/ml for rumen of goats and chicken manure 1.0 $x10^{6}$ cfu/ml. The total EM4 mold inoculant produced is 3.0 x $10^{5}$ cfu/ml.

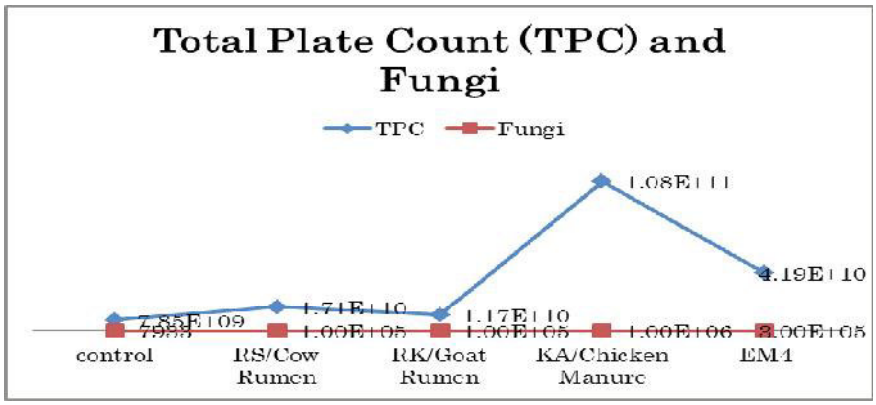


Figure 3. Result of Total Plate Count (TPC) and Fungi of Kitchen Waste Compost

The composting process occurs in a wide range of pH. The optimum pH for the composting process ranges from 6.5 to 7.5. The standard quality of pH is defined according to the World Bank, International, PT Pusri and Market is 7.5. The results of compost derived from kitchen waste treated with different inoculants showed an average pH of 7.

Figure 5 shows the mineral of kitchen waste compost. Inorganic ash content is a component that remains after the material is heated at a temperature of 600°C. Generally the ash content varies depending on the inoculants used. Average of ash content of compost in five treatments was 2.3%. Ash content of compost was 2.2%, 2.19%, 2.44%, 2.26% by using rumen of cows and goats inoculant, chicken manure, EM4 respectively. In composting process, ash increased due to degradation of organic compounds into inorganic compounds (Sriharti and Salim, 2007).

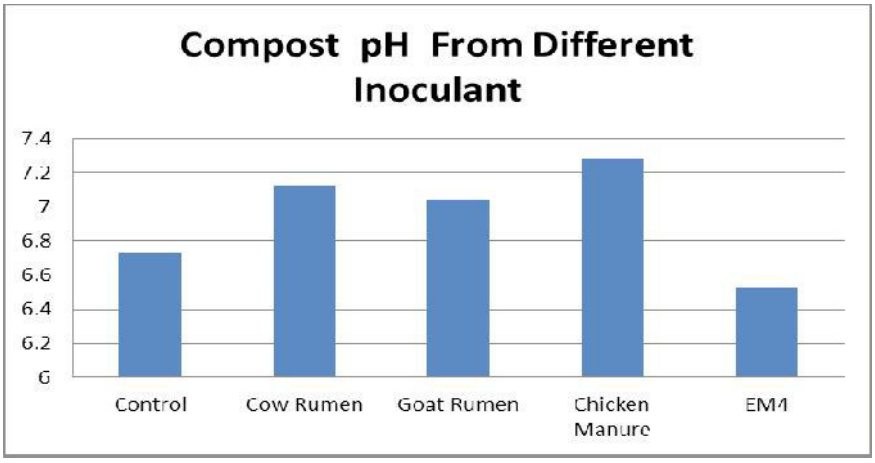


Figure 4. Kitchen Waste Compost pH from Different Iinoculant

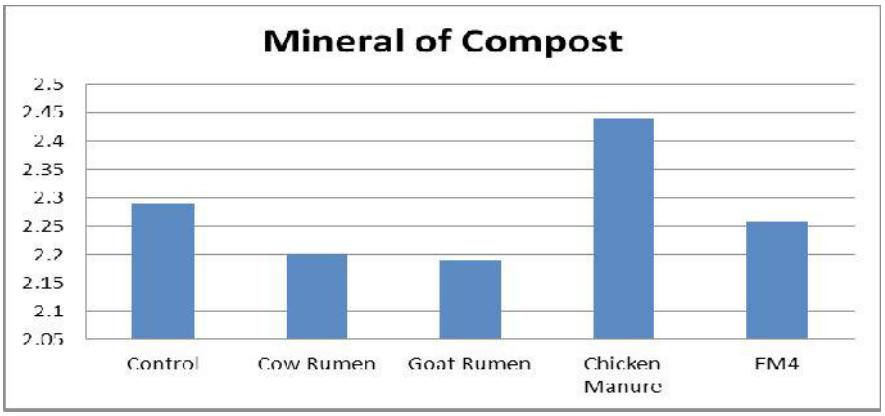


Figure 5. Mineral of Kitchen Waste Compost

## CONCLUSION

1. The average moisture content of rumen of cows, goats and EM4 ranges from 90%, while the water content of chicken manure ranges from 60%. pH inoculant is categorized st neutral pH.
2. TPC results showed that the highest concentration of bacteria found in chicken manure inoculant around 1.1 x $10^{11}$ cfu/ml while the total fungi produced by chicken manure is 3.4 x $10^{4}$ cfu/ml.
3. In compost, TPC results showed that the highest concentration of bacteria found in household waste inoculants treated with chicken manure is 1.1 x $10^{11}$ cfu/ml while the total fungi produced is 1.0 x $10^{6}$ cfu/ml.
4. The average pH of kitchen waste inoculant with different treatment is 7 while the ash content of domestic waste inoculants with different treatment is 2.3%. The kitchen waste inoculants treated with chicken manure has a low ash or high mineral of 2.44%.

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# HEAVY METAL BIOREMEDIATION LEAD (Pb) TO AGRICULTURAL LAND ON THE EDGE OF LAKE BUYAN BALI WITH PLANT *Sansevieria lorentii*

**Ni Luh Suriani**

*Biology Department, Faculty of Mathematics and Natural Sciences, Udayana University*

*niluhsurianisuriani@yahoo.com*

**ABSTRACT**

This study aims to determine the effectiveness of *Sansevieria lorentii* in the absorption of Pb contained in the agricultural land at the edge of Lake Buyan, and how much lead is down from the land. The research was conducted at the Analytical Laboratory, Udayana University. Pb concentration was determined using AAS. The results showed that after 1 month, the concentration of Pb in *Sansevieria lorentii,* grown in media of agricultural land taken from the edge of Lake Buyan with an average Pb content of 208.4 mg/kg, increased by an average of 44.9 mg/kg with a value of effectiveness of 34.3% and the average reduction of Pb concentration was 39.5 mg/kg with a value of 19.3% effectiveness. The result showed that *Sansevieria lorentii* can absorb Pb not only from the air, but also from the soil.

# THE IDENTIFICATION AND INVESTIGATION OF PARASITIZATION RATE OF PARASITOIDS OF HAIRY CATERPILLAR *LYMANTRIA MARGINATA* WLK. (LEPIDOPTERA: LYMANTRIIDAE) IN BULELENG BALI

I Wayan Susila[*)] And I Putu Sudiarta
*Faculty of Agriculture, Udayana University*
*[*)]Corresponding author E-mail: w1sus@yahoo.com*

**ABSTRACT**

The population of hairy caterpillar was break down in Bali early year 2011 with the high population in some regency in Bali as well as Buleleng. The hairy caterpillar was reported attack some crop plant. On the other hand the problem was caused by hair caterpillar is also skin injury of human. To control hair caterpillar was impossible used chemical pesticide, because very dangerous for human. One way is using natural enemies, sac as parasitoid. To utilize the parasitoid the identification and investigation and parasitization rate of parasitoid is needed. The experiment of Identification and Investigation of parasitization rate of parasitoid of hairy caterpillar, *Lymantria marginata* Wlk. (Lepidoptera: Lymantriidae) was conducted on March 2011 in Laboratory of Integrated Pest Management, Faculty of Agriculture Udayana University. The objective of this experiment is in other to know the kind and parasitization rate of parasitoid of hairy caterpillar *L. marginata* in Buleleng Regency. The result indicated that 47% from the 160 pupal *L. marginata* collected was parasitized by parasitoid. Kind of parasitoid which emerges from of pupal *L. marginata* was family *Tachynidae* and *Brachymeria* sp.

***Keywords:*** *Lymantria marginata*, parasitoids, parasitization rate

# POSTER PRESENTATIONS
# HEALTH

# THE IMPLEMENTATION OF MODULE-JIGSAW COOPERATIVE LEARNING STRATEGY, IMPROVING THE PREVENTATIVE BEHAVIOR TOWARDS DENGUE HEMORRHAGEIC FEVER

**Dewa Nyoman Oka**
*Jurusan Penddidikan Biologi Fakultas Pendidikan MIPA IKIP Saraswati*
*Jalan Pahlawan Nomor 2 Tabanan-Bali*
*Correspondence Author: Dewanyoman_oka@yahoo.co.id*

**ABSTRACT**

The increasing number of Dengue hemorrhagic fever (DHF) cases causes the wider spread of dengue virus carried by *Aedes* mosquitos. This indicates that the preventive behavior toward DHF is still low. The aim of this research was to investigate if (1) the implementation module *jigsaw* CLS improved students' behavior toward prevention of DHF; (2) the implementation of module *jigsaw* CLS improved the students' behavior better than *jigsaw* CLS and conventional learning did. The applied research design was the real experimental design, which was called *Randomized Pre and Posttest Control Group Design*. Results of analysis showed that the implementation of *jigsaw* CLS did not significantly improve the students' behavior in DHF prevention while the implementation of module *jigsaw* CLS was able to improve the students' behavior better than *jigsaw* CLS and conventional learning did.

***Keywords**:* module *jigsaw* CLS, behavior, DHF prevention

## INTRODUCTION

The number of DHF cases keeps rising in Indonesia. In its first case in 1968 there were only 50 cases and in 1999 there were 21,134 cases. In 2010 it reached 140,000 cases. The increasing number of Dengue hemorrhagic fever (DHF) cases causes the wider spread of dengue virus population brought by *Aedes* mosquitos. It shows us that preventive behavior toward DHF is still low.

In relation to this phenomenon Sintoroni (2007) stated that if the community do not conduct integrated and consistent control, DHF cases will continue to rise year after year. This is caused by the nature of global environment condition, which accelerates the spreading of mosquitos and viruses.

Cahyo (2006) stated that level of education and knowledge about DHF are the factors that cause the respondents incapable to do DHF prevention. Level of knowledge about DHF can also influence the respondents' seriousness concerning DHF prevention and its clinic, medic and social consequences.

Furthermore, Lawrence Green (1980) stated that behavior is influenced by three factors, namely: (1) *predisposing factors*, which are in the form of knowledge, attitude, belief, faith, values, etc; (2) *enabling factors*, which are in the form of physical environment, availability or unavailability of health facilities like community health center, medicines, contraception, toilet, etc; (3) *reinforcing factors* are in form of paramedics and other officers' behavior, which are the role model groups for community.

Cooperative learning strategy is a group learning strategy, which is recently attracting attention and suggested by education experts. Slavin (1995) proposed two reasons; first, several results of research prove that cooperative learning implementation can increase achievement of study, increase socializing ability, develop acceptance to self and others' weaknesses, and increase self-esteem. Second, cooperative learning fulfils students' needs, which are to learn to think, to solve problems, and to integrate knowledge with skill.

The strength of CLS *jigsaw* is that it can increase the students' responsibility for their own learning process and also others' learning process. The students do not only study the given materials but are also ready to share the materials they have studied with the other members of the group. They also must improve their cooperative way in learning the materials given to them (Yasa, 2009).

The well-structured module can give many benefits, namely: (1) learning process can reach its maximum result; (2) the students are more active in the learning process in which they have some problems or assignments to do; (3) it can give immediate feedback so that the students can know the results; (4) the learning process is directed because the modules have clear objectives; and (5) involvement of teachers in

learning process is very minimum (Nasution, 2009).

Based on what mentioned above, questions, then, can be identified as follows: (1) can *jigsaw* CLS implementation improve students' behavior toward DHF prevention?; (2) can module *jigsaw* CLS implementation improve students' behavior toward DHF prevention?; (3) can module *jigsaw* CLS improve students' behavior better than *jigsaw* CLS toward DHF prevention?

## METHODS

The research design used in this research is the real experiment design *Randomized Pre* and *Posttest Control Group Design*. The target population in this research was all students of SMPN 2, SMPN 3, and SMPN 4 Abiansemal. The covered population was all students in grade 9 of 2, SMPN 3, and SMPN 4 Abiansemal that follow UKS (health school unit) extracurricular, whose biology scores in grade 8 semester 2 were relatively the same and whose pre-test scores were minimum 30 and maximum 50. The samples were 32 persons per group/per school that were chosen through *simple random sampling* from the covered population. Since there were three groups involved, the total number of samples for each group was 3 x 32 persons = 96 persons.

Variables in this research can be divided into (1) free variable, which is *jigsaw* CLS and module *jigsaw* CLS; (2) bound variable, which is the understanding of the students about DHF prevention and students' behavior toward DHF; (3) controlled variable, which is the biology scores grade VIII semester 2 and students' early knowledge about DHF prevention.

This research commenced with developing module materials of DHF. The first group was given conventional learning, the second group was given *jigsaw* CLS and the third group was given module *jigsaw* CLS. The collected data from each group were analyzed in order to draw the conclusions.

## RESULTS AND DISCUSSION

The normality of students understanding and behavior data as well as understanding and behavior improvement toward DHF prevention after and before DHF learning were tested by *Shapiro-Wilk* test with significance level $\alpha = 0.05$. Data were normally distributed if p value from *Shapiro-Wilk* was $\alpha = 0.05$. Based on the normality test, data of understanding before learning, understanding after learning, behavior before learning, behavior after learning, and understanding improvement toward DHF prevention were distributed normally with p value > 0.05 from *Shapiro-Wilk*. Conversely, normality test result for data behavior improvement toward DHF prevention was not distributed normally with P value < 0.05.

The data homogeneity of research subject was examined with *Leven's Test* with significance level $\alpha = 0.05$. The result of homogeneity across the strategies was not homogenous ($p<0.05$) for behavior improvement toward DHF prevention. Conversely,behavior data before and after the learning had homogenous variant ($p>0.05$).

The comparability was seen from different condition of all learning strategies from the level of behavior before the learning strategies were conducted. The analysis of the three strategies' differences was made by using *One-Way Anova* test. The result is presented in Table 1:

Table 1. Analysis on Different Scores of Behavior before the Implementation of the Three Strategies

| Source of Variation | Average | SD | Score F | Score p |
|---|---|---|---|---|
| Behavior Scores of the Students Before the Implementation of the Strategies | 60.34 | 7.50 | 0.557 | 0.575 |

The result of analysis on different average scores of behavior before the implementation of the three strategies is not significantly different (P > 0.05). Since the initial behavior scores have no significant difference, the scores after the implementation are, then analyzed.

Different impacts of behavior improvement toward DHF prevention were made by comparing the students' behavior improvement among the three learning systems using KRUISKAL-Wallis because the data improvement was not distributed normally. In this analysis, behavior improvement is used as bound variable while treatment is used as free variable. Results of analysis are presented in Table 2.

**Table 2.** *Kruskal-wallis Analysis of* Behavior Scores' Impacts on The Students' Behavior Improvement after the Implementation

| Learning Strategy | Sample | The Average Scores ± SD | The Average Rank | Chi-square | p Value |
|---|---|---|---|---|---|
| Conventional Learning | 32 | 4.44±4.04 | 34.77 | 32.874 | 0.000 |
| *Jigsaw* CLS | 32 | 5.69±5.72 | 39.42 | | |
| Module *Jigsaw* CLS | 32 | 13.78±7.71 | 71.31 | | |
| Total | 96 | | | | |

The results of Kruskal Wallis analysis show that there was one different strategy from the other two strategies that had improvement behavior in average because Chi-squre from Kruskal Wallis analysis was 32.874 with p value = 0.000

From the further analysis using *Mann-Whitney* test that is presented in Table 3, it was found that conventional learning and *jigsaw* CLS gave insignificantly different behavior improvement toward DHF prevention with p value > 0.05. Conversely, module *jigsaw* CLS gave higher behavior improvement toward DHF prevention than conventional learning did ($p<0.05$). Besides, module *jigsaw* CLS gave higher behavior improvement toward DHF prevention than *jigsaw* CLS did ($p<0.05$).

**Table 3.** *Mann-Whitney* Analysis of the Disparity of Average Behavior Improvement Scores among the Learning Strategies

| Learning Strategy | Sample | The Average Scores ± SD | The Average Rank | Mann-Whitney | P value | The Improvement disparity |
|---|---|---|---|---|---|---|
| Conventional Learning | 32 | 4.44±4.04 | 31.09 | 467.000 | 0.542 | 28.2 % |
| *Jigsaw* CLS | 32 | 5.69±5.72 | 33.91 | | | |
| *Jigsaw* CLS | 32 | 5.69±5.72 | 22.02 | 176.500 | 0.000 | 142.2% |
| Module *Jigsaw* CLS | 32 | 13.78±7.71 | 42.98 | | | |
| Conventional Learning | 32 | 4.44±4.04 | 20.17 | 117.500 | 0.000 | 210.4 % |
| Module *Jigsaw* CLS | 32 | 13.78±7.71 | 44.83 | | | |

The implementation of module *jigsaw* CLS gave higher understanding improvement than *jigsaw* CLS did. This is because *jigsaw* CLS divides general learning information into smaller and simpler components. In this way, students can concentrate more on the learning process because of the focused teaching materials which are easier to understand. In addition, the students can discuss gradually and systematically on matters starting from group discussions, to discussions with experts and finally to class discussions. Therefore, the students (1) do not depend very much on the instructors. Instead, they can improve their own thinking ability, get information from many sources, and learn from the other students; (2) can improve their ability in expressing ideas and opinion verbally and compare theirs with the other students ; (3) can help the students to respect others, to realize that they have limitation and to accept differences.

In addition, module *jigsaw* CLS implemented the learning process exactly the same as *jigsaw* CLS did. The DHF module with *jigsaw* CLS consists of three modules: (1) module 1 is about Aedes Aegypty; (2) module 2 is about DHF about infection and prevention; and (3) module 3 is about dengue virus infection. Each module has 4 subtopics which are systematically, chronologically and logically composed. The language used is simple and understandable with attractive pictures. In this way, the students will be interested to learn so that the learning process can reach its maximum results. Each subtopic has its worksheets (lembar kegiatan pebelajar) containing topic discussion and questions for the students to answer. This will challenge the students and be more active in learning process. The aims of learning mentioned in the introduction of each module will direct the students to discuss the materials and answer the questions in the worksheets provided. The tests with their key answers and scoring methods to know students' mastery on the materials speed up evaluation process and the students are able to quickly know the results of learning. This is supported by Nasution (2009), who said that a well- arranged module could give many advantages, among other things are; (1) it can maximize the learning process, (2) students are more active in the learning process because they have problems and assignments to do, (3) it gives a quick feedback to students so they can know the results as soon as possible, (4) learning activities are directed because the module contains clear objectives, and (5) instructors' involvement in learning process is limited.

A team from WHO mentioned several strategies to improve behavior, they were as follows: (1) Using Force/Power. Community is forced to have behavior as expected. This is, for example, by making rules that must be obeyed by the people. This will create a quick change of behavior but it may not last long as the change does not or has not come from one's own awareness. (2) Giving Information. Giving information about the way to prevent DHF will increase community's knowledge. They will, then, have the awareness they are expected to have with their knowledge. This process of behavioral change will take longer time but such change will remain more permanently as it is based on the community's awareness (not on force). (3) Discussion and Participation. This is an improved strategy of the two strategies above. Information distribution about DHF prevention is not conducted in one way but in two ways..

Furthermore, Green (1980) stated that behavior is influenced by three factors, namely: (1) predisposing factors, which are in the form of knowledge, attitude, belief, faith, values, etc; (2) enabling factors, which are in the form of physical environment, availability or unavailability of health facilities like community health center, medicines, contraception, toilet, etc; (3) reinforcing factors, which are in form of paramedics and other officers' behavior, which are the role model group for community. On the other side, Sunaryo (2004) stated that motivation is a locomotive to reach certain objectives consciously or unconsciously. Motivation can emerge from an individual or from environment. The best motivation comes from one's own self (intrinsic motivation), not extrinsic motivation. Moreover, Sunaryo proposed several ways can be implemented to motivate someone, namely (1) motivating by force. It is motivating someone by law enforcement or force so that the motivated one can do what he/she has to do (2) motivating by enticement. It is motivating by persuading or giving present so that the motivated one can do as expected. (3) motivating by identification. It is motivating by raising awareness so that the motivated one is willingly do something from his/her self-awareness.

From the explanation posed above, it can be concluded that through the implementation of module *jigsaw* CLS, the improvement of the students' understanding can be perfectly and deeply gained so that they have their intrinsic motivation in order to do preventive actions toward DHF. As a result, their behavior can be a role model for others.

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# CLASSIC HODGKIN'S LYMPHOMA SIMULATING ANAPLASTIC LARGE CELL LYMPHOMA

**I Gusti Ayu Sri Mahendra Dewi**
*Anatomical Pathology Department of Medical Faculty*
*Udayana University/ Sanglah Hospital Denpasar*
*Correspondence author: mahendradewi@rocketmail.com*

**ABSTRACT**

Classic Hodgkin's lymphoma (HL) and Anaplastic Large Cell Lymphoma (ALCL) may share some morphologic features that account for 2.6% cases, so responsible for diagnostic difficulties in conventional histopathologic examination. This study report a case mixed cellularity HL in a 16-year old woman, with main complain was the present of masses at right and left neck. Additional complain were fever, night sweats, fatigue, loss of appetite and body weight, cough and dyspnea. Chest x-ray and thorax computed tomography (CT) scan showed a mass at mediastinum with right and left pleural effusion. Abdominal ultrasonography showed hepatosplenomegali and ascites. Clinically this patient diagnosed as non-Hodgkin's Lymphoma (NHL) stage II B. The conclusion from histopathological examination is malignant lymphoma, with differential diagnosis are mixed cellularity HL and ALCL. Immunohistochemical examination with CD20 staining showed negative result, so the possibility diagnosis is ALCL derived from T cells, with differential diagnosis is mixed cellularity classical HL. Further immunohistochemical examination with Hodgkin cell marker CD15 showed paranuclear positivity in most of large atypical cells and CD30 positive in few atypical cells. Tc cell marker (CD8) positive in part of small lymphocytes. Final diagnosis is mixed cellularity HL. Immunohistochemical examination is very important to differentiate HL from NHL for further treatment modality.

***Keywords:*** mixed cellularity, HL, ALCL

## INTRODUCTION

Malignant lymphoma is the term given to tumors of the lymphoid system, specifically of lymphocytes and their precursor cells (Rosai, 2004). The malignant lymphomas can be divided into two major categories, the Hodgkin's Lymphoma (HL) and non-Hodgkin's Lymphoma (NHL) (Rosai, 2004; Aster, 2005). Anaplastic large cell lymphoma (ALCL), a type of NHL may share some morphological features to classic HL, so to diagnosed these two different cases from morphologic feature only is often difficult (Vanhentenrijk et al., 2006; Willenbrock et al., 2006; Tamaru et al., 2007; Gualco et al., 2008). ALCL and classic HL are origin from different cell type. ALCL is tumor of peripheral (post-thymic) T-cell lymphoma and also known as Ki-1 lymphoma (Aster, 2005) but classic HL is of B-cell origin (Stein et al., 2000; Willenbrock et al., 2006). ALCL and HL have biologically distinct entities. In one study the difficulties to diagnose this cases account for 2.6% (Vassallo et al., 2006). The Revised European American Lymphoma classification uses the term Hodgkin's-like anaplastic large cell lymphoma (HD-like ALCL) for borderline cases with features of both ALCL and classical HL (Asano et al., 2007). Some literatures use the term ALCL-like Hodgkin's Lymphoma for HL that has similar morphologic features to ALCL (Vanhentenrijk et al., 2006). Clinically, accurate diagnosis between HL and NHL is important for modality of therapy (Aster, 2005).

This study report a case mixed cellularity HL that shares some morphologic features to ALCL.

## MATERIALS AND METHODS

The specimens were origin from excisional biopsy of right and left region of neck lymph nodes. Macroscopic examination was done. The specimen from lymph nodes of right neck was 2.0 x1.0 cm in size and left neck was 5.0 x 3.0 cm in size, round to ovoid shape, on cut section showed grey-white surface and firm consistency. Microscopic slides were made and stained by conventional staining Hematoxyllin-Eosin (HE), confirmed by immunohistochemistry CD 20, CD 15, CD 30 and CD 8 staining, by strepavidin biotin method, using monoclonal antibodies specific. The diagnosis was made according to histopathologic examination stained by Hematoxyllin-Eosin, supported by clinical, macroscopic examination and immunohistochemistry staining.

## RESULTS AND DISCUSSION

Patient was female, 16 years old, with main complain was the present of masses at her right and left neck. She also felt fever, night sweats, fatigue, and loss of appetite and body weight, cough and dyspnea since a month ago. On physical examination the tumor located at right and left neck, 2.0 x1.0 cm and 5.0 x 3.0 cm in size, respectively, fixed and painless. Chest x-ray showed the present of tumor at mediastinum with right and left pleural effusion. Further thorax computed tomography (CT) scan revealed a mass at anterior mediastinum, 11x14x15 mm in size, discrete and regular border, adhere to aorta, with right and left pleural effusion. Abdominal ultrasonography showed hepatosplenomegali and ascites. Clinical diagnosis is NHL stage II B.

Excision of lymph nodes was done and submit for conventional histopathology examination and immunohistochemistry staining. Macroscopically, the specimens consist of two lymph nodes, the lymph nodes from right neck was 2.0 x1.0 cm in size and left neck was 5.0 x 3.0 cm in size, round to oval shape, on cut section showed grey-white surface, and firm consistency. Microscopically, the lymph nodes composed by large highly atypical cells showing marked pleomorphism, irregular nuclear membrane, one to multiple nuclei, some multilobated, 1-3 prominent nucleoli, the cytoplasm is abundant and eosinophilic, simulate Hodgkin's cells, another infiltrate cells are lymphocytes, neutrophils, histiocytes, and eosinophils. This patient diagnosed as malignant lymphoma (Figure 1). Pleural effusion was examined three times in different time. Seeding of malignant lymphoma cells were found in pleural effusion fluid.

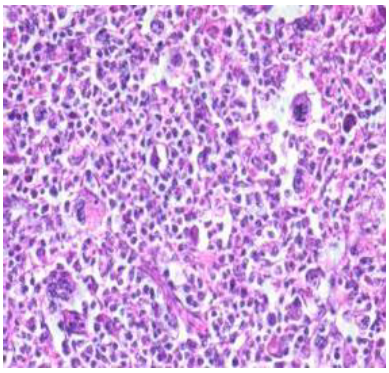

Figure 1
Mixed cellularity HL. Examination by conventional histopathogy, morphologically is difficult to differentiate with ALCL (HE, 400x).

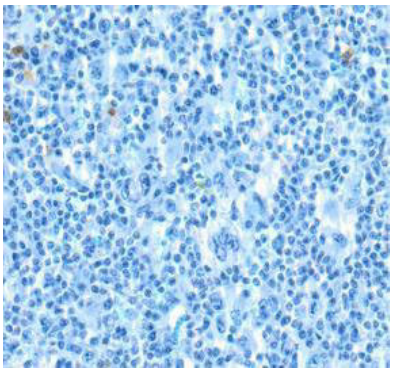

Figure 2
Mixed cellularity HL. CD20 immunostain showed negative result (CD20, 400x).

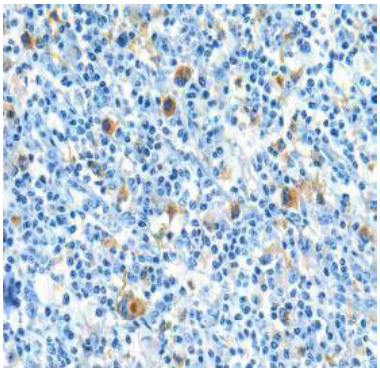

Figure 3
Mixed cellularity HL. CD15 immunostain showed paranuclear positive result in most of large atypical cells (CD15, 400x).

Immunohistochemical examination with CD20 showed negative result (Figure 2), so the possibility diagnosis is ALCL derived from T cells, with differential diagnosis is mixed cellularity classical HL. Further immunohistochemical examination with Hodgkin cell marker CD15 showed paranuclear positivity in most of large atypical cells (Figure 3) and CD30 positive in few atypical cells (Figure 4). Tc cell marker (CD8) positive in part of small lymphocytes (Figure 5). The conclusion of immunohistochemistry examination was mixed cellularity HL.

Generally HL affect male more often than female about 1.5: 1 except nodular sclerosis type (Rosai, 2004; Aster, 2005). There is biphasic incidence, with peak incidence in young adulthood and adult more than 55 years old (Aster, 2005). This case occur in young female.

This disease may present in a variety of ways, about 90% of HL cases being painless enlargement of superficial cervical lymph nodes. HL begin in a single lymph node or chain of nodes and spreads first to anatomically contiguous lymphoid tissues and result in a fusion of the involved nodes. The lymph nodes

involved by HL usually enlarged, sometimes massive, the consistency varies from soft to hard depending on the amount of fibrosis. On cut section usually homogen but areas of necrosis may be present (Rosai, 2004). Direct invasion of skin, skeletal muscle and other sites, spleen, liver and bone marrow, can occur (Rosai, 2004; Aster, 2005). Spread to mediastinum lymph nodes are particularly common in the nodular sclerosis, but also can occur in mixed cellularity and lymphocyte depletion (Rosai, 2004). This case present as enlargement of lymph node that begin at neck. The involvement of mediastinum lymph node, evaluated by chest x-ray and CT scan also present. No metastasis in spleen, liver and bone marrow, but present of pleural effusion seeding.

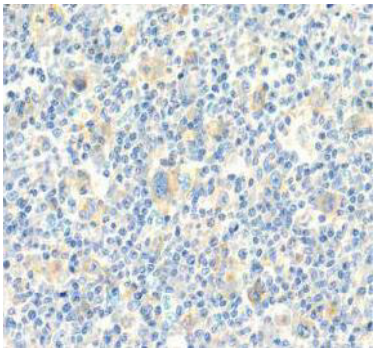

Figure 4
Mixed cellularity HL. CD30 immunostain showed paranuclear positive result in few of large atypical cells (CD30, 400x).

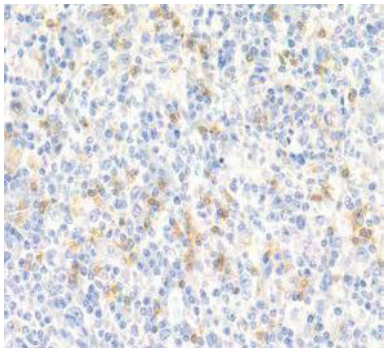

Figure 5
Mixed cellularity HL. CD8 immunostain showed positive result in part of small lymphocyte cells (CD8, 400x).

The lymph nodes enlargement accompany by fever, night sweats and loss of weight, so-called B symptoms, occur in approximately 25% of HL cases. The presence of B symptoms influences the clinical staging. Patients with spread disease (stage III and IV) or mixed cellularity and lymphocyte depletion are more common have systemic symptoms. Abdominal involvement more common occur in patient with B symptoms and mixed cellularity or lymphocyte depletion type (Rosai, 2004). Staging was made according to Ann Arbor classification for clinical staging for Hodgkin's disease (Rosai, 2004; Aster, 2005). Clinical final diagnosis of this case was HL stage II B cause by the involvement of two lymph node regions of the same side of the diaphragm, i.e. the neck and mediastinum lymph nodes accompany by constitutional symptoms, i.e. fever, night sweats and loss of weight.

Microscopically, HL characteristic by the present of Reed-Sternberg cell, the neoplastic cell origin from B lymphocyte, with characteristic background of non-neoplastic reactive inflammatory cell of various types. Mixed cellularity HL comprises 20-30% of all cases of HL (Rosai, 2004; Aster, 2005). A large number of eosinophils, plasma cells, granulocytes and histiocytes and atypical mononuclear cell, admixed with numerous classic Reed-Sternberg cells. Focal necrosis may be present, but fibrosis should be minimal or absent (Rosai, 2004; Aster, 2005; Vassallo et al., 2006). Microscopically, ALCL has a polymorphic infiltrate, with a variable admixture of neutrophils, lymphocytes, histiocytes and highly atypical and pleomorphic cells, the nuclei are often horseshoe shaped and multiple, nucleoli are prominent. Cells indistinguishable from Reed-Sternberg cells may be present, the cytoplasm is abundant and eosinophilic (Rosai, 2004). This case composed of cells morphologically is difficult to differentiate between ALCL and mixed cellularity HL, so immunostaining is very important to make an accurate diagnosis.

In the vast majority of cases, the neoplastic Reed-Sternberg cells are derived from germinal center or post-germinal center B cells, but in rare cases derived from T cells transformation. The tumor cells express CD15 and CD30. Most of small lymphocytes at the background are T cells. CD20 are positive for marrow pre-B cells and mature B cells. CD15 are positive for granulocytes and Reed-Sternberg cells and its variant in classic HL. CD30 are positive for activated B cells, T cells and monocytes, and also expressed by Reed-Sternberg cells and its variant in classic HL (Aster, 2005). ALCL are positive for CD30

(Ki-1) immunostaining (Rosai, 2004; Vassallo et al., 2006; Hirsch et al., 2008) but negative for CD15 and CD20 (Vassallo et al., 2006; Ziarkiewicz et al., 2006). CD 30 stimulation of ALCL cells was found in one study, but not responsive for Hodgkin cells (Hirsch et al., 2008). If difficult to distinguish ALCL and HL from morphologic features by H & E stain, it is advisable to include EMA and CD15 staining in the first line panel. If EMA-positive and CD15-negative, continue to ask for anaplastic lymphoma kinase (ALK) staining. If ALK-positive, the diagnosis is ALCL (Vassallo et al., 2006). ALK positivity was detected in 90.7% cases (d'Amore et al., 2007) CD30 and CD15 are expressed in Reed-Sternberg cells in 80% of the cases (Rosai, 2004).

The etiology of HL remains unknown, but Epstein-Barr virus (EBV) suggested plays an important role. EBV genomes have been identified in Reed-Sternberg cells in up to half of the cases, especially in the mixed cellularity subtype, young patients, and/or in developing countries (Rosai, 2004). Genetic predisposition and immunologic deficiency are also evidence. In mixed cellularity HL, EBV genomes have been identified in Reed-Sternberg cells in 70% cases, whereas EBV genomes not identified in most of ALCL cases (Aster, 2005).

Treatments of HL are radiation and chemotherapy, the choice being largely depended on the stage of the disease. Successful therapy results in disappearance of the tumor cells (Rosai, 2004).

The present of a large number of lymphocytes was associated with a good prognosis. Reed-Sternberg cells, eosinophils and plasma cells had no prognostic value. Traditionally, lymphocyte predominant and nodular sclerosis type had the best prognosis, whereas mixed cellularity had intermediate prognosis and lymphocyte depletion has the worst, but it also associated with clinical stage (Rosai, 2004).

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# EXTENDING TIME OF KOMBUCHA TEA FERMENTATION IMPROVES THE GROWTH OF INHIBITION CAPACITY OF *Escherichia coli*; THE PRODUCER OF EXTENDED SPECTRUM BETA LACTAMASES (ESBL) BY *IN VITRO* METHOD

**Ni Putu Aryadnyani[1*], I Dewa Made Sukrama[2], I Wayan Putu Sutirta Yasa[2]**
*[1] Department of Analyst of Health, Stikes Wira Medika Bali, Denpasar Bali*
*[2] Faculty of Medicine, Udayana University, Denpasar Bali*
**Corresponding author: Aryadnyani85@gmail.com*

**ABSTRACT**

One of the reasons that bacteria turn out to be resistant is that the bacteria are able to produce Extended Spectrum Beta lactamases (ESBL) that can be bewildering in selecting antibiotic for treatment, hence conventional way of medications is recommended as alternative, e.g, substances function as antimicrobe. This research aims to investigate whether fermented *kombucha tea* can inhibit the growth of *Escherichia coli*, that produces Extended Spectrum Beta Lactamases (ESBL), by *in vitro* method, as well as to investigate whether the length of fermentation affect the inhibition capacity. This research used five groups of experiments, i.e; control group, 6 days fermentation, 10 days fermentation, 14 days fermentation and 18 days fermentation, each treatment was repeated six times. The inhibition zone was measured in millimeter by using Vernier callipers. The data were analyzed by *One Way Anova* method. It showed the value of $p<0.001$, which indicated that the inhibition capasity of *kombucha tea* against *Escherichia coli*, that produced ESBL, was distinctly significant for each group. To test the different groups, further test was undertaken with Least Significant Difference–test (LSD). The results of the LSD-test indicated that there are significantly differences between *kombucha tea* fermented for six days compared with *kombucha tea* with length of fermentation of 10 days, 14 days and 18 days ($p<0.05$).

***Keywords:*** *Escherichia coli, ESBL, kombucha tea*

## INTRODUCTION

Digestive system is vulnerable to infection by microorganism. Common infection of digestive system is caused by class of Enterobacter, especially by *Escherichia coli.*

Primary treatment for infection is antibiotic, nevertheless a large number of bacteria become resistance toward antibiotic. One of the reason is that the bacteria able to produce *Extended Spectrum Beta Lactamases* (ESBL) (Al-Zahrani and Akhtar, 2005), which is classified as enzyme with potential to breakdown oxyimino B-lactams therefore it is resistant against antibiotics belong to beta lactam group (Rupp and Fey, 2003). *Escherichia coli* is a bacterium that has potential to produce ESBL, consequently, which narrowing the selection of antibiotics for treatment (Serefhanoglu et al., 2009).

Traditional medication can be used as alternative treatment for infection. Nowadays, tea has been known as a fermented product by symbiosis of fungi and bacteria. One of the popular one is kombucha tea (Naland, 2008). Other names of kombucha are manchurian tea mushroom, hung ca ku, cajnyj kvas, heldenpilz, mandarin tea mushroom, fungus japonicum, tea kwass, olinka, mogu, kargasok tea, zauberpilze, olga spring, jamur super, jamur dipo, teh kombu, tea of immortality (Naland, 2008), Medusomyces gisevii (Jayabalan et al., 2010), fungo-japan, pitchia fermentants, cembuya orientalis, tschambucco, volga spring, champinon de longue vie, kwassan, champagne of life (Cavusoglu dan Guler, 2010).

Kombucha tea contains abundance of substances that is good for human body, mainly: vitamin B complex, vitamin C, folat acid, glucoronat acid, acetic acid, hyaluronic acid, chondroitin sulphuric acid, lactic acid, polyphenol and usnic acid (Hidayat et al., 2006; Naland, 2008). It is intended to investigate whether the tea is able to inhibit the growth of *Escherichia coli*, that produce extended spectrum beta lactamases (ESBL), by in vitro method, and whether the length of fermentation will increase the inhibition capacity.

## MATERIALS AND METHODS

### Preparation of Kombucha Tea

To make 1000 ml of tea, it was firstly refrigerated, and put into glass. Then, 100 ml of kombucha tea starter was added. The starter was originated from previous fermented tea. It was covered by clean linen for 6 days, 10 days, 14 days, and 18 days respectively. The resulting tea were filtered, and 25 ml each were put into the clean and sterilized bottle (Hidayat et al., 2006; Naland, 2008).

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### Identification of Bacteria *Escherichia coli* ESBL Producer

Bacteria isolate is inoculated on selective media (MCA and EMB), it was then biochemically tested using Indol, methyl red, voges proskouwer, cimon citrate, urea, TSI, its motility was also tested and confirmation test of ESBL producer was carried out using double disk synergy test.

### Inhibition Capacity Test of Kombucha Tea against *Escherichia coli* ESBL Producer

The isolate of *Escherichia coli,* the ESBL producer, was inoculated on MHA media, paper disk which contain kombucha tea was laid on the media, it was then incubation for 16-18 hours at 37°C. Inhibition zones (in mm) were measured using Vernier Calliper (Davis and Stout, 1971).

**Figure 1.** The Process of Preparing Kombucha Tea

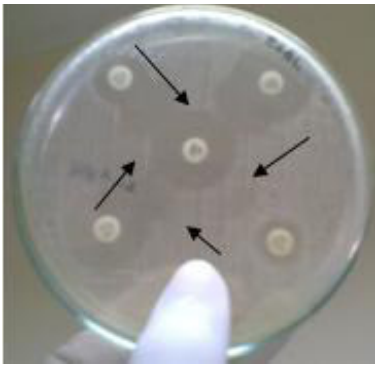

**Figure 2.** The Result Test of Double Disk Synergy Test

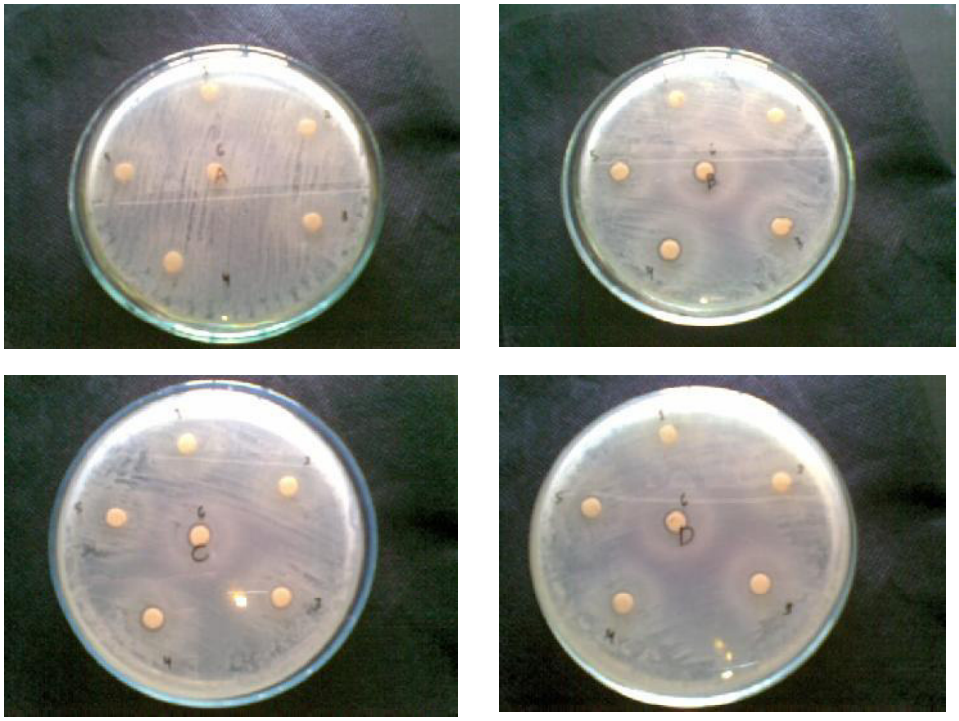

**Figure 3.** Zone of Inhibition of Kombucha Tea Against *Escherichia coli* (ESBL producer) Between the Group After Giving Treatment

## RESULTS AND DISCUSSION

There is inhibition zones of kombucha tea against the growth of *Escherichia coli* (ESBL producer). The average deviation between the groups of treatment are shown in the table below:

Table 1. Diameter Average Zone of Inhibition of Kombucha Tea against *Escherichia coli* (ESBL producer) Between the Groups after Treatment

| Group | Different rate | P |
|---|---|---|
| Control and 6 days fermented | 1.917 | 0.001* |
| Control and 10 days fermented Control and 14 days fermented | 3.350 | 0.001* |
| Control and 18 days fermented | 3.300 | 0.001* |
| 6 days and 10 days fermented | 3.333 | 0.001* |
| 6 days and 14 days fermented | 1.433 | 0.001* |
| 6 days and 18 days fermented | 1.383 | 0.001* |
| 10 days and 14 days fermented | 1.417 | 0.001* |
| 10 days and 18 days fermented | 0.050 | 0.805 |
| 14 days and 18 days fermented | 0.017 | 0.934 |
| | 0.033 | 0.869 |

* significantly different

Kombucha tea which is produced by Saccaromyces sp and Acetobacter sp is a healthy drink which contain antimicrobial capacity because it contain organic acids mainly: glucoronic acid, lactic acid, acetic acid, succinic acid, gluconic acid as a fermented product, which is effective to destroy microorganism such as virus and fungi (Frank, 1995).

The result showed that, 6 days fermented tea produced average zone inhibition of 6.47 mm, 10 days fermentation produced average inhibition zone of 7.90 mm, 14 days fermentation produced average inhibition zone of 7.85 mm, and 18 days fermentation zones produced average zone of 7.88 mm. The result indicated that there is a distinct difference between inhibition capasity of 6 days fermented tea and 10 days fermented tea.

It was discovered that the kombucha tea fermented for 10 days, the sugar had been used up by the bacteria and yeast, consequently, the volume of organic acid will be maximally produced in 10 days and its concentration will remain constant eventhough the fermentation is continued until 14 and 18 days. The result shows that the optimal length of fermentation of kombucha tea is 10 days.

There is no meaningful difference of inhibition among the kombucha tea fermented for 10 days, 14 days, and 18 days. This may be caused by the sugar content of tea has been used up by the bacteria and yeast in kombucha tea after 10 days fermentation, therefore the levels of organic acids in fermentation produced was maximum at 10 days and then it will be constant eventhough the fermentation is continued for up to 14 days and 18 days. Those results showed that the length of fermentation of kombucha tea is optimal at 10 days.

## CONCLUSION

It was concluded that kombucha tea fermented for 6 days, 10 days, 14 days, and 18 days have inhibition capacity against *Escherichia coli* (ESBL producer). The increasing of inhibition occurred within 6 days to 10 days, 14 days and 18 days.

## ACKNOWLEDGEMENT

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# ROLE OF P53 IN CELLULAR AGING

**Ni Putu Sriwidyani**
*Pathology Department, Faculty of Medicine, Udayana University*

**ABSTRACT**

p53 is called a "guardian of the genome". p53 senses DNA damage, and assist in DNA repair by causing cell cycle arrest and inducing DNA repair genes. A cell with damaged DNA that cannot be repaired is directed to undergo apoptosis. Senescence is thought a permanent of cell cycle arrest. Mutation of TP53 gene will lead the cell to malignant transformation.

***Keywords***: p53, aging, apoptosis, senescence.

## INTRODUCTION

Aging is thought to be the result of genetically programmed and environmentally inflicted accumulation of damage to tissues and cells. Mechanisms of decrease of active cells are apoptosis and cellular senescence (Finger, 2008). p53 is called a "guardian of the genome" (Zilfou and Lowe 2009). p53 is a major regulator of apoptosis and senescence. Mutation of this gene is also has a major contribution to carcinogenesis of many cancer.

## DISCUSSION

### p53, Cell Cycle, and Apoptosis

Proliferating cells progress through a series of checkpoints and defined phases is called the cell cycle. The cell cycle consists of presynthetic growth phase 1 (G1 phase), a DNA synthetic phase (S phase), premitotic growth phase (G2 phase), and mitotic phase (M phase). Quiescent cells are in physiologic state called G0 (Kumar *et al.*, 2010) (Figure 1).

Entry and progression of cells through the cell cycle are controlled by changes in the levels and activities of family protein called cyclins. Cyclins accomplish their regulatory functions by complexion with constitutively synthesized proteins called cyclin-dependent kinases (CDK). After cell division, cyclins are degraded, until there is a new growth stimulus and synthesis of new cyclins, the cells do not undergo further mitosis. Cyclins activation is also regulated indirectly by inhibitors of CDK (CDKI) (Kumar *et al.* 2010).

Other protein that has important role in cell regulation that works in collaboration with cyclins and their kinases is p53. Normal p53 in non stressed cells has a short half life. This short half life is due to an association with MDM2, a protein that targets it for destruction. When the cell is stressed with assault on its DNA, p53 undergoes post transcriptional modifications that release it from MDM2 and increase its half life. p53 becomes activated as a transcription factor. Genes whose transcription is triggered by p53 can be those that cause cell cycle arrest and those that cause apoptosis. (Kumar *et al.* 2010)

p53 mediated cell cycle arrest occurs late in the G1 phase and is caused mainly by p53 dependent transcription of CDKI-CDKN1A (p21). CDKN1A inhibits cyclin/CDK complexes and prevents phosphorylation of Retinoblastoma (RB), a DNA-binding protein. RB protein is expressed in every cell type, where it exists in active hypophosphorylated and an inactive hyperphosphorylated state. In its active state, RB serves as brake from G1 to S phase of the cell cycle. In hyperphosphorylated RB, the break is released, and cells enter S phase and continue to divide. A pause in cell cycling gives the cells time to repair DNA damage. p53 also helps the process by inducing GADD45 (growth arrest and DNA damage) protein that help in DNA repair. If DNA damage is repaired successfully, p53 up-regulates transcription of MDM2, which then down regulates p53, relieving cell cycle block. If during the pause DNA damage cannot be successfully repaired, normal p53 directs the cell die by triggering apoptosis. p53 induces apoptosis-inducing genes transcription such as BAX (Kumar *et al.*, 2010) (Figure 2).

The P53 tumor suppressor gene is one of the most commonly mutated genes in human cancer. If there is a DNA damage in p53 mutant cell, cell repair will not occurred. Clone expansion and additional mutations will leading to malignant transformation in p53 mutant cell (Rodier *et al.* 2007).

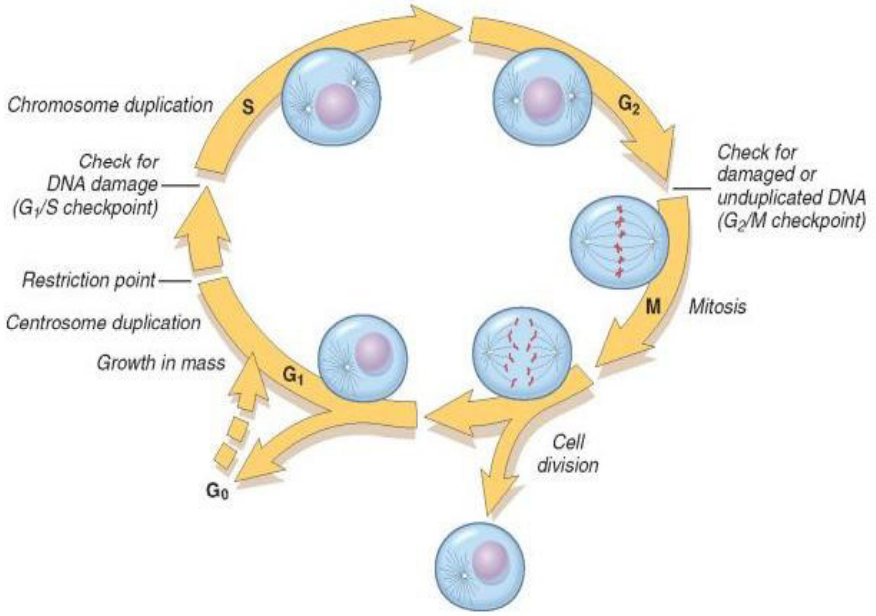


Figure 1. Cell cycle and its checkpoints (Kumar *et al.* 2010)

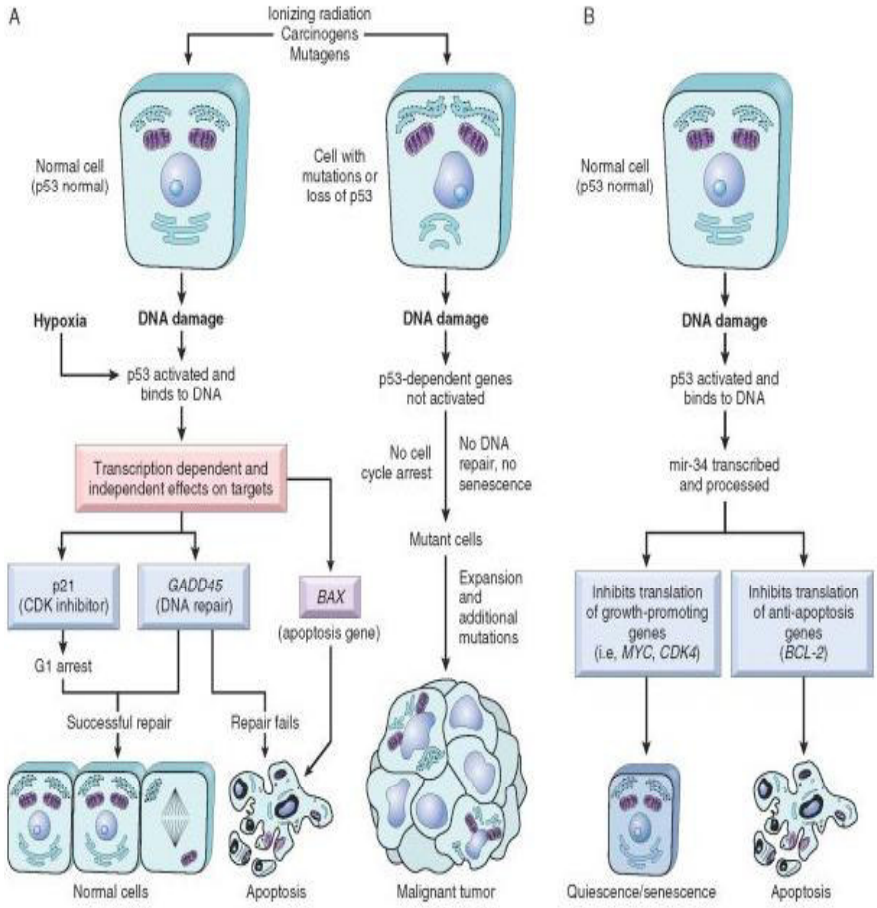


Figure 2. The role of p53 in maintaining the integrity of the genome (Kumar *et al.* 2010).

**p53 and Cellular Senescence**

Cellular senescence is the irreversible exit of the cell cycle and can be caused by various stimuli. Senescence occurring at the end of the proliferative life span of normal cells is a response to telomere shortening (Rodier and Campisi 2011).

Telomeres are regions of repeat DNA that cap the terminal ends of chromosomes, preventing inter-chromosomal fusion and ligation. In many organisms, including humans, this repeat sequence is 5'-TAGG-3'. (Milewski, 2010) Telomeres shorten with each cell division in the majority of somatic cells. When telomeres reach a critically short length, cell senescence program induced.

There is strong evidence that telomeres primarily mediate cellular senescence via the action of p53. The C terminus of p53 is known to recognize and interact with damage to telomeric DNA. Telomeric damage is likely to accumulate over time. Thus p53, being sensitive to telomeric damage, may activated when the level of this damage reaches a critical point (Kumar *et al.* 2010).

Once activated, p53 undergoes several modifications, enabling it to accumulate within the nucleus. One of the key post translational modifications is phosphorylation by ataxia-teleangiectasia protein, which serves to protect p53 from HDM-2 mediated degradation. Being a transcription factor, p53 then binds to specific target genes within nucleus. One of the key target genes that activates is p21, a gene whose protein orchestrates cell cycle arrest (Kumar *et al.* 2010).

Ordinary, withdrawal from the cell cycle is temporary and cells continue mitosis upon repair of the damaged DNA. Telomeric-induced p53, however, causes cells to produce proteins that repress the genes required for re-entry into the cell cycle. These cells are now viewed as senescent and their continued expression of genes inhibiting cell cycle re-entry. It means they are in a permanent growth arrest states. These cells are recognizable in vitro and in vivo as they express biomarkers such as β galactosidase. They also undergo other complex changes in gene expression pattern and begin to secret various factors, including inflammatory cytokines, matrix metalloproteinases and growth factors (Kumar *et al.* 2010).

Senescence cells contribute to aging in a many ways. The level of telomerase activity in stem cells is limited. This means that over time, stem cells activate cellular senescence and stem cell pools get irreversibly depleted. This lead to a progressive loss of tissue regeneration capacity which compromises organ structure and function. The low level of telomerase activity and depletion of stem cell polls responsible for tissue atrophy that occurs during aging process (Spike and Wahl 2010).

## CONCLUSION

There are two mechanisms of decline of active cells in aging, apoptosis and cellular senescence. P53 is a central regulator in both mechanisms. Cells with DNA damaged will undergo cell cycle arrest to permit cells to repair. If repair is defective, the cells will lead to apoptosis. In cellular senescence, p53 recognizes and interacts with damage to telomeric DNA. Telomeric-induced p53 causes cells to produce proteins that repress the genes required for re-entry into the cell cycle and the cells become senescent.

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# ANTIBACTERIAL TERPENOID COMPOUNDS FROM RHIZOME OF *Curcuma zedoaria* (Berg). Roscoe

**I Made Sukadana[1*] and Sri Rahayu Santi[2]**
[1]*Posgraduate School of Biomedicine Udayana University*
*Researcher in Chemistry Department Faculty of Mathematic and Natural Sciences Udayana University.*
[2]*Researcher in Chemistry Department Faculty of Mathematic and Natural Sciences Udayana University.*
**Correspondence Author: imsukadana@yahoo.com*

**ABSTRACT**

This paper describes the identification of antibacterial terpenoid compounds from rhizome of *Curcuma zedoaria* (Berg) Roscoe. An amount of 23.45 g of concentrated extract obtained from 600 g dry powder of rhizome was macerated in ethanol. This extract was dissolved in a mixture of ethanol-water (7:3) and then was partitioned with n-hexane and chloroform. It was evident that only chloroform extract contained terpenoid and active against *Staphylococcus aureus*. Separation of crude terpenoid using column chromatography resulted in four fractions ($F_A$ – $F_D$) and the fraction of $F_A$ was observed to contain terpenoid and active against *Staphylococcus aureus* and *Eschericia coli*. Further identification of $F_A$ fraction using Gas Chromatograph Mass Spectometry (GC-MS) showed that the fraction were a combination of at least five compounds, namely zingberene, β-sesquiphellandrene, tumerone, α-tumerone, and α-limonene.

***Keywords****: Curcuma zedoaria* (Berg) Roscoe*., identification, antibacterial, Staphylococcus aureus* and *Eschericia coli*

## INTRODUCTION

One of the phytopharmacologic approaches relied on etnobotany, screening bioactive compound of plant is based on its use as traditional medicine by certain society (Farnsworth, 1996). Traditional medicine represents one of Indonesia's cultural assets and has been empirically proven from generation to generation (Kardinan and Taryono, 2003).

A plant which can be exploited as traditional medicine is *Curcuma zedoaria* (Berg) Roscoe. The rhizome of this plant is applicable to ulcers, constipation, sore throat, inflammation of the liver, thyroid, menstrual pain, vaginal discharge, acne, boils, stimulant medication, worming, diarrhea, antiviral, cough, chest pain, indigestion, launched bleeding blood, cervical cancer, ovarian cancer, cancer lung, liver cancer, breast cancer, leukemia, and lung disorders such as asthma, tuberculosis, and bronchitis (Heyne, 1987; Hembing, 2005; Satya, 2007). This is possibly because of the content of chemical compounds of the rhizome, especially those belong to secondary metabolic compounds such as monoterpene (antineoplastic), sesquiterpene with major component in essential oils. Moreover, kurzerenone, curcumin (as antiinflamation and antioxidant), epikurminole (antitumor), kurkuminole (hepatoprotector), zingeberene, saponin, flavonoid, and polyphenol (Dalimartha, 2003; Novalina, 2003; Hembing, 2005; Syukur, 2004) have also been identified in the rhizome of the plant. Nevertheless there is no research explaining the correlation between the compounds in the rhizome with the traditional healing, although many people have proven the effect and benefit of rhizome of *Curcuma zedoaria* (Berg) Roscoe.

The result of phytochemical screening to ethanol extract of the rhizomes shows that it contains terpenoids (major content), flavonoid, and polyphenol compounds. According to chemotaxonomy approach, plants from the same genus or family contain substances with similar chemical structures and bio-activities. It is known that rhizome of *Curcuma longa L*, posses antibacterial and antifungal activities (Shikha *et al*, 2010; Santosh et al., 2007), hence *Curcuma zedoaria* (Berg) Roscoe is expected to contain a compound which has the same activity, since both plants fall in the same genus. Based on the traditional medicine practices and chemotaxonomy approach *Curcuma zedoaria* (Berg) Roscoe can suppress bacteria. Moreover, results of phytochemical screening tests indicate that the rhizome of *Curcuma zedoaria* (Berg) Roscoe mainly contain terpenoid compounds. Therefore it is crucial to investigate the chemical contents of the rhizome *Curcuma zedoaria* (Berg) Roscoe and their bio-activity as an antibacterial agent. In this paper the isolation process of terpenoid compounds from the rhizomes and the antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* are described.

## MATERIALS AND METHODS

### Materials

Materials used in this research are: rhizome of *Curcuma zedoaria* (Berg) Roscoe, obtained from Badung market Bali and taxonomycally identified by LIPI's Kebun Raya "Eka Karya" Bali. Chemicals used are ethanol, n-hexane, chloroform (p.a and technical), concentrated sulphuric acid, anhydrous acetic acid, ethylacetate, p.a, potassium bromide, aquadest, silica gel 60, and silica gel $GF_{254}$.

Equipments used include a set of glass wares, analytical balance, blender, knife, rotary vacuum evaporator, UVs lamp (254 and 366 nm), thin layer and column chromatographs, desiccators, test tubes, testing dishes, Pasteur pipettes, volumetric pipettes, GC-MS.

### Methods

Six hundred grams of dried rhizome powder of *Curcuma zedoaria* (Berg) Roscoe was macerated using 8 L ethanol (EtOH). Maceration process was conducted 4 times at 24 hours each. The ethanol extract was evaporated using rotary vacuum evaporator to obtain a concentrated EtOH extract. The EtOH extract was dissolved in a mixture of EtOH-$H_2O$ (7:3). The non-polar components was fractionated using n-hexane. Furthermore EtOH-$H_2O$ extract is evaporated and fractionated using 400 mL chloroform. Each fractions was then evaporated to obtain concentrated water extract and chloroform extract. Each concentrated extract was tested for terpenoid content with the LB test. Both extracts contained terpenoid compound. The result of antibacterial test toward *Staphylococcus aureus* and *Escherichia coli,* showed only chloroform extract was active as antibacterial at 1000 ppm. Furthermore the concentrated chloroform extract was fractionated by column chromatograph using 100 g of silica gel 60 and a n-hexane-ethylacetate (1:1) mixture as the mobile phase. Each fraction was tested by the LB test and those containing terpenoids were pooled and purified to obtain a relative pure isolate. It was once again tested against antibacterial test toward *Staphylococcus aureus* and *Escherichia coli*. It was then analysed using GC-MS to determine the components.

## RESULTS AND DISCUSSION

The result of maceration about 600 g rhizome dried powder of *Curcuma zedoaria* (Berg) Roscoe using 8 L ethanol was about 23.45 g brown concentrated ethanol extract containing terpenoid (result of LB test). After fractionation 9.79 g of concentrated n-hexane extract, 21.97 g of concentrated chloroform, and 1.48 g water extract were resulted.

The LB tests showed that only two extracts contained terpenoid, and none was the water extract. The antibacterial test toward *Staphylococcus aureus* and *Escherichia coli* showed that only chloroform extract was active as antibacterial toward *Staphylococcus aureus* with inhibition zone of 2 mm at 1000 ppm (Figure 1).

Separation of 1.02 g of the chloroform extract using column chromatograph yielded 4 fractions (Faction $F_A$-$F_D$). The LB test into these fourth fractions showed that all of fractions contained terpenoid, but only fraction $F_A$ it was a major componentr. Purity test carried out by TLC at various mobile phases of fraction $F_A$ shown pure relatively. Furthermore, the result of antibacterial test shown that fraction $F_A$ could inhibit *Staphylococcus aureus* within zone 1 mm and 4 mm at concentration 500 and 1000 ppm respectively also zone 0.5 mm and 2 mm toward *Escherichia coli* at same concentrations. (Figure 2).

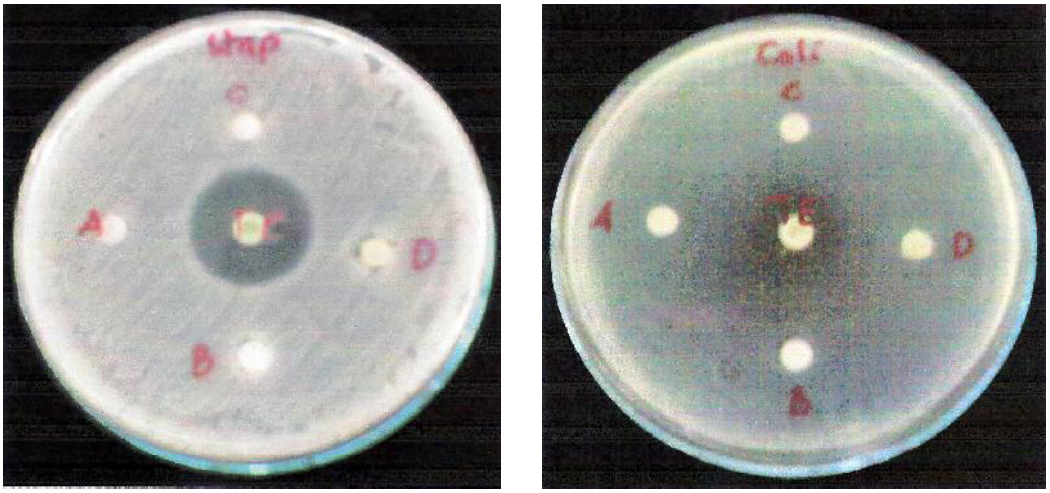


Figure 1. Antibacterial test of n-hexane and chloroform extract toward *Staphylococcus aureus* (I) and *Escherichia coli* (II). A. solvent n-hexane; B. solvent chloroform; C. 1000 ppm n-hexane extract; D. 1000 ppm chloroform extract; and TE. 30 µg tetracycline

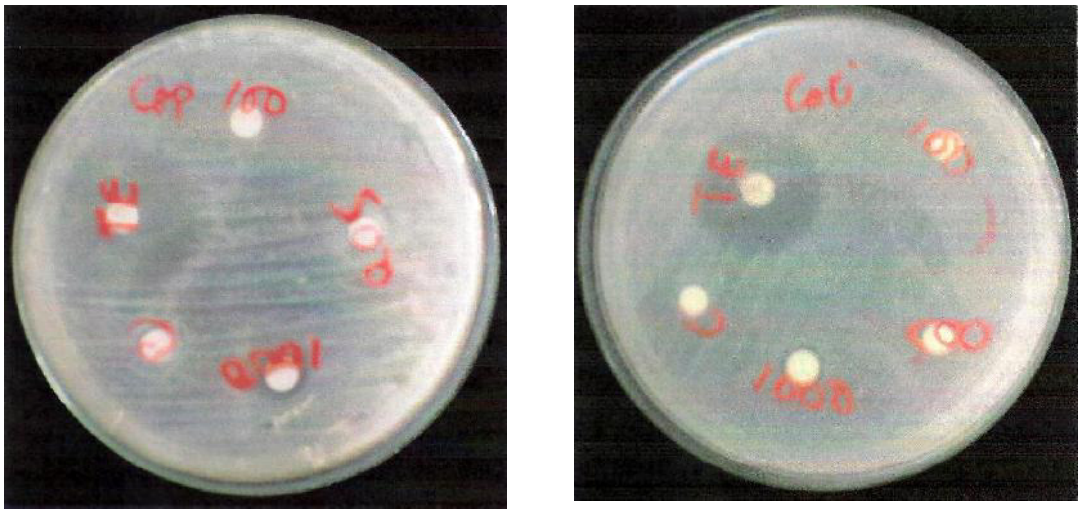

Figure 2. Antibacterial test of fraction $F_A$ toward *Staphylococcus aureus* (I) and *Escherichia coli* (II). at concentration 100, 500, 1000 ppm, C. solvent ethanol, and TE. 30 µg tetracycline

The result of analysis using the Gas Chromatograph Mass Spectometry (GC-MS) showed that the isolate (Fraction $F_A$) were combination of least five compounds namely zingberene, β-sesquiphellandrene, artumerone, α-tumerone, and α-limonene.

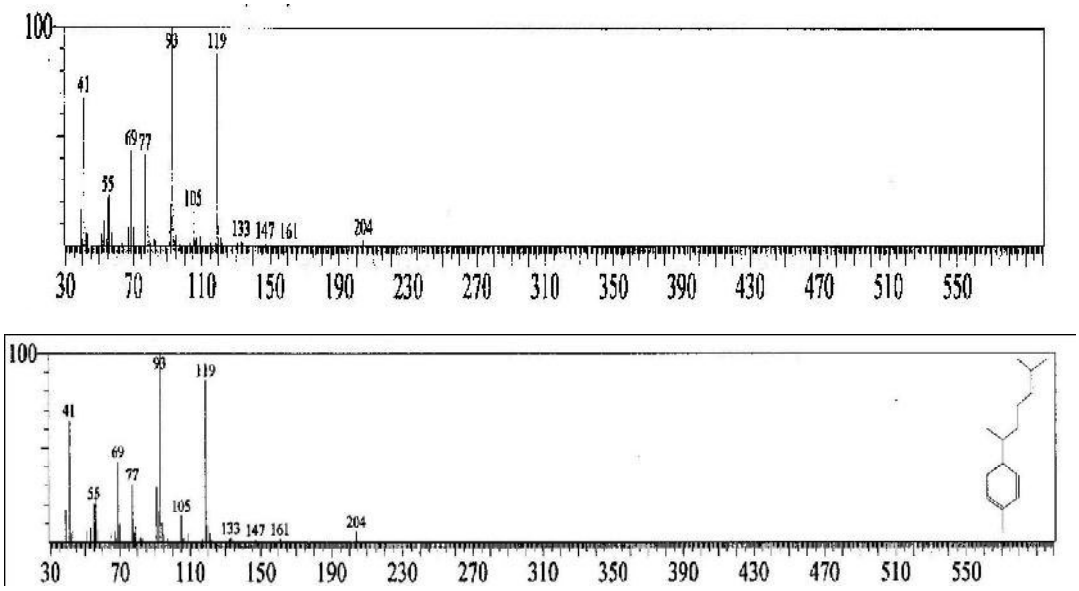


Figure 3. Mass spectrum and structure of zingeberene

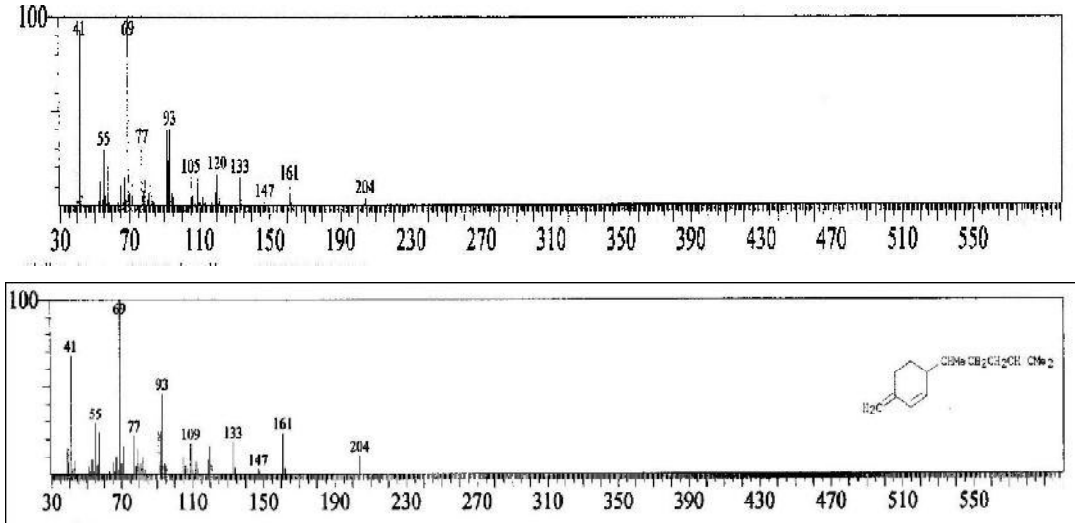


Figure 4. Mass spectrum and structure of β-sesquiphellandrene

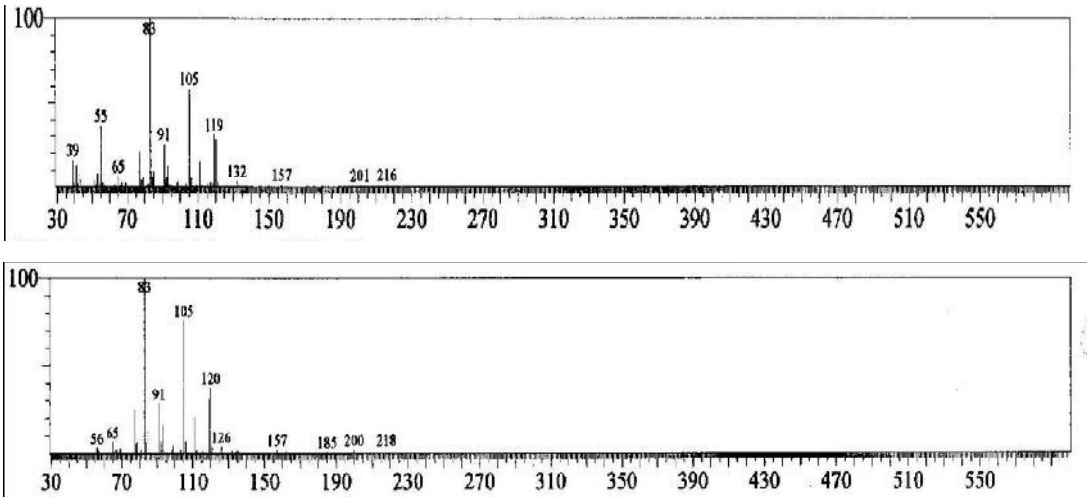


Figure 5. Mass spectrum and structure of artumerone

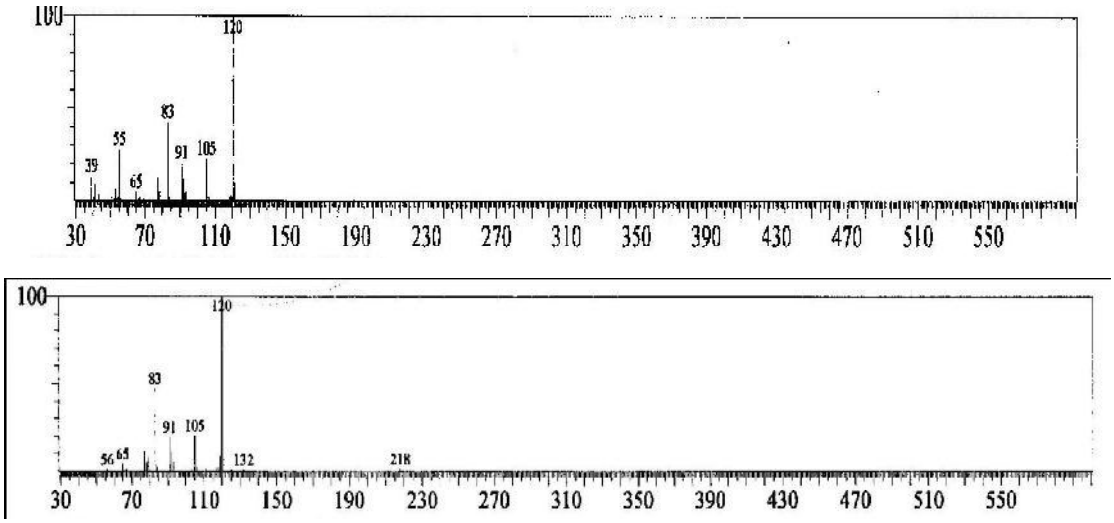


Figure 6. Mass spectrum and structure of α-tumerone

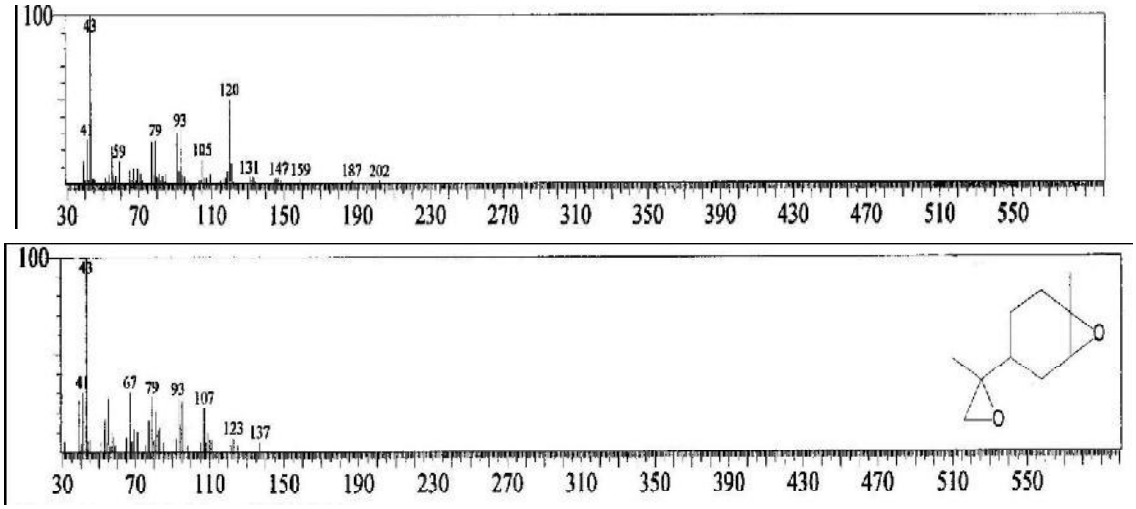


Figure 7. Mass spectrum and structure of α-limonene

## ACKNOWLEDGEMENTS

We would like to express our gratitude to Ni Nyoman Yuni Aswindarti, S.Si for her excellent technical assistance in the preparation of sample and support in Laboratory, also to Dra. Iryanti Eka Suprihatin, M.Sc., Ph.D who supported this paper.

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# AMPLIFICATIONS OF TERMINAL ENDS OF NEWCASTLE DISEASE VIRUS GENOME BY RAPID AMPLIFICATION OF CDNA ENDS (RACE)

**Anak Agung Ayu Mirah Adi[1*], Nyoman Mantik Astawa[2], and Yasunobu Matsumoto[3]**
*[1]Pathology Laboratory [2]Virology Laboratory, Faculty of Veterinary Medicine,*
*Udayana University - Jln. PB Sudirman Denpasar Bali.*
*[3] Laboratory of Global Animal Resource Science, Department of Global Agricultural Sciences, Graduate School of Agricultural and Life Sciences, the University of Tokyo,*
*1-1-1 Yayoi, Bunkyo-ku, Tokyo, Japan.*
**Correspondence author: mirah638@yahoo.co.id*

**ABSTRACT**

Leader and trailer (terminal ends) of ribonucleic acid (RNA) sequences are important in characterizing novel Paramyxovirus as they contain important signals for replication and transcription their genomes and therefore important in understanding the process of viral evolution. Conventional polymerase chain reaction (PCR) is normally unable to amplify these regions. Recently, rapid amplification of cDNA Ends (RACE) is a PCR-based technique that has been developed to amplify these regions in order to determine RNA terminal sequences. In this study, the leader and trailer, of the viral RNA genome of Newcatle disease Virus (NDV)/Bali-/07 were amplified. The leader and trailer sequence of the viral genome was determined using 3'-RACE and 5'-RACE method respectively. With this method both leader and trailer of NDV/Bali-1/07 can be amplified, without using a highly cost RACE kit.

***Keywords***: genome, leader, trailer, RACE

## INTRODUCTION

Fragments of viral RNA genome can be amplified by the reaction of reverse transcriptase polymerase chain reaction (RT-PCR). However fragment terminal ends (leader and trailer) of viral RNA genomes could not be amplified by RT-PCR. The methods developed for this purpose is rapid amplification of cDNA Ends (RACE)( Bertioli, 2000). Several RACE methods have been developed and continue to be evaluated for a simple and inexpensive method (Bertioli, 2000; Li et al., 2005; Liu et al., 2010). In this study we attempted to modify an inexpensive RACE method of Li et al. (2005) for amplifying the leader and trailer of NDV/Bali-1/07 genome.

## MATERIALS AND METHODS

### Viral Propagation and RNA Isolation

NDV/Bali-1/07 (acc. number AB426628) was propagated in 9-day-old embryonated SPF chicken eggs. NDV RNA isolation was performed by standard Trizol method.

### Amplification of leader and trailer of the viral genome

The leader sequence of the viral genome was determined using 3'-RACE method. Viral RNA was ligated with a 3'_end cordecypin-blocked adaptor DT88 (5'-GAAGAGAAGGTGGAAATGG CGTTTTGG-3') using T4 RNA ligase (TaKaRa-Takara Bio Inc._Siga Japan) following procedures as described Zhou et al. (2005) with slightly modification: 1µg of RNA, 20 pmol of DT88, 2µl of 10× RNA ligase buffer, 20U of T4 RNA ligase, and RNase-free water to a final volume of 20 µl were mixed and incubated at 37°C for 1 h. A 2 µl -aliquot was taken out to make cDNA using 20 pmol of primer DT89 which is complementary to the adaptor (5'-CCAAA ACGCCATTTCCACCT TCTCT TC-3') (Zhuo et al., 2005). The reaction was conducted at 65°C for 60 min followed by inactivation at 85°C for 5 min. The resulting adaptor-ligated cDNA was then amplified using the primer DT89 and NDV specific primer F1rBali (Table1). The hemi-nested PCR was performed using 1µl of 1:100 diluted primary PCR products as template, 20 pmol each of DT89 and a nested gene-specific primer F1rBali2 (Table 1). The resultant PCR product was cloned and positive clones were sequenced using M13 forward and M13 reverse primers.

The sequence of the leader region was determined.

The trailer sequence was determined using 5'-RACE method. Firstly primer F25s (Table 1) was used to reverse trancribed viral RNA into cDNA. The cDNA, were treated with the Wizard(R) SVG gel and PCR Clean Up system (Promega-Japan). The cDNA was then polydATP-tailed and polydGTP-tailed using T4 terminal deoxynucleotidyl transferase according to the manufacturer's (TaKaRa-Bioinc-Japan). The PCR reaction using template both the polydATP-tailed and polydGTP-tailed cDNA, were performed independently using the same primer sense F30S Bali (Table 1) and primer forward Oligo–dT (5'-ACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTV-3') and Oligo–dC (5'-.ACCACGCGTATCGATGTCGACTCCCCCCCCCCCCCCD-3') respectively. The obtained amplicons were then cloned and positive clones were sequenced using M13 forward and M13 reverse primers.

**Table .** Oligonucleotides used to generate PCR products in 3'-5' RACE method, to obtain leader and trailer of NDV/Bali-1/07 sequences

| Primer name | Nucleotide sequence |
|---|---|
| @F25s $^{12000\text{-}12019}$ | 5′-aatagctcatatgtcgccac-3′ |
| @F29s$^{14001\text{-}14020}$ | 5′-ctgtatcattgctgcattgt-3′ |
| *F30sBali1$^{14490\text{-}14511}$ | 5′- ttctgt gcagaaagt ttagt -3′ |
| *F30S Bali2$^{14910\text{-}14931}$ | 5′-tatacttgactcgtgctcaaca-3′ |
| *F1r Bali1 $^{540\text{-}521}$ | 5′-tgacaaacggggtaccgt-tg-3′ |
| *F1r Bali2$^{244\text{-}223}$ | 5′-ctatccctgatctcactgt-3′ |

@Primers were designed based on the genome sequence of the NDV/LaSota(NCBI number AF77761)..*Primer were designed using the obtained genome sequence of NDV/Bali-1/07.
Numbering in superscript indicates the positions on NDV/LaSota genomic sequence

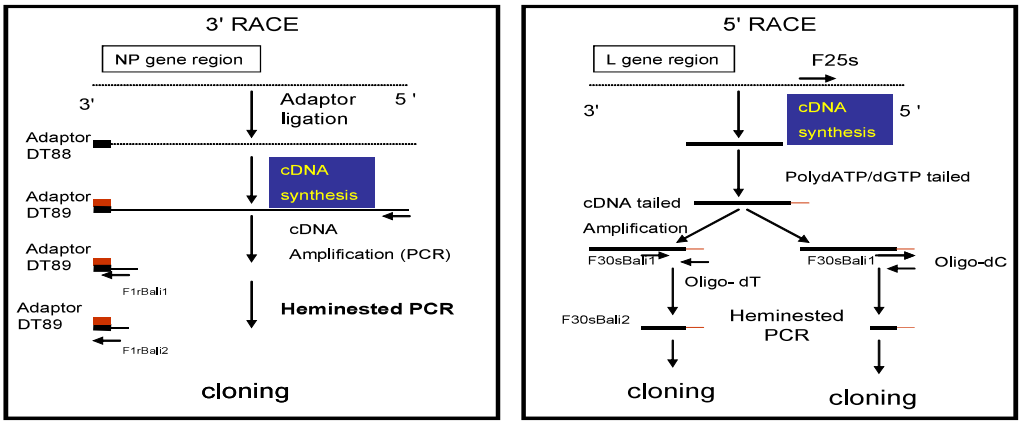


Figure1. Schematic diagram of 3'-RACE (A and 5'-RACE (B). The viral RNA genome (dotted line) is presented in the 3 ' and 5 ' direction. The cDNA is represented in solid line. In 3'-RACE viral RNA was ligated with adaptor DT88, followed by cDNA synthesis using primer DT 89 which complementary to adaptor DT88. For 5'-RACE, cDNA synthesis was performed first, the cDNA was then polydATP-tailed and polydGTP-tailed. The remaining step PCR and heminested PCR are same for the both 3'-RACE and 5'-RACE, with the exception of the primers used.

## RESULTS AND DISCUSSION

Previously to amplify terminal end of NDV/Bali-1/07s we has been attempted using RACE method of Schutze et al.,(1995) that had been modified by de Leeuw and Peeters (1999). This method was able to amplify the trailer but was failed to amplify the leader of NDV/Bali-1/07 (unpublished data). By modifying

RACE method of Li et al. (2005) especially in the viral specific primers both leader and trailer of NDV/ Bali-1/07 could then be amplified (Fig.2A-B). For 3'- RACE in the first PCR,using sense primer DT 89 and reverse primer F1rBali1, the PCR product of 1500 bps was clearly visible in the agarose gel following electrophoresis (Fig.2A line 2). As well in the heminested PCR using reverse primer F1rBali2, PCR product of 250 bp was also clearly visible (Fig.2B line 3).

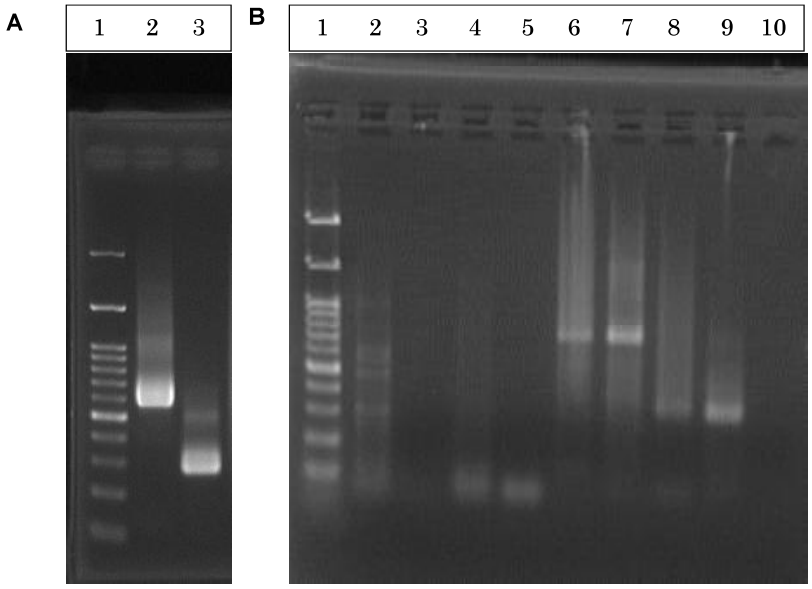


Figure 2. Amplification Result of 3'- RACE (A) and 5'-RACE (B); (2A). Adaptor ligated cDNA was amplified using Bali-1/07 NP gene specific primers. Line 1. DNA marker 100 bp., line 2.First PCR using primers DT89 and F1rBali-1, line 3.Heminested using primers DT 89 and F1r Bali2.(2B). Polyadenilated cDNA was amplified using primer specific L gene of LaSota strain and Bali-1/07 strain. Line 1. Marker 100 bp, line 2.Bali_1 TdT/dA/F29S- oligo dT, line 3.no template/ F29S- oligo dT, line 4. Bali_1 TdT/dG/ F29S- oligo dC, line 5.no template/ F29S- oligo dC, line 6**.** Bali_1 TdT/dA/F30SBali□□oligo dT, line 7. Bali_1 TdT/dG/ F30SBali- oligo dC, line 8. Bali_1 TdT/dA/F30SBali2- oligo dT, line9. Bali_1 TdT/dG/ F30SBali2- oligo dC, line 10. no template/ F30SBali2- oligo dC.

For the 5'-RACE by using primer specific L gene of LaSota strain (F29s) as a sense primer, both polydATP-tailed and polydGTP-tailed cDNA were failed to amplify(Fig.2B. Line 2-4). However by using Bali-1/07 L gene specific primer, first PCR and heminested PCR using polyadenilated cDNA as template, both could produce PCR product of 700 bp.(Fig.2B.Line 6-7) and 260 bp. (Fig.2B.Line 8-9) respectively.

The length of 3'-leader and 5'-trailer were 55 and 114 nt, respectively, these length were similar with those of other NDV strain (Fig. 3 A-B). The genome terminal ends sequences are highly conserved and there is complementary between the 3'- and 5'- termini (Fig.3C). These conserved terminal sequences, especially the first 12–13-nt, are believed to contain the genome and anti-genome promoters essential for replication and transcription (Lamb and Kolakofsky, 2001), and are useful markers for classification of new viruses and studying virus evolution in the family (Wang et al., 2003).

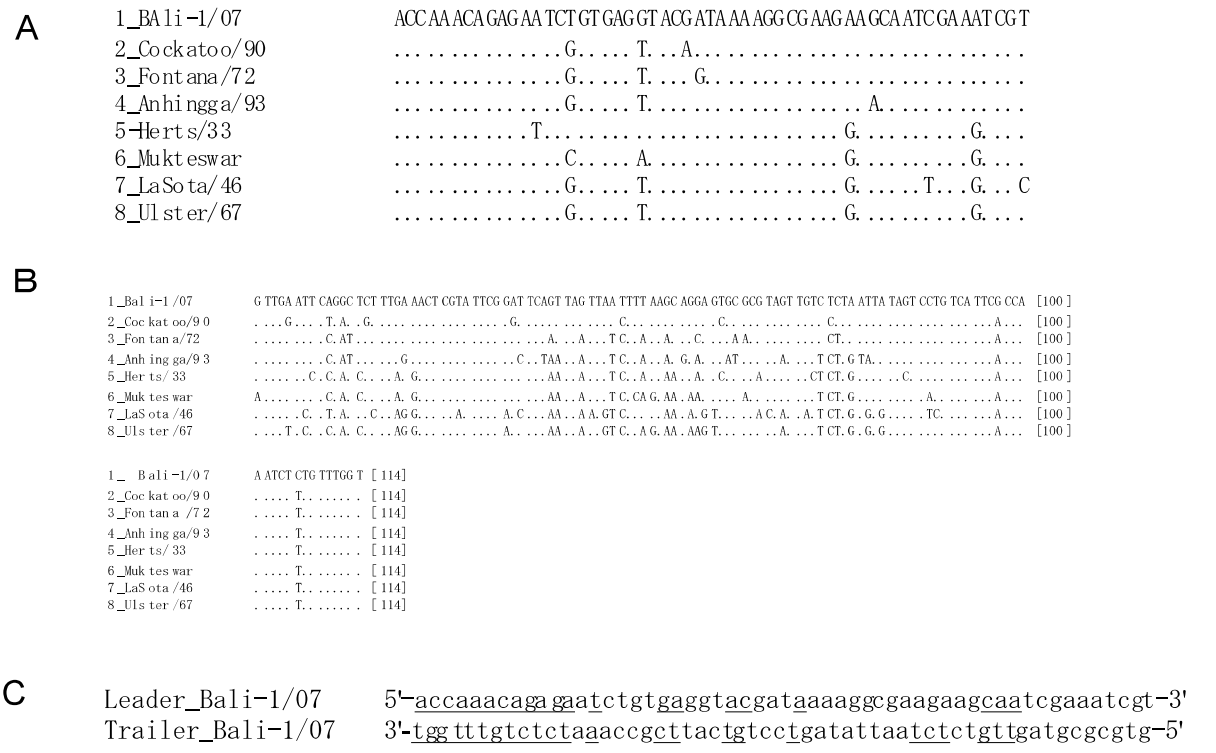


Figure 3. Alignment of the 5' leader and 3' trailer of NDV /Bali-1/07 isolate with several NDVs. The sequences are presented as CDNA in the 5 ' and 3 ' direction. (A) Alignment of the leader. (B) Alignment of the trailer.(C).Paired nucleotides at 5' and 3 ' end of Bali-1/07 cDNA are underlined.

## CONCLUSION

With this method both the leader and trailer of NDV/Bali-1/07 can be amplified, without using a highly cost RACE kit. The length of 3'-leader and 5'-trailer were 55 and 114 nt, respectively, these length were similar with those of other NDVs strain.

## ACKNOWLEDGEMENT

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# THE ROLE OF p16INK4a IN CELLULAR SENESCENCE

**I Gusti Ayu Dewi Ratnayanti*, I Gusti Ngurah Mayun, Sri Wiryawan, Ika Wahyuniari, Ni Made Linawati, I Wayan Sugiritama, Arijana**
*Histology Department, Medical Faculty, Udayana University*
**Correspondence author: mynameisratna@yahoo.com*

**ABSTRACT**

Cellular senescence is the end stage of cellular development that leads to senescence of tissue and aging of organism. The mechanism of cellular senescence has not been fully identified. One of the key proteins is tumor suppressor protein p16INK4a. This protein is a CDK inhibitor of the cell cycle. The presence of this protein stalls the cell cycle progression in G1/S phase and promotes the cell to enter the senescent stage. The mechanism of this protein to induce senescence is mediated by p38MAPK-p16INK4a-pRb/E2F signaling. Triggers of senescence through p16INK4a can be initiated by telomeric and non telomeric event. This protein may be the determinant factor in cellular senescence, but further study is needed to elaborate the role of this protein, especially to understand the significant of this protein in preventing and promoting cellular senescence.

## INTRODUCTION

Aging of the organism is a complex process involving and affecting not only physical but psychological being as well. The process of aging has not been understood clearly. Cellular senescence has been thought as the underlying mechanism or organismal aging. The accumulation of senescence cell will lead to disruption of the tissue physiological function and manifest in age related diseases (Chen *et al*., 2007a; Cox, 2009). The process of cellular senescence has not been elaborated completely. Understanding the process may give insight in finding the way to maintain the healthy life span.

Tumor suppressor protein p16INK4a is a significant player in cell cycle control and check point (Vermuelen *et al*., 2003). In correlation with the involvement of this protein in cell cycle, it is the main protein that determines the senescence fate of cell. This review is aimed to describe the role of this protein in the mechanism of cellular senescence.

## CELL CYCLE

Cell cycle is a series of event during cell division. This complex process is related to cell growth and proliferation, DNA repair mechanism, and cell response to trauma and cancer. Somatic cells cycle in two phases, interphase and mitosis. The first phase takes the longest period consist of three stages, which are G1 (gap 1), S (synthesis), and G2 (gap 2). In this phase, preparation, DNA replication, and error checking are tightly regulated to permit cell entering next phase of cell cycle. Division of nucleus and cytoplasm occurs in the late phase resulting in two identical cell descendants. Mature cells in their terminal differentiation and inactive dividing cells are in the G0 phase or known as quiescent cell (Alberts *et al.* 2003; Gartner & Hiatt, 2008).

Cell cycle is regulated by cyclins and cyclin dependent kinases (CDKs). Cyclin is group of proteins that controls phosphorylation of CDK, whereas CDK induces the cell cycle progression. Cyclin possesses signal to locate nucleus and direct the complex of cyclin and CDK to the nucleus (Alberts *et al.*, 2003). Types of cyclin controlling the cell cycle include cyclin A, B, C, D1,2,3, and E. CDKs involved in cell cycle consist of CDK 1,2,4,6 and 7 (Vermeulen *et al.*, 2003, Alberts *et al.*, 2003). The complex of cyclin-CDKs phosphorylates retiblastoma tumor suppressor protein (pRb). The deactivation of pRb causes dissociation of pRb-E2F transcription factor complex. The free E2F subsequently triggers the transcription of its target genes, including cyclins and CDKs, required in the next phase of cell cycle (Huevel, 2005).

Cell cycle can be inhibited by CDK inhibitors or Wee 1 and Myt 1 kinase. CDK inhibitors in the INK4 family are p16, p15, p19, and p18. Another CDK inhibitors belong to Cip/Kip family, such as p21, p27,and p57. Wee 1 and Myt 1 target point is progression of G2 to M phase, whereas CDK inhibitor primarily targets the G1/S phase as well as G2/M phase (Vermuelen *et al*., 2003).

## CELLULAR SENESCENCE

Cellular senescence refers to dysfunctional end state of cell development. In this so called condition, cell cycle is irreversibly arrested (Jun & Goligorsky, 2006). Cellular senescence is argued to be responsible for the aging of organism, because as the cell senesce, its biological functions decline. Cell is a living unit of organism, thus, accumulation of dysfunctional cell will affect the function of tissue and ultimately the performance of organism as a whole (Chen *et al.* 2007a). It has been found that accumulation of senescent cells increases with age in various tissue of animals and human (Campisi, 2000; Herbig *et* al., 2006; Cox, 2009; Wang *et al.*, 2009).

Senescence of cell occurs as a result of telomere shortening in cell division. Continuing cell division causes telomere to finally reach its critical length and induces cellular senescence. This process is known as replicative senescence. Senescence can also occur prematurely by various stimuli such as oxidative stress, activated oncogene, chemotherapeutic agent and DNA damage (Chen, *et al.*, 2007b; Burton, 2009). The identified mechanism of cellular senescence can be divided into telomere dependent and telomere independent pathway. Telomere dependent pathway is related to telomere shortening and instability that could induce senescence through cascade of signaling (Erusalimsky and Kurz, 2006; Chen *et al*., 2007b). The condition is also observed in telomerase inactivity (Minamino and Komuro, 2007). Both dependent and independent pathways involve the activation of ataxia telengiectasia mutated/ataxia telengiectasia and Rad-3-related (ATM/ATR) or mitogen activated protein kinase (p38MAPK). Activation of ATM/ATR stimulates accumulation of p53, and then it can stimulate senescence through increase expression of p21. This signaling is primarily as a response of DNA damage. Though, senescence had been observed without obvious DNA damage response, through activation of p21 or p16 (Chen *et al*., 2005; Chen *et al*., 2007; Rodier and Campisi, 2011; Zhang *et al*., 2012).

## THE ROLE OF P16INK4A IN CELLULAR SENESCENCE

p16INK4a is a tumor suppressor gene that is also known as multiple tumor suppressor-1 (MTS-1). The gene is located in the INK/ARF locus of chromosome 9p2 which codes several proteins through alternative splicing, including p16INK4a, p19ARF, and p14ARF. The protein of the gene is also called p16INK4a (Huschtscha and Reddel, 1999).

Increase expression of this protein is associated with cellular senescence. Level and stability of p16INK4a is found to increase in replicative cellular senescence (Wang *et al*., 2005). The high expression of p16INK4a is also observed in senescent cell of epithelial cell, fibroblast, T lymphocyte, and endothelial cell (Huschtscha and Reddel, 1999; Brodsky *et al*., 2005; Jun and Goligorsky, 2006). Regarding the consistence expression of p16INK4a in cellular senescence, this protein is used as senescence marker (Rodier and Campisi, 2011). The role of this protein in aging is outlined by evidence of clearance of this protein can prevent age associated condition such as cataract, loss adipose tissue and muscle of aged mouse (Baker *et al*., 2011).

Induction of p16INK4a expression has been observed in telomeric and non telomeric-related event in cells. Telomeric induction includes telomere loss, structural instability, and decrease of telomerase function (Zhang *et al*., 2012). Non telomeric event that could increase expression of p16INK4a are culture stress, oncogenic stress, ionizing radiation, DNA damage or oxidative stress (Serrano *et al*., 1997; Chen *et al.*, 2007b; Darbro, 2007; Takahasi *et al*., 2007). It has been shown that the induction of p16INK4a in cellular senescence is mediated by p38MAPK in telomeric or non telomeric pathway (Yuan, 2006; Zhang *et al*., 2009; Spallarossa, 2010). p16INK4a inhibits the cell cycle by directly binds CDK 4,6 and prevents its association with cyclin. The blocking of cyclin D/CDK 4,6 complex formation in G1 phase will impede CDKs activation. CDKs are unable to phosphorylate the pRb protein. Thereby, stability of pRb-E2F complex is maintained and protein E2F cannot perform its function in subsequent cyclins and CDKs transcription. The resulted gene silencing will prevent the progression of cell cycle to enter the S

phase. The cell cycle stops and cell becomes senescent (Beausejour *et al*., 2003; Takahashi *et al*., 2007; Rayess *et al*., 2012). The role of p16INK4a in cellular senescence is summarized in figure 1. As describe previously, cellular senescence does not exclusively involve p16INK4a in the process, but also mediated by p53-p21-pRb signaling. The differentiation of the p53 or p16INK4a pathway has not been elucidated. It is unclear whether both pathways could independently exert senescence or concert in action. Eventhough, it seems that cellular senescence process is maintained primarily by p16INK4a. The dominant role of this protein is showed by adequate level of this protein prevents reversal of senescence by p53 inactivation (Beausejour *et al*., 2003). It is supported by another study that found telomere associated senescence could not be induced in the condition of p16INK4a deficient (Zhang *et al*., 2012).

## CONCLUSION

Tumor supressor protein p16INK4a is a main regulator in cell cycle progression. Increase expression of this protein is closely associated with cellular senescence. Cellular senescence stimulation is mediated through p38MAPK-p16INK4a-pRb signaling. This protein plays dominant role in cellular senescence mechanism. Modulation of this protein can be further studied as it is a promising target in the prevention of aging and its associated disease.

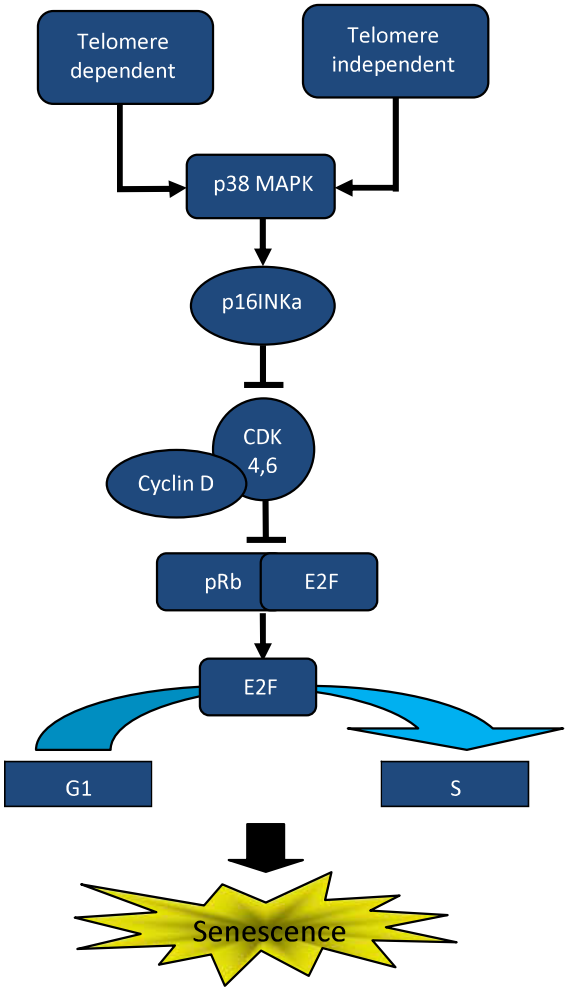


Figure 1. The role of p16INK4a in cellular senescence. See text for explanation

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# ROLE OF TLR2 ON MYCOBACTERIUM TUBERCULOSIS INFECTION

**I Nyoman Wande[1*], I Wayan Putu Sutirta Yasa[1], and Jusak Nugraha[2]**
[1]*Medical Faculty, Udayana University*
[2]*Medical Faculty, Airlangga University*
**Corresponding author: wande_nyoman@yahoo.com*

**ABSTRACT**

Tuberculosis is a major public health problem, about 10 million new cases diagnosed each year, causing a death toll of 2 million victims. Innate immunity plays an important role in the host defense against *M.tuberculosis*, and the first step in this process is recognition of *M.tuberculosis* by cells of the innate immune system. Toll-like receptors (TLRs) have been revealed to be critical for the recognition of pathogenic microorganisms including mycobacteria. TLR2 is believed to be important in the initiation of innate host defense through its stimulatory effects on TNF-α production in macrophages. The TLR2 gene is located on chromosome 4q32 and is composed of two noncoding exons and one coding exon. More than 175 SNPs for the human TLR2 have been reported. By use of Northern blots, it is found that TLR2 is expressed as 4- and 4.4-kb mRNAs in heart, brain, and muscle. Further studies are necessary to determine the TLR2 expression and polymorphisms gene associated with *M.tuberculosis* infection and complicated tuberculosis.

***Keywords:*** TLR2, Innate immunity, Tuberculosis

## INTRODUCTION

Indonesia is now ranked fifth in the country with the burden of tuberculosis (TB) in the world. The estimated prevalence of all TB cases was 660,000 and the estimated incidence amounted to 430,000 new cases per year (WHO, 2010). The number of TB deaths was estimated 61,000 deaths/year. The incidence of TB in humans is closely related to the incidence of infection Immunodeficiency Human Viruses (HIV), the presence of HIV infection, impaired immune system, making it easier infected with Mycobacterium tuberculosis (*M. tuberculosis*). Indonesia is a country with the acceleration of the highest HIV epidemic among countries in Asia. Nationally, estimates of HIV prevalence in the adult population was 0.2%. A total of 12 provinces have been declared as priority areas for intervention with HIV and an estimated number of people living with HIV/ Acquired Immunodeficiency Syndrome (AIDS) in Indonesia around 190,000-400,000. National estimates of HIV prevalence in new TB patients was 2.8%. picture of Multi-Drug Resistance (MDR)-TB is estimated at 2% of all new TB cases (lower than estimated in the region of 4%) and 20% of TB cases with re-treatment. It is estimated there are approximately 6,300 cases of MDR TB each year (Kemenkes RI, 2011).

The first stage in the activation of the body's natural defences begins with pattern recognition of pathogens. Pathogen-associated molecular pattern (Pamp) of M. tuberculosis recognized by pattern recognition receptors (PRRS), specifically, will spur the production of proinflammatory cytokines and chemokines, phagocytosis and killing of mycobacteria, and present the antigen (Kleinnijenhuis et al., 2011). PRRS consists of three members include the toll-like receptor (TLR), NOD-like receptor (NLR) and RIG-like receptor (RLR). TLR Location is at the cell membrane (plasma) and the endosomal membrane, while the location of the NLR and RLR are intracellular (Ma'at, 2009).

TLR membrane receptors are divided into two groups. The first group consists of TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, TLR11, TLR12 and TLR13 are typical TLR cell surface. The second group consists of TLR3, TLR7 and TLR9 is found primarily in the membrane endosome. In phylogenetic, TLR divided into six families, namely TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11. TLR recognize and are activated by a variety of Pamp, such as bacterial DNA, LPS, peptidoglycan, teikhoat acid, flagellin, gyre, viral dsRNA, and zimosan fungi. For example, TLR2 recognizes peptidoglycan and teikhoat acid from Gram-positive bacteria, whereas TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacterial cell walls. Activation of TLR, triggers the expression of various cytokines such as IFN-γ, IL-2, IL-6, IL-8, IL-12, IL-16 and TNF-α (Pasare and Medzitov, 2005).

The aims this article to determine molecular aspects of Toll-like receptor 2 (TLR2), to know the different of TLR gene polymorphisms are present in M. tuberculosis infection, and the role of TLR2 in M. tuberculosis bacterial infection.

## METHODS

This scientific writing was done by review article and meta-analysis, which concluded a scientific journal and books, made comparisons between various journals or publications, and then created in the form of a review article.

## DISCUSSION

### Toll-Like Receptor 2

Toll-like receptors (TLRs) are pattern recognition family receptors (PRR), which consists of 12 members in mammals. TLR expressed on the surface of the cell membrane or membrane vesicles endocyte, especially at the cell immunity such as macrophages and dendritic cells (Akira, 2003). Toll-like receptor 2 is called TLR2 is present in human proteins encoded by TLR2 genes. TLR2 is also expressed as CD282, plays a role in the immune system. TLR2 is a membrane protein and a receptor, expressed on the surface of some cells, to recognize foreign substances and forward the signal to the appropriate system of immune cells (Rock et al., 1998).

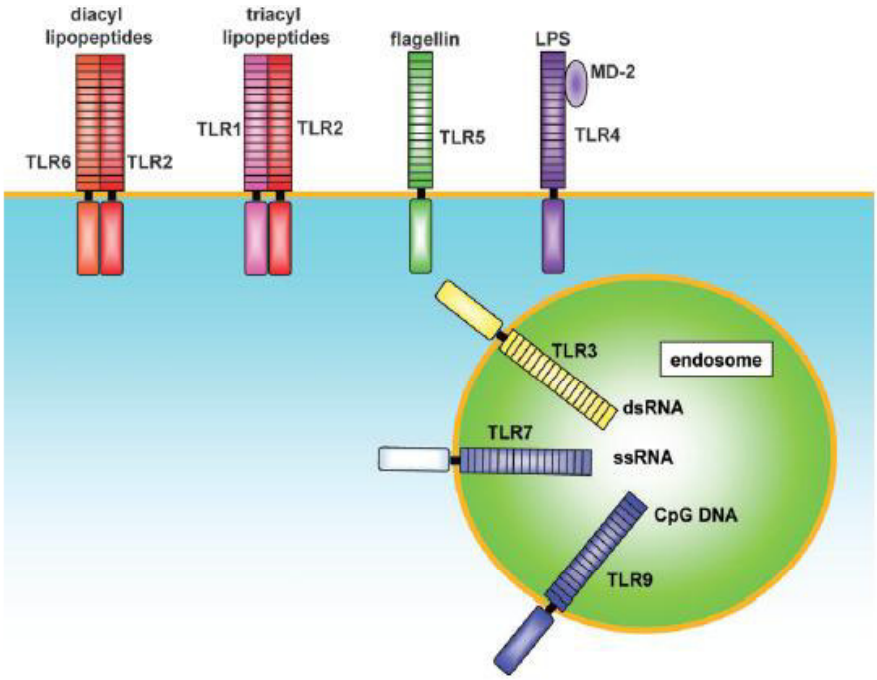


Fig. 1. TLR2 is essential in the recognition of microbial lipopeptides. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR9 is essential in CpG DNA recognition. TLR3 is implicated in the recognition of viral dsRNA, whereas TLR7 and TLR8 are implicated in viral-derived ssRNA recognition. TLR5 recognizes flagellin. The TLR family members recognize specific patterns of microbial components (Takeda and Akira, 2005).

TLR2 can recognize several microbial components such as lipoprotein/lipopeptide of different types of pathogens, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from

mycobacteria, glycosylphosphatidylinositol anchors from *Trypanosoma cruzi*, phenol-soluble modulin from *Staphylococcus epidermidis*, zymosan from fungi and glycolipids of *Treponema maltophilum* (Takeda et al., 2003). Other than that reported TLR2 can recognize LPS from non-enterobacteria such as *Leptospira interrogans*, *Porphyromonas gingivalis* and *Helicobacter pyroli*. TLR2 can recognize microbial components widely predicted by two aspects of the mechanism. The first aspect, TLR2 forms heterophilic dimmers with others of TLR such TRL6 and TRL1. The second aspect, TLR2 cooperate with other receptors such as Dectin-1, a lectin family receptor for fungal cell wall containing β-glucan (Takeda and Akira, 2005).

**Overview of Biochemistry**

By structural analysis, it is known that the human TIR of TLR2 and TLR1, about 50% are identical in amino acid containing central 5-stranded parallel beta-sheet surrounded by five alpha helices on both sides. It is said that the interference with the TIR structure or mutation can eliminate the signal by disrupting recruitment of MyD88 (Xu et al., 2000).

**Mapping**

With the TLR2 gene mapping method of fluorescent *in situ* hibridization, Chaudhary et al. in 1998 was show located on chromosome 4q31.3-Q32. With the same method, Rock et al., in the same year refine discovery, was obtain located on chromosome 4q32.

Characteristics of human TLR2 (Gene bank 2011) shows its position on chromosome 4q32 (positions 154 605 441 .. 154 627 243) (NC_000004) with gene length: 21,803 bp DNA (NC_00004), the number of amino acids making up the protein: 784 aa (NP_003255), number of exons : 3 exon (NG_016229), the number of intron 2 intron (NG_016229) and mRNA length: 3417 bp mRNA (NM_003264). Characteristics of TLR2 in *Mus musculus*: (Gene Data Bank 2011) note that the position on the chromosome: 83,640,194 .. 83,645,530 (NC_000069) with gene length: 5337 bp DNA (NC_000069), the number of amino acids making up the protein: 784 aa (NP_036035), the number of exons : 3 exon, intron number: 2 intron and mRNA length: 2874 bp mRNA (NM_011905).

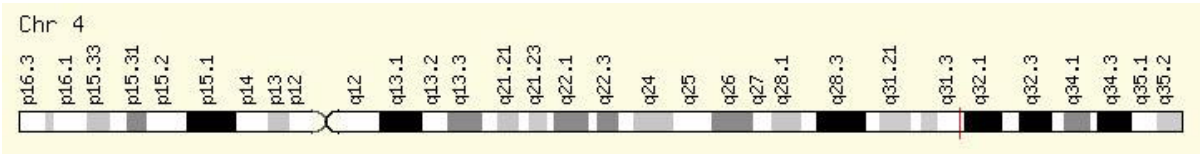


Fig. 2. TLR2 *Gene in genomic location* (Anonym, 2011).

**Genomic structure of TLR2 in mice**

Comparison of genomic DNA clones were isolated on chromosome along with the published cDNA sequence of mouse TLR2, TLR2 mice revealed that the gene consists of three exons, separated by two introns. Exon sequences are given in capital letters, and intronic sequences in lowercase. All intersections are expected to contain a GT and AG donation (Wang et al., 2001).

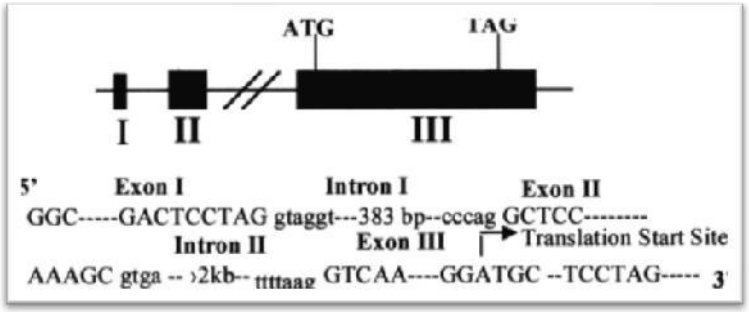


Fig.3. genomic structure of TLR2 in mice (Wang et al., 2001).

**TLR2 expression**

TLR2 is expressed on microglia, Schwann cells, monocytes, macrophages, dendritic cells, polymorphonuclear leukocytes (PMN), B cells and T cells, including regulatory T cells (CD4 +, CD25 + regulatory T cells). In some cases, there are in a state of TLR2 heterodimer (molecular combinations) as pairs with TLR1 or TLR6. TLR2 is also found in airway epithelium, lung alveoli, renal tubules, and the bowman's capsula in renal corpuscle. In the skin TLR2 found on keratinocytes and sebaceous glands (Wikipedia, 2011).

The role of TLR in Mycobacterium tuberculosis infection

After the interaction between of specific M. tuberculosis with TLR, signalling pathways through the adapter molecule myeloid accelerated differentiation primary response protein 88 (MyD88). Furthermore, IL-1 receptor-associated kinases (IRAQ), TNF receptor-associated factor (TRAF) 6, TGFβ-activated protein kinase 1 (TAK1) and mitogen-activated protein (MAP) kinase signalling cascade recruited in the activation and nuclear translocation from transcription factor such as nuclear transcription factor (NF)-κB (Takeda and Akira, 2004). This situation causing a transcription of genes involved in the activation of the body's natural defences, especially in the production of proinflammatory cytokines such as TNF, IL1β, and IL-12, and nitric oxide (NO) (Akira, 2003).

TLR known to play a role in the introduction stage of the M. tuberculosis TLR2, TLR4, TLR9 and possibly TLR8. TLR2 forms heterodimers with TLR1 or TLR6. Heterodimer is aimed at the introduction of the wall of glycolipid mycobacteria such as glycoproteins mycobacterial LAM, LM, 38-kDa, and 19 kDa, lipoprotein phosphatidylinositol mannoside (PIM), triacylated (TLR2/TRL1), or diacylated (TRL2/TRL6). (Means et al., 2001; Jones et al., 2001; Thoma-Uszynski et al., 2001). TLR2 is believed to be very important role in the early stages of the body's stimulation natural immune response through the production of TNFα by macrophages (Bafica et al., 2005). TLR2 and TLR6 plays an important role in stimulating the production of IL-1β (Klinnijenhuis et al., 2009). TLR2 also plays an important role in the expenditure of IL-12 by macrophages, but not dendrityc cells (Pompey et al., 2007). In mice with a deficiency TRL2 showing granuloma formation is not perfect, and when infected with high doses of M. tuberculosis bacteria, the mice clearly show increased susceptibility to infection than normal mice. TLR2 deficient mice also showed impaired control of chronic M. tuberculosis infection (Drennan et al., 2004).

More than 175 human TLR2 SNPs have been reported. In cohort conducted in Turkey, found an association between Arg753Gln and susceptibility to tuberculosis (Ogus et al., 2004), while the opposite result is found in the two cohorts were conducted in Asia which did not find any mention of a specific polymorphism in the population (Xue et al., 2010). Cohort conducted in Tunisia said that Arg677Trp associated with susceptibility to tuberculosis, but the results obtained with these dubious pseudo gene is found on the site of the SNP (Ben-Ali et al., 2004; Malhotra et al., 2005). Studies on the polymorphism in the TLR2 gene still needs to be done since there are many differences in the results obtained from previous studies. 597CC genotype of TLR2 associated with susceptibility to tuberculosis, especially in the form of widespread infection (type miliary and meningitis) caused by a particular M. tuberculosis genotype families ("the Beijing genotype"), a condition found in cohort conducted in patients from Vietnam (Thuong et al., 2007; Caws et al., 2008). In the cohort were conducted in Korean population, polymorphisms high on repetition guanine-thymine, which is located at 100 bp, at the beginning of TLR2 translation start site in intron 2, correlates with the activity of promoter and expression of TLR2 on CD14 + PBMCs (repetition of short, lead to a weak promoter activity and expression of TLR2 low) in both pulmonary infection with M. tuberculosis or with non-tuberculosis ( Yim et al., 2008). Another genotype variation found in cohort studies conducted in populations of Taiwan, said that TLR2 expression is influenced by the presence of insertions / deletions in the -196 to -174 were associated with tuberculosis, whereas other studies showed a possible effect of progression toward systemic symptoms. Many studies conducted in TLR2 polymorphisms are associated with changes in susceptibility to tuberculosis, but it needs further confirmation (Velez et al., 2010; Chen et al., 2010). Changes in the TLR2 gene showed a change in expression of TLR2 against M. tuberculosis that could ultimately affect the body's response to tuberculosis infection.

TLR2 expression on the local network such as lung tissue needs further study because there are many differences of opinion on TLR2 expression on the development of tuberculosis infection. Polymorphism in the TLR2 gene in patients with tuberculosis in Indonesia still need to be revealed further in the role of TLR2 in tuberculosis infection is very important in determining the body's natural immune response of the host.

## CONCLUSIONS

TLR known to play a role in the introduction stage of the M. tuberculosis TLR2, TLR4, TLR9 and possibly TLR8. TLR2 forms heterodimers with TLR1 or TLR6. TLR2 gene located on chromosome 4q32 and compiled by two noncoding exons and one coding exon. TLR2 is expressed on microglia, Schwann cells, monocytes, macrophages, dendrityc cells, polymorphonuclear leukocytes (PMN), B cells and T cells, including regulatory T cells (CD4 +, CD25 + regulatory T cells). TLR2 is believed to be very important role in the early stages of the body's stimulationαnatural immune response through the production of TNF effect by macrophages. The existence of polymorphisms in the TLR2 gene associated with susceptibility is expected to contract tuberculosis.

Given the important role of TLR2 against M. tuberculosis infection, need to do a study on TLR2 expression in lung tissue cells especially macrophages in various clinical conditions such as active tuberculosis, latent tuberculosis and tuberculosis in systemic diseases such as diabetes mellitus and immunodeficiency. The existence of TLR2 gene polymorphisms in M. tuberculosis infection needs to be studied further because it was suspected role in the development of pulmonary tuberculosis.

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# SAPONINS APPLICATION AS ANTICHOLESTEROL

**Bogoriani, N.W.**
*Chemistry Department, Faculty of Math and Natural Sciences, Udayana University*

**ABSTRACT**

Saponins are phytosterol compounds from different types of plants. Saponin derived from the Latin word "*sapo*" which means it contains a stable foam when dissolved in water. Saponins have detergent or surfactant properties due to their structure naturally contains triterpene or steroida glycone that is hydrophobic (fat soluble) and contains one or more sugar chains as glikon that are hydrophilic (soluble in water). Saponin triterpenoid widely found in Leguminose, Araliaceae and and Caryophyllaceae. While steroid saponins widely distributed to the family Liliaceae, Agavaceae and Dioscoreaceae.

Based on several studies that have been conducted, showing that the saponins from different plant species have biological activitys anti-cholesterol. Cholesterol-lowering activity of saponins as has been shown in animal studies and clinical trials in humans.

***Keywords***: saponins; anticholesterol; cholesterol, surfactant

## INTRODUCTION

At this time the public interest against the natural products has increased due to its biological activity is very beneficial for animal and human health. Saponins are plant glycosides molecular structure consisting of steroid or triterpene aglycone called sapogenin and glycone (one or more sugar chains) (Abe *et al*., 1993; Osbourn, 2003; Guclu-Ustundag *et al*., 2007; Vincken *et al*., 2007).

Saponin derived from the Latin word 'sapo' which shall mean containing stable foam when dissolved in water. The ability of saponins foam caused by a combination of sapogenin that are hydrophobic (fat soluble) and the sugar chain is hydrophilic (soluble in water) (Naoumkina *et al*., 2010). Saponin triterpenoid widely found in Leguminose, Araliaceae and Caryophyllaceae (Huhman and Sumner, 2002; Dixon and Sumner, 2003; Sparg *et al*., 2004; Suzuki *et al*., 2005). While steroid saponins widely distributed to the family Liliaceae, Agavaceae and Dioscoreaceae (Mimaki and Sashida, 1996; Sparg *et al*., 2004).

According to some literature, suggesting that saponin from different types of plants havebiological activity as lowering cholesterol. Cholesterol-lowering activity of saponins as blood or plasma have been tested in experimental animals (Malinow *et al*., 1977; Story *et al*., 1984; Dixit and Joshi 1985; Oakenfull and Sidhu, 1990; Harword *et al*., 1993; Hosttetman and Marston, 1995; Matsuura, 2001; McAllister *et al*., 2001; Afrose *et al*., 2010) and clinical trials in humans(Bing Ham *et al*., , 1978; Oakenfull and Sidhu, 1990; Chapman *et al*., 1997; Kim *et al*., 2003). The low heart disease that occurs in Batemi and Maasai tribes of East Africa despite rather eat foods that contain cholesterol or saturated fat, because the food you eat contains saponins and soluble dietary fiber in water (Chapman *et al*., 1997).

In this paper we will discuss about the saponin, saponin separation techniques, identification of saponin, saponin as an anti-cholesterol activity.

## MATERIALS AND METHODS

Research was carried out by literature study.

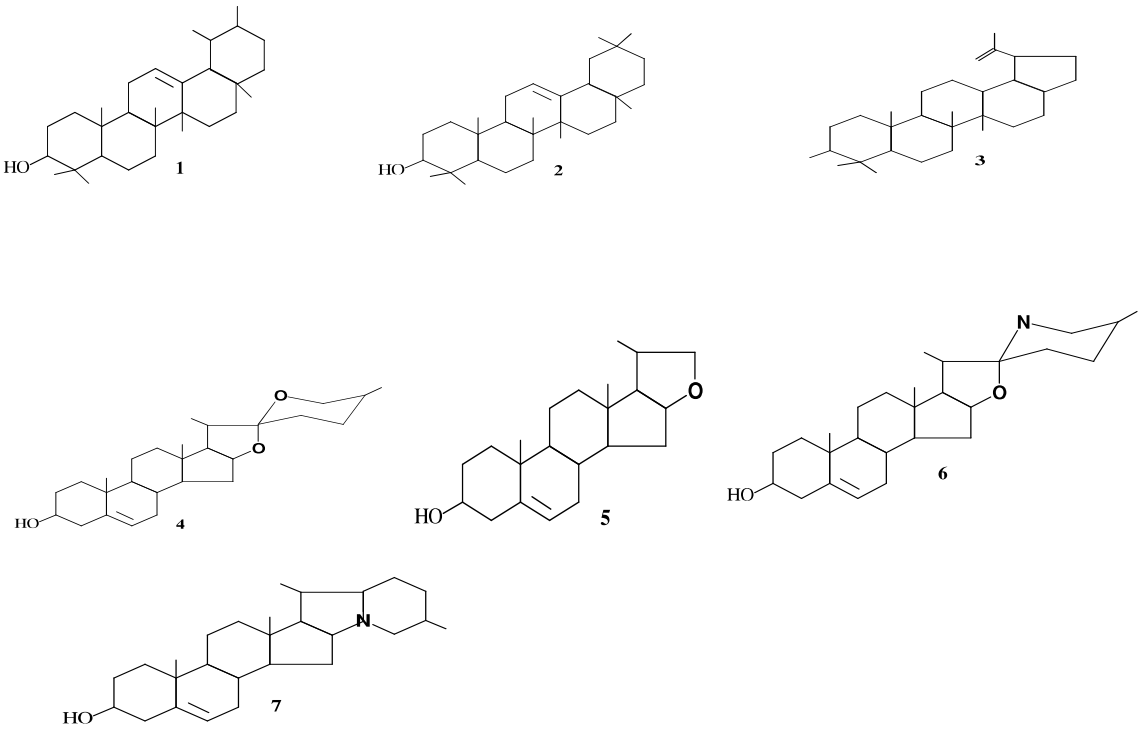
## DISCUSSION

### Saponins

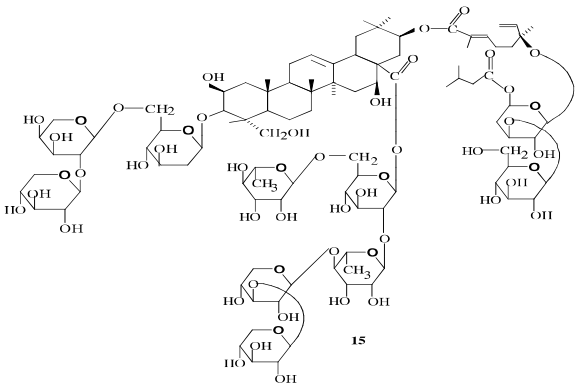
Saponins in plants has been stimulated by the need for a readily available source of sapogenin and can be converted in the laboratory into animal sterols are efficacious essential (e.g. cortisone, estrogen contraceptives, etc.). Compounds that have been used include hekogenin of *Agave*, diosgenin, and yamogenin type of *Dioscorea* (Harborne, 1987).

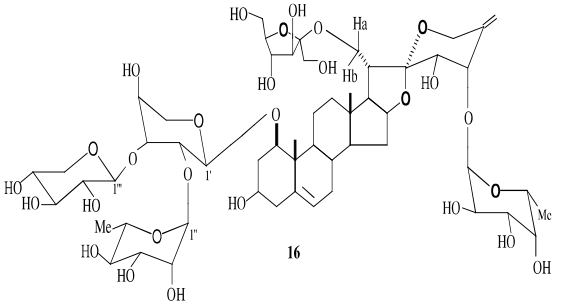
Saponins have been reported more than 100 plant families, and in some marine sources such as star fish and sea cucumber (Hostettmann and Marston, 1995). Steroid saponins mainly found in monocotyledons (such *Agavaceae, Dioscoreaceae* and *Liliaceae*), and triterpene saponins mainly on dicotyledons (Leguminosae, Araliaceae, Caryophyllaceae) (Sparg *et al.,* 2004). The main food sources of saponins are legumes (soy, beans, peanuts, red), are also found in wheat, *allium* species (leek, garlic), asparagus, tea, spinach, sugar beet, and yam (Price *et al.,*, 1987). Soap bark tree (*Quillaja saponaria*), fenugreek (*Trigonella foenum-graceum*), alfalfa (*Medicago sativa*), brown horse (*Aesculus hippocastanum*), licorice (*Glycyrrhiza glabra*), soapwort (*Saponaria officinaux*), Mojave yucca (*Yucca schidigera*), *Gypsophila* genus (such as *Gypsophila paniculata*), sarsaparilla (*Smilax regelii* and other closely related species of the genus *Smilax)* and ginseng (*Panax* genus) is the main source of non-food saponin used for medical and industrial applications (Hostettmann and Marston, 1995; Balandrin, 1996).

Groups of compounds are surface active compounds, can be detected by their ability to form foam and can hemolysis red blood cells, making it toxic to mammals that enter the bloodstream. Saponin is a complex class of natural compounds, large molecular mass, which is a plant glycosides, which would result if the total hydrolyzed aglycone and sugar. Aglycone derived from saponins called sapogenin. Based on the sapogenins group, saponins can be classified as triterpene saponins (sapogenins is triterpene), steroid saponins (sapogenins is a steroid), and alkaloids steroidal saponins (sapogenins are steroid alkaloids) (Mahato *et al*., 1982; Hostettmann and Marston, 1995). Triterpene sapogenins can be classified as a group of α -amirin(1), β-amirin (2), and lupeol (3). The second difference carbon skeleton between α -amirin and β-amirin located from the position of substituents attached to the C-20.One of the methyl group attached α-amirin, while two methyl groups attached to C-20 β-amirin. Characteristics of triterpene saponin is having a hydroxy group at C-3 and the methyl group at position C-4, C-8, C-10, C-14, C-17 and C-20, for lupeol compound containing isopropyl group on C -19. Sapogenin steroids can be classified as a group spirostanol (4), and furostanol (5). Sapogenin steroid alkaloids can be classified as a group solasodin (6) and solanidin (7). Characteristics of steroid saponins are bound hydroxyl group at C-3 and the methyl group at position C-10 and C-13.



Glycon that binds to the sapogenin generally composed of one or more sugar units, so that the structure of saponin is a natural compound complex structure with a large molecular mass. One example of saponin with a lot of sugar units triterpene saponins (example: gimnocladus G of *Gymnocladus chinensis*) (15) and steroid saponins (example: Recurvosida E of stem *Nolina rucurvata* (16) (Konoshima, 1995; Takaashi *et al*., 1995 ).




According Chandel and Rastogi (1980); Mahato *et al.* (1982); Chen and Snyder(1993), the sugar bound with sapogenin glycosides include: D-glucose, D-galactose, D-glukoronat acid, D-ribose, D-xylose, L-arabinose, L-fukosa, and L-ramnosa may include monosaccharides, disaccharides, oligosaccharides and polysaccharides. Sugar cluster is generally attached to the C-3 or can be attached to the C atom to another. This causes difficulty in identifying or determining the structure of saponin. Saponins are a class of highly polar compounds and polarity increases in proportion with the number of units that make up glycone sugar. Glycoside bond orientation is α-and β-glycoside (Figure 1).

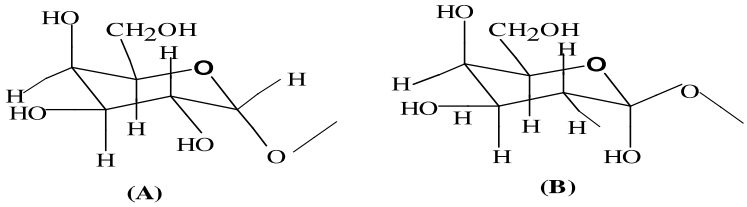



Figure 1. α-glycoside bond orientation (A) and β-glycoside (B).

**Saponins Isolation Techniques**

Saponins are a glycoside plant that can form a stable foam when shaken with water (Mahato *et al*., 1982). Because having a hydroxyl group derived from sugar, the saponin is a polar compound, so in general saponin soluble in polar solvents such as ethanol, methanol, butanol, water and others. The presence of sugar bound to the likely cause saponins more soluble in water, which is a better solvent for glycosides. Instead, aglycone (sapogenin) less polar tend to be more soluble in solvents such as ether and chloroform.

Fresh herbs are an ideal starting material for analyzing saponin, although dry samples that have been stored carefully for years may still be able to give a satisfactory result. However, the plant material is long, is the tendency to turn into aglycone glycoside in the influence of mushrooms, and the aglycone were sensitive to oxidation.

Isolation of saponin compounds is generally done by the method of extraction, either by maceration, percolation, and soxlet reflux, using a solvent that can dissolve the compound saponin. Because saponin compounds are generally soluble in polar solvents, then the extraction process for the purpose of screening and isolation of most use methanol or ethanol. This is because this solvent is dissolving compounds ranging from less polar to polar. Extract condensed methanol or ethanol content of its compounds are then separated

by fractionation techniques, which are usually based on the increase of solvent polarity. Many solvents with increasing polarity were used to isolate saponin compounds, such as methanol or ethanol extract condensed successively fractionated with n-hexane or petroleum ether, n-butanol (Saijo *et al*., 1983; Jia, Zhong-Jian *et al*., 1992); fractionated with n-hexane, chloroform (Woo *et al*., 1983); extracted with petroleum ether, diethyl ether (Encarnacion *et al*., 1981); successfully washed with diethyl ether, chloroform, acetone and finally dissolved in methanol, filtered and the filtrate was added $Et_2O$ or ethylacetate excess sediment forming saponins (Nakanishi *et al*., 1981; Mandloi *et al*., 1981); fractionated with ethylacetate, n-butanol (Konoshima *et al*., 1981), and fractionated only with n-butanol (Takaashi *et al.,* 1995; Mimaki *et al*., 1997; 1998). Less polar saponin fraction obtained from n-butanol and water fractions obtained from a highly polar saponin (Zhong-JianJia and Ju Young, 1992). Precipitation of saponins can be done by adding diethylether or ethyl acetate, which is a measure of dialysis that can be done to remove the water-soluble molecules such as sugar. Water is a less efficient extraction solvent for saponins but has the advantage of being cleaner extracts.

Extraction procedure must be in a state of cold as possible because saponins may change or transformation as occurred: saponin-esterification on acid saponin during treatment with alcohol, hydrolysis of the ester group and the enzyme hydrolysis during extraction with water.

**Identification of Saponins**

Qualitatively to indicate the presence of saponins in the sample material can be done with the foam test. Furthermore its sapogenin groups can be determined by color reaction using Liebermann-Burchard reagent. Based on the color of the form, if the form of red or purple color indicates triterpene saponin, whereas when forming green or blue color indicates steroid saponins (Farnsworth, 1966).

Conventionally, saponin structure elucidation through derivatization and degradation studies (Chen and Snyder, 1993; Qiu *et al*., 1999). Saponin derivatization through methylation or acetylation reaction. Saponin degradation through hydrolysis reaction and the total or partial hydrolysis. Hydrolysis of saponins can be done by an enzyme, alkaline, or acidic. which produces sapogenin and sugar. Hydrolysis under acidic hydrolysis produces total or partial depending on the concentration acid hydrolysis, time, and temperature. In particular the results of hydrolysis is to identify the total saponin and sapogenin glycone. Glycone inter glycosidic bond position and between glycone and sapogenin, identified by metylated reaction followed by hydrolysis reaction in total sugar units that make up glycone. The part that is not methylated in each sugar unit is bound side. The results of total and partial hydrolysis reaction as shown in Figure 2.

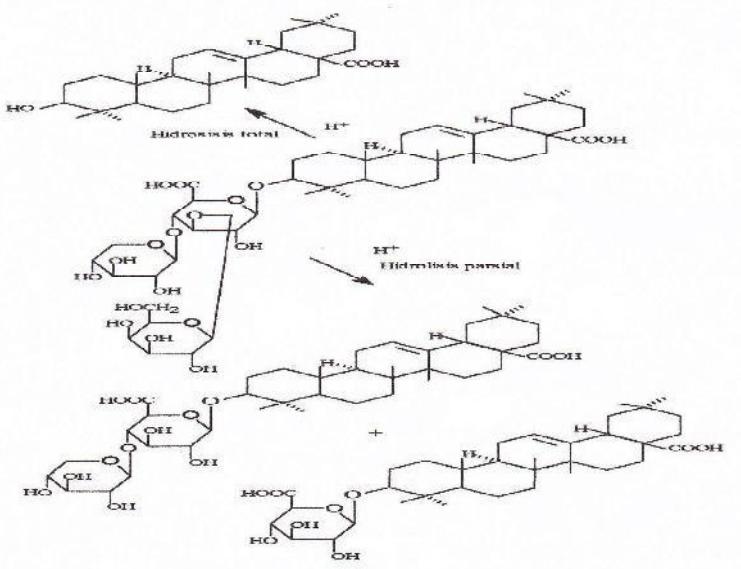



Figure 2. Total and partial hydrolysis reaction (Shibata, 1977)

According Mimaki *et al*. (1997); Mahato and Nandy (1991), the elucidation of the structure of saponin was observed separately and the sapogenin part glycone after hydrolysis. Part glycone identified using gas chromatography (GC) or a combination of gas chromatography-mass spectroscopy (GC-MS). Carbon bonded to the sapogenin glycone circuit can be determined by the price of carbon chemical shifts before and after hydrolysis. Carbon chemical shifts are glycosylated sapogenin is more paramagnetic (5-12 ppm) compared with the hydroxylated carbon, but carbon chemical shift of carbon glycosylated neighbors more diamagnetic (1-6 ppm) than carbon hydroxylated (Mimaki *et al*. 1998).

Sapogenin grouping can be done by calculating the amount of angular methyl proton resonance peaks in the spectrum of the RMP and the amount of angular methyl carbon resonance peaks (primary carbon) and quaternary carbon saturated at RMC spectrum. The spectrum of steroid sapogenin RMP will provide two angular methyl proton resonance peaks with singlet multiplicity in chemical shift (δ) 0.5 to 2.0 ppm, and sometimes gives a resonance peak secondary methyl doublet multiplicity in chemical shift (1-2 ppm), whereas the spectrum of RMC will provide two primary carbon resonance peaks at δ30-45ppm, and two quaternary carbon peaks saturated atδ 6-35 ppm (Agrawal *et al*, 1985, Mimaki *et al*., 1997; 1998). Triterpene sapogenin spectrum RMP will provide 5-7 angular methyl proton resonance peaks(δ0.5 - 2.0 ppm) with singlet multiplicity, whereas the peaks spectrum of RMC will provide primary carbon resonance peaks 5-7 (δ10-35 ppm) and 5-6 quaternary carbon peak ((δ 30-45 ppm) (Mahato and Kundu, 1994, Yu *et al.* 1994)

Based on advances in technology, especially nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (SM), the elucidation of the structure of saponin completely do without hydrolyze part glycone (Yayli and Findlay, 1999; Qiu *et al*., 1999). The number of units that make up glycone sugar can be determined by calculating the amount of carbon anomer resonance peaks in the spectrum of RMC (δ 90-112 ppm) (Agrawal, 1992; Mimaki *et al*., 1997; 1998).

Glycone inter glycosidic bond orientation or inter glycone and sapogenin can be determined from the coupling constant prices (J) visinal anomer proton (J $H_1$-$H_2$) and the price of J C-H anomer of glucose units that make up glycone. Price J $H_1$-$H_2$ of α -O-glycosidicorientationwas 0-5 Hz ($H_1$ equatorial-$H_2$ axial) and for the orientation of β -O-glycosidic is 6-14 Hz($H_1$ axial-$H_2$ axial) (Agrawal, 1992; Burger *et al.,* 1998). Proton signals of anomeric from α-D-glukoside, α-D-manosida, α-L-ramnosida and β-L-arabinosidaδ-L-arabinoside generally appear at lower field (5.0 to 6.0 ppm ) than the anomerβ-and α- ( is 4.5 to 5.0 ppm)(Mahato *et al*., 1982).The difference between J C-H anomer orientation price α-and .β-glycosidic for sugar all units that make up glycone is 10 Hz. Price J C-H anomer sugar unit L-ramnosa for α -glycosidic orientation is 164-168 Hz (C-H equatorial) and for α -glycosidic orientation is152-158Hz (C-H axial) (Kasai *et al.,* 1979; Chen and Snyder, 1993). Price J C-H anomer sugar unit besides L-ramnosa for α-glycosidic orientationis 168-174 Hz (CH equatorial), and for orientation β-glikosidic is 158-164 Hz (C-H axial) (Mahato *et al*., 1982; Agrawal, 1992).

**Saponins Activity as Anti-Cholesterol**

Cholesterol-lowering activity of saponins as has been shown in animal studies(Malinow *et al*., 1977; Story *et al*., 1984; Dixit and Joshi,1985; Oakenfull and Sidhu, 1990; Harword *et al*, 1993; Hosttetman and Marston,1995; Matsuura, 2001; McAllister *et al*., 2001; Afrose *et al*., 2010;) and clinical trials in humans (BingHam *et al*., 1978; Oakenfull and Sidhu, 1990; Chapman *et al*., 1997; Kim *et al*., 2003).

Several saponins have been shown to inhibit the intestinal absorption of cholesterol and to reduce plasma cholesterol levels in a variety of experimental animal models (Sauvaire *et al*., 1991; Harwood *et al*., 1993;Hosttetman and Marston, 1995). Koch (1993) indicated that the cholesterol-lowering effect of garlic preparations may be due to its saponin content.

Two of these saponins, tisqueside and pamaquestide, were able to inhibit cholesterol absorption in rabbits by □50% (Morehouse *et al*., 1999). Interestingly, pamaquestide was about an order of magnitude more potent than tisqueside with comparable efficacy. The cholesterol/saponin ratio in the excreted lipids ranged from 0.2 to 0.4 for tisqueside-treated animals and 1.5 to 5.2 for pamaqueside-treated animals. This near-stoichiometric

ratio suggests that these saponins interact directly with cholesterol or a few molecules of cholesterol, and they inhibit absorption via that mechanism rather than interfering with a transporter itself (Table 1 and Figure 3)

TABLE 1. Effects of saponin CAIs on plasma and hepatic cholesterol levels and body weight changes in cholesterol-fed rabbits

| Group (n) | [$^3$H]Cholesterol Recovery | Hepatic Cholesterol | Plasma Cholesterol | Body Weight Change |
|---|---|---|---|---|
| | % | *mg/gm liver* | *mg/dl* | g |
| Control (8) | 29.3 ± 8.1 | 7.0 ± 0.6 | 244 ± 184<br>*83 ± 37* | 227 ± 28 |
| Pamaqueside-treated | | | | |
| 2.5 mg/kg (5) | 22.1 ± 5.4[a] | 6.3 ± 2.0 | 159 ± 85<br>*77 ± 17* | 239 ± 58 |
| 5 mg/kg (5) | 13.1 ± 5.1[a] | 5.0 ± 1.1[a] | 103 ± 54<br>*78 ± 21* | 284 ± 82 |
| 12.5 mg/kg (5) | 11.1 ± 4.4[a] | 3.8 ± 0.6[a] | 55 ± 24[a]<br>*86 ± 21* | 295 ± 76 |
| 25 mg/kg (5) | 8.8 ± 2.8[a] | 3.5 ± 0.3[a] | 44 ± 13[a]<br>*88 ± 22* | 215 ± 70 |
| Tiqueside-treated | | | | |
| 25 mg/kg (5) | 26.9 ± 7.0 | 6.8 ± 0.5 | 278 ± 140<br>*102 ± 44* | 189 ± 108 |
| 50 mg/kg (5) | 26.1 ± 6.5 | 6.6 ± 1.4 | 231 ± 104<br>*78 ± 25* | 144 ± 70 |
| 125 mg/kg (4) | 10.8 ± 1.9[a] | 4.0 ± 1.0[a] | 54 ± 24[a]<br>*78 ± 35* | 179 ± 37 |
| 250 mg/kg (5) | 7.7 ± 3.1[a] | 3.8 ± 0.4[a] | 52 ± 13[a]<br>*82 ± 21* | 221 ± 82 |

Rabbits were fed a 0.4% cholesterol, 10% peanut oil diet for 5 days and then grouped on the basis of their TPC values. All groups were then switched to a 0.13% cholesterol, 3.3% peanut oil diet with or without added CAI for 23 days (tiqueside) or 24 days (pamaqueside). The number of animals per treatment group is shown in parentheses. A dose of 2.5 mg/kg is approximately equal to 0.01% (w/w). Cholesterol recovery data are the percent of an orally adminstered dose of radio labeled cholesterol recovered in the plasma and liver of rabbits at necropsy. Plasma cholesterol data are from the terminal blood sample. Data in italics are TPC values for each treatment group prior to the start of cholesterol feeding. Body weight change was calculated from the start of CAI treatment. Values are means 6SD [a]*P* , 0.05 vs. controls (Morehouse *et al.,*1999*)*.

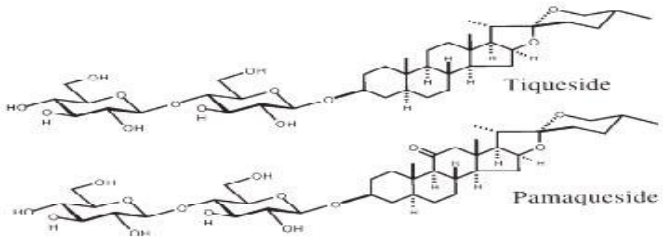



Figure 3. Structures of synthetic saponin cholesterol absorption inhibitors(Morehouse *et al.,*1999*)*.

Group B soyasaponins (Figure 4) lowered plasma total cholesterol (by 20%),non-HDL cholesterol(by 33%), triglycerides (by 18%), and the total cholesterol-to-HDL-cholesterol ratio in female hamsters(by 13%) (Table 2)( Lee *et al*., 2005).

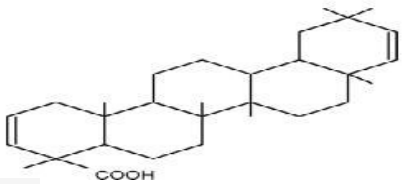


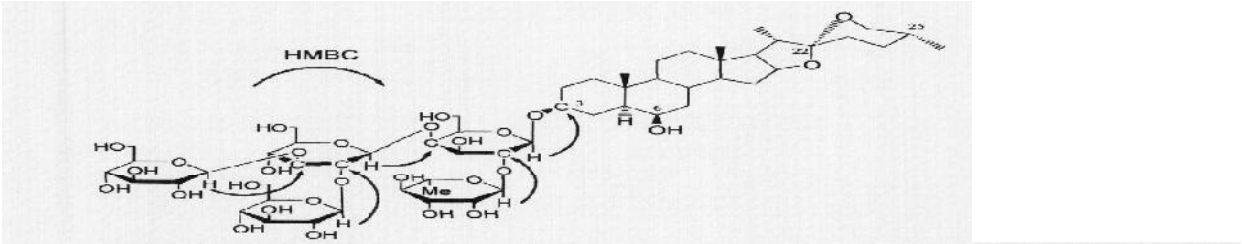

Figure 4. The putative structure of soyasaponin metabolite(Lee *et al*., 2005) .

Table 2. Group B Soyasaponins Lowered Plasma Total Cholesterol and Non-HDL Cholesterol in Female Hamsters[a](Lee *et al*., 2005) .

| Treatment | Total cholesterol (mM) | HDL cholesterol (mM) | Non-HDL cholesterol (mM)[b] | Ratio of total cholesterol to HDL cholesterol | Triglyceride (mM) |
|---|---|---|---|---|---|
| Casein | 6.33 ± 0.32[c] | 3.75 ± 0.53 | 2.58 ± 0.31[c] | 1.69 ± 0.04[c] | 2.67 ± 0.24[c] |
| Group B soyasaponin | 5.14 ± 0.69[d] | 3.43 ± 0.69 | 1.71 ± 0.39[d] | 1.50 ± 0.10[d] | 2.18 ± 0.17[d] |

[a] Values represent means ± SD. $n = 10$. Within a column, means with different superscripts are different ($P < 0.05$).
[b] Represents the LDL + IDL + VLDL fractions (by difference: total – HDL).

Koch (1993) indicated that the cholesterol-lowering effect of garlic preparations may be due to its saponin content. Plant saponins have been shown to inhibit cholesterol absorption from the intestinal lumen in experimental animals, and consequently to reduce the concentration of plasma cholesterol. This may be the result of a complex formation with cholesterol in the digestive tract or a direct effect of plant saponins on cholesterol metabolism (Hosttetmann and Marston, 1995). steroid saponins (Figure 5) may also account for the cholesterol-lowering effect of garlic. Matsuura (2001) found that the saponin fractions from garlic lowered plasma total and LDL cholesterol concentrations without changing HDL cholesterol levels in a hypercholesterolemic animal mode.




$^{1}H$-$^{13}C$ long-range heteronuclear multiple-bond correlation (HMBC) observed for the sugar moiety of Compound 20.

Figure 5. Steroid saponins in Garlic for cardiovascular diseases (Matsuura, 2001)

Alfalfa saponin was reported to be hypocholesterolemic and antiatherosclerotic. This extract (I) produced the most significant decrease in total cholesterol and LDL-cholesterol by 85.1 and 88%, respectively, of the corresponding levels in hypercholesterolemic rabbits. The beneficial effect of alfalfa preparations against hypercholesterolemia and atherosclerosis was further evaluated by histopathological examination of the aorta wall for the assessment of atherosclerosis in presence and absence of the alfalfa intake. Remarkable inhibition of progression of intimal thickening by hypercholesterolemia was noticed upon concomitant administration with all forms of alfalfa extracts, as shown in Figures 6 and 7 (Khaleel *et al*., 2005).

The administration of PS produced profound effects on the control of obesity and lipid metabolism, which resulted in LDL-cholesterol reduction. PS (Figure 8.) also caused a remarkable reduction in calorie intake, which was highly correlated to the body weight loss. These results suggest that PS has a greater role in antiobesity, hypolipidemia, and liver protection than previously thought. Hence, PS could be a potential therapeutic alternative in the treatment of obesity and hyperlipidemia (Zhao *et al*.,2005)

The supplementation of karaya saponin, *R. capsulatus*, and the combination of karaya saponin and R. capsulatus suppressed the incorporation of (14)C from 1-(14)C-palmitic acid into the fractions of total lipids, phospholipids, triacylglycerol, and cholesterol in the liver in vitro ($P < 0.05$). These findings suggest that the hypocholesterolemic effects of karaya saponin and *R. capsulatus* are caused by the suppression of

the cholesterol synthesis and the promotion of cholesterol catabolism in the liver in Figure 9 (Afrose *et al*., 2010).

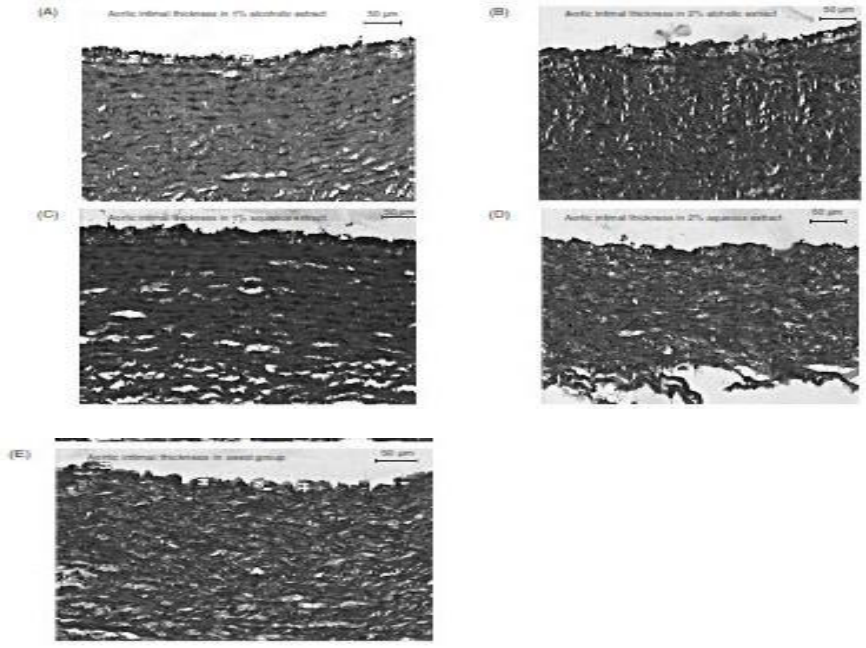


**Figure 6.** Photomicrographs of sections in ascending aorta of alfalfa a treated hypercholesterolemic rabbits. Hx and E, 100□.(A) 1% alcoholic extract; (B) 2% alcoholic extract; (C) 1% aqueous extract;(D)2% aqueous extract; (E) seed. (Khaleel *et al*., 2005)

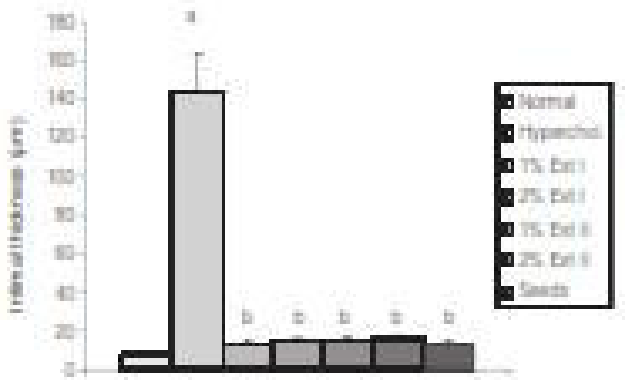


**Figure 7.** Intimal thickness of aorta in normal, hypercholesterolemic,1% extract I, 2% extract I, 1% extract II, 2% extract II, and seeds groups. Values are means ± SEM. Each mean represents the average reading of 10 sections for each aorta for 7 animals (i.e. each reading is an average of 70 values). [a]Significant difference from normal at $p < 0.05$. [b]Significant difference from hypercholesterolemic control at $p < 0.05$ . (Khaleel *et al*., 2005)

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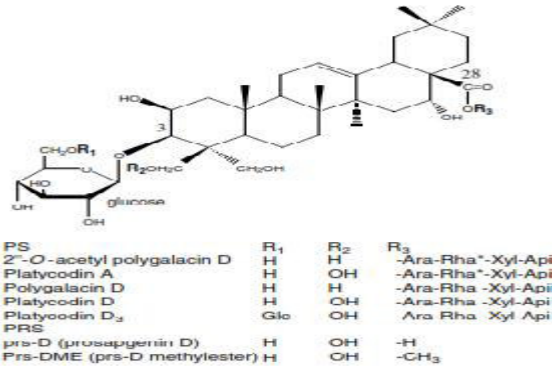

| PS | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 2″-*O*-acetyl polygalacin D | H | H | -Ara-Rha*-Xyl-Api |
| Platycodin A | H | OH | -Ara-Rha*-Xyl-Api |
| Polygalacin D | H | H | -Ara-Rha -Xyl-Api |
| Platycodin D | H | OH | -Ara-Rha -Xyl-Api |
| Platycodin $D_3$ | Glc | OH | Ara Rha Xyl Api |
| PRS | | | |
| prs-D (prosapygenin D) | H | OH | -H |
| Prs-DME (prs-D methylester) | H | OH | $-CH_3$ |

Figure 8. Structure of platycodin saponis(PS) and PRS. Ara: Arabinose, Rha: rhamnose, Rha*: acetylated rhamnose,xyl: xylose, Api: apiose (Zhao *et al*.,2005)

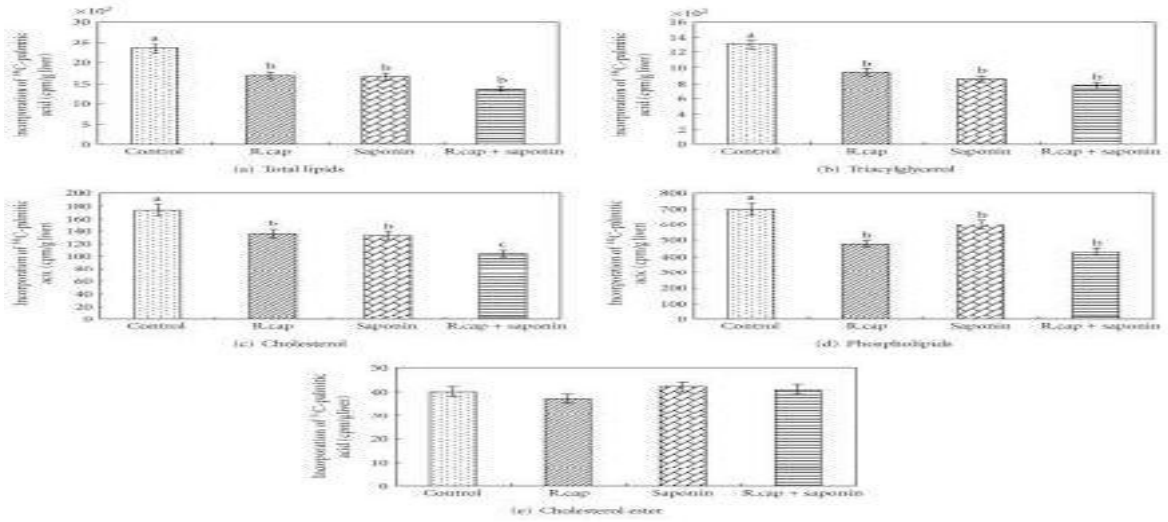


Figure 9.Effect of dietary karaya saponin and *R. capsulatus* on the incorporation of 1-$^{14}$C-palmitic acid into hepatic (a) total lipids, (b) triacylglycerol, (c) cholesterol, (d) phospholipids, and (e) cholesterol ester fraction. Differences were tested by Duncan multiple-range test. $^{a\text{-}c}$Values with different superscripts differ significantly ($P$< .05); "a", "b", "c" indicate significant difference from each other. Values are mean ± SD, $n$ = 10 laying hens (Afrose *et al*.,2010)

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# THE STUDY OF BCL-2 EXPRESSION OF RED FRUIT OIL ETHANOL EXTRACT ON BREAST CANCER CELL LINE T47D

**Agung Wiwiek Indrayani[1], Ida Ayu Ika Wahyuniari[2], I Wayan Sugiritama[2] , and I Gusti Ayu Artini[1]**
*[1]Department of Pharmacology, Faculty of Medicine, Udayana University Denpasar*
*[2]Department of Histology, Faculty of Medicine, Udayana University Denpasar*

**ABSTRACT**

For many years, cancer treatment has widely used chemical substances, hormonal therapy, antibiotics, radiation and surgery. However, these types of treatment have caused many severe side effects, which is also difficult to avoid. One of the alternative treatments suggested recently was using phytopharmaca, by finding out the chemical substance of plants that had potential effect as a new drug. The aim of our study is to investigate the effect of red fruit oil ethanol extract to the Bcl-2 expression in breast cancer cell line T47D. This study was conducted in vitro, using immunocytochemistry methods. For analyzing Bcl-2 expression, we used five divided doses of red fruit oil ethanol extract, then evaluated using imunocytochemistry after 24 hours of incubation. Our study showed that the Bcl-2 expression at five divided doses of red fruit oil ethanol extract (0.625; 0.3125; 0.1562; 0.0781; and 0.0391 µL/mL) were 6.67; 19.67; 31.67; 39,33; and 49,33 per 100 cells, respectively. When compared to positive control (doxorubicin), the Bcl-2 expression at five divided doses (1.5625; 0.7812; 0.3906; 0.1953; and 0.0976 µL/mL) were 14.67; 23.33; 25.67; 30.33; and 44.67 per 100 cells, respectively. The result was statistically significant ($p < 0.001$). In conclusion, red fruit oil ethanol extract has potential effect on reducing Bcl-2 expression in breast cancer cell line T47D in vitro.

***Keywords***: *Red fruit oil ethanol extract, Bcl-2 expression, breast cancer cell line T47D, in vitro*

## INTRODUCTION

As we know, nowadays cancer has become the second cause of death after cardiovascular disease in all gender worldwide. The proportion of death caused by cancer has continued to be risen for several years in all countries.[1] Among all type of cancers, breast cancer was the second most frequent type of cancer found in Indonesian after cervical cancer.[2]

For many years, phytopharmaca has been widely used as alternative treatment, by investigating chemical substances on plants potentially used as drug. Moreover, thirty five food from plants that had chemopreventive effect against cancer has been studied by American National Cancer Institute.[3] Indonesia was the second country in world that had many plants as sources of phytopharmaca.[4]

Bcl-2 expression demonstrated cell's inability to protect itself from apoptosis.[5] In almost all types of cancer, there would be an increased expression of antiapoptotic protein such as Bcl-2 and Bcl-XL. Conversely, proapoptotic protein such as Bax and Bad would be decreased.[6] Bcl-2 family protein was an important regulator of apoptotic pathways. Bcl-2 expression indicated the cell failure to protect itself due to an excessive proliferation of cancer cell.[5]

Red fruit (*Pandanus conoideus* Lam) has been widely used empirically in human in Papua to treat cancer, diabetes, hypercholesterolemia and coronary heart disease. Previous studies have revealed that red fruit oil ethanol extract have cytotoxicity and antiproliferative activity as well as increasing apoptotic on cervical cancer cell line SiHa. It has been suggested from the previous studies that red fruit oil ethanol extract has demonstrated inhibition of cyclooxygenase 2 (COX-2) enzyme expression on breast cancer cell line T47D.[7,8] COX-2 was known to be able inducing carcinogenesis by reducing the level of proapoptotic protein Bax and increasing the anti apoptotic protein Bcl-2. Therefore the apoptotic process on epitel cell of mammary gland would be diminished and subsequently promote tumorigenesis.[6]

The aim of this study is to investigate the effect of red fruit oil ethanol extract on Bcl-2 expression of breast cancer cell T47D *in vitro.* Hopely, this study would give some scientific information regarding the potency of red fruit as chemopreventive agent.

## METHODS

Our study was an experimental study using post test with control group design. Our study was conducted in LPPT (*Laboratorium Penelitian dan Pengujian Terpadu*) Gadjah Mada University. This study was using breast cancer cell line T47D, red fruit oil ethanol extract (*P. conoideus* Lam) and doxorubicin as positive control. Activity of the studied substances on inhibiting Bcl-2 expression was observed by means of immunocytochemistry staining.

Red fruit oil ethanol extract was prepared using maseration method. Immunocytochemistry was conducted using 3% hydrogen peroxidase in methanol, goat serum, primary antibody for Bcl-2, *Phosphate Buffer Saline* (PBS) (pH 7.4), avidine, biotin, secondary antibody IgG biotinylated, avidine conjugate of *horsedish peroxidase*, chromogen 3,3-diaminobenzidine, deionated water and hematoxyline.

Bcl-2 expression on breast cancer cell after treatment of red fruit oil ethanol extract and doxorubicin was observed under light microscope. Cells expressing Bcl-2 demonstrated as brown cell and cytoplasm. It was expressed in term of cells expressing Bcl-2 per 100 cells. The result was analyzed using Anova test with 95% confidence interval.

## RESULTS AND DISSCUSSION

We compare the number of cells expressing Bcl-2 after given red fruit oil etanol extract and doxorubicin with each five dose variation. The morphological feature of cells expressing Bcl-2 in red fruit oil, doxorubicin and negative control group were shown on Figure 1 and 2.

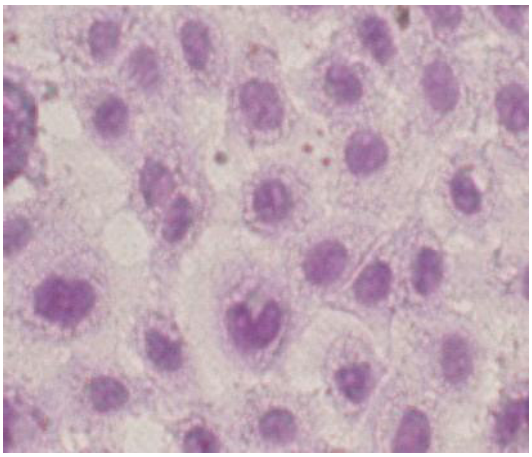

Figure 1. The morphological feature of breast cancer cells given red fruit oil etanol extract 0,6250 µL/mL

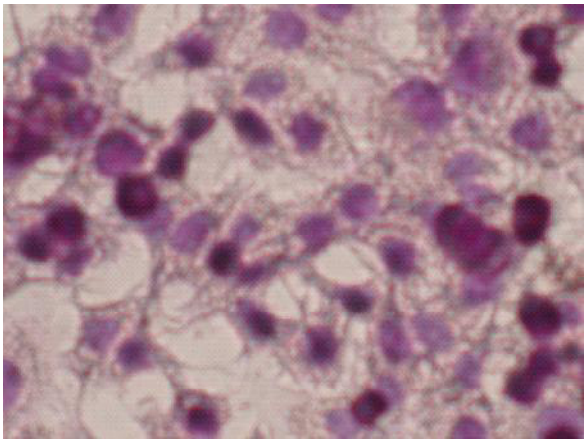

Figure 2. The morphological feature of cells in negative control group

**Positive result of Bcl-2 expression was demonstrated as brown nuclei and cytoplasms**

From the result, we could see that as the concentration of red fruit oil etanol extract increased, the Bcl-2 expression would decrease. In the negative control, it has been shown that the number of breast cancer cell T47D expressing Bcl-2 was relatively higher than other groups. Our result was obviously shown that red fruit oil ethanol extract would reduce Bcl-2 expression on breast cancer cell line T47D.

Our study revealed that the Bcl-2 expression at five divided doses of red fruit oil ethanol extract (0.625; 0.3125; 0.1562; 0.0781; and 0.0391 µL/mL) were 6.67; 19.67; 31.67; 39.33; and 49.33 per 100 cells, respectively. When compared to positive control (doxorubicin), the Bcl-2 expression at five divided doses (1.5625; 0.7812; 0.3906; 0.1953; and 0.0976 µL/mL) were 14.67; 23.33; 25.67; 30.33; and 44.67 per 100 cells, respectively. This result was statistically significant ($p<0.001$) (Figure 3).

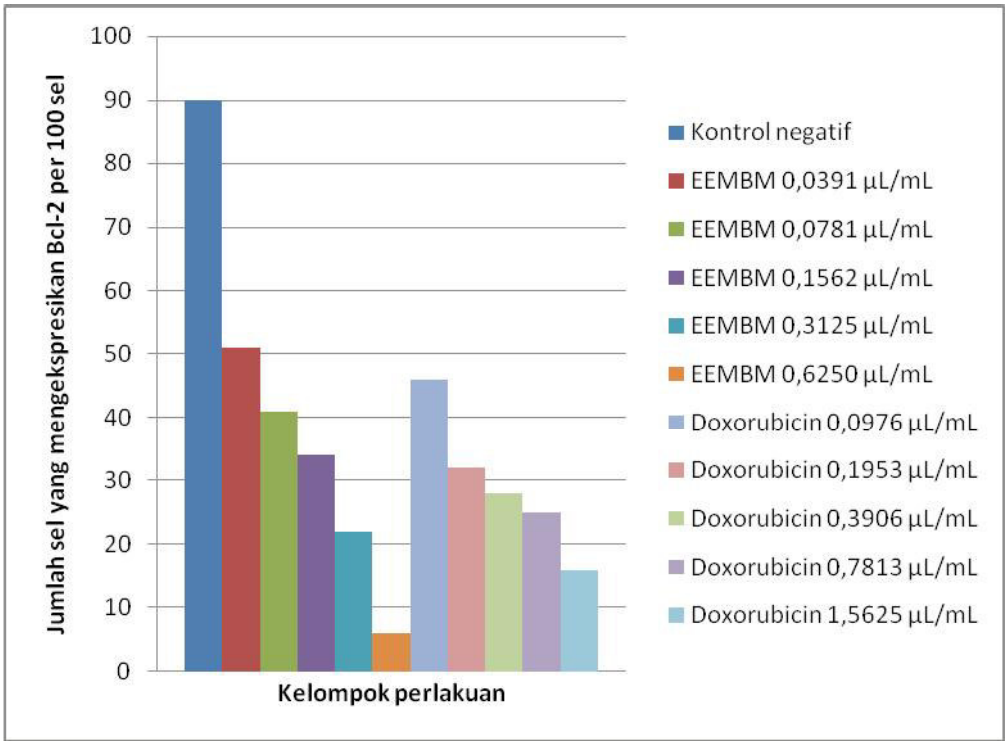


Figure 3. The number of breast cancer cell T47D expressing Bcl-2 per 100 cells in red fruit oil ethanol extract and doxorubicin group after 24 hours of incubation (observation using light microscope with 40X magnification)

Induction of apoptosis on cell line cancer by direct activation of Bcl-2 by plant product has broght new hope for the treatment of cancer where these concepts showed us how the molecule of Bcl-2 kept the integrity of mitochondria and how the pro apoptosis protein BH3 makes damage of the cell.[9] The same thing was shown by red fruit oil ethanol extract on breast cancer cell line T47D that had capacity to influence the expression of Bcl-2. Our study showed that ethanol extract of red fruit oil decreased the expression of Bcl-2. The higher the dosage of ethanol extract red fruit oil given on cell line breast cancer, the lesser expression of Bcl-2 was shown. This result was statistically significant ($p < 0.001$).

It has been assumed from the previous studies that red fruit contained many important substances including 12,000 ppm of total carotenoid, 11,000 ppm of total tocopherol, 700 ppm of betacaroten, 500 ppm of alpha tocopherol, 58% oleic acid, 8.8% linoleic acid and 2.0% decanoic acid.[10,11] Alpha tocopherol and beta carotene was assumed to be able preventing carcinogenesis because oncogenesis could be resulted from free radicals which attack DNA. Alpha tocopherol was assumed to be able capturing reactive oxygen or nitrogen spesies as well as inducing apoptosis.[12]

Induction of apoptosis on tumor cell by direct activation of Bcl-2 pathway was thought to bring new hope for developing new anticancer agent with minimal side effects on normal tissues. As we know previously, chemotherapy treatment was a systemic treatment which might also affecting normal tissues. Moreover, chemotherapy-induced toxicity on normal tissues was related to its antiproliferative activity which subsequently destruct DNA and initiate apoptosis. This side effect commonly observed on bone marrow, epithel of gastrointestinal tract and hair follicle.[1,13]

The Bcl-2-like molecules have been assumed to have an ability for maintaining mitochondrial integrity whereas proapoptotic protein BH3 have been assumed to destroy cells.[9] The Bcl-2 expression has caused many stress variation that would subsequently responsible for the failure of Bcl-2 in causing cell apoptosis.[5]

## CONCLUSION

In conclusion, red fruit oil ethanol extract has potential effect on reducing Bcl-2 expression in breast cancer cell line T47D in vitro.

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# THE ROLE OF INTERLEUKIN 22 IN LUNG TUBERCULOSIS AND ITS MOLECULAR ASPECTS

**Ni Made Linawati[1], Dewa Made Sukrama[1], Made Mertaniasih[2], and Mantik Astawa[3]**
*[1]Medical Faculty of Udayana University, Bali. [2]Medical Faculty of Airlangga University, Surabaya.*
*[3]Faculty of Veterinary Medicine Udayana University, Bali. Correspondence author: linawandira@yahoo.com*

**ABSTRACT**

Tuberculosis caused by *Mycobacterium tuberculosis* is a major health problem with 10 million new cases are diagnosed each year. Immunopathogenesis pulmonary tuberculosis is involving the role of the innate immune responce as a defense against *Mycobacterium tuberculosis* infection. The methods in this paper is review article, about the recent research in interleukin 22, the molecular aspect and its related with tuberculosis. Natural killer (NK) cells has an important role in controlling tuberculosis through secretion of interleukin 22 or IL-22 which stimulates phagolysosome fusion of macrophages infected with *Mycobacterium tuberculosis*. In human, gene encoding IL-22 located on chromosome 12q15, 5257 bp gene in length, 5 exons and 4 introns, the location of the start codon in the sequence of bases no. 54 and a stop codon at base sequences no.4703. IL-22 is produced by activated NK cells and Th17. Formation of this cytokine resembles production of proteins that generally pass through the process of transcription, translation, post translational modifications and secreted by regulated exocytosis with paracrine characteristic. It can be concluded that interleukin 22 was produced by NK cells stimulate phagolysosome fusion in macrophages infected by *Mycobacterium tuberculosis* and gene encoded IL-22 located on chromosome 12q15.

***Keywords**:* Interleukin 22, Lung tuberculosis

## INTRODUCTION

In 2010, World Health Organization or WHO showed Indonesia is number fifth in the country with the highest TB burden in the world. The problem of tuberculosis in Indonesia was exacerbated by growing epidemic Immunodeficiency Human Virus (HIV), the prevalence of HIV in new TB patients was 2.8%. Multi Drug Resistance (MDR) tuberculosis is estimated at 2% of all new TB cases and 20% of tuberculosis cases with re-treatment and there are approximately 6,300 cases of MDR tuberculosis each year (Anonym 1, 2010; Anonym 2, 2011). Recent research suggests NK cells through the secretion of IL-22 can enhance the activation of macrophages infected with *Mycobacterium tuberculosis* by enhancing phagolysosomes fusion so it can kill the microbe with acid lytic enzymes (Dhiman *et al.,* 2009; Zhang et al., 2011). Genes encoding human IL-22 is located on chromosome 12q15, while in mice is located on chromosome 10. The gene encoding IL-22 that will be translated into IL-22 (Anonim 3. 2011). IL-22 is also a family cytokine IL-10. Signal sent from IL-22 is passed through the IL-22 receptor that pairs with receptors IL-10β, IL-10β receptors which are ubiquitously expressed whereas IL-22 receptor specifically expressed on epithelial tissue and are believed mediates epithelial innate response (Zheng, 2008). NK cells as the major source of IL-22 are important in regulating the function of mucosal epithelial cells, maintaining barrier integrity, and protection from bacterial infection in the lungs and stomach. At the time of primary infection of the respiratory tract, IL-22 has role in protection against viral and bacterial infections including *Mycobacterium tuberculosis* (Walzer et al., 2007; Guo et al., 2010; Connelley et al., 2011). This review was aimed to describe the role of Interleukin 22 in lung tuberculosis and Its molecular aspect.

## DISCUSSION

*Mycobacterium tuberculosis* infection were spread perinhalation, in the balance of the immune system can manifest into three main conditions: first; Healthy, second; Suffering pulmonary tuberculosis, and third; Containment of *Mycobacterium tuberculosis* which is seem healthy. The condition in which immune system eliminated Mycobacterium it will be a state one, but if it can not be eliminated Mycobacterium can be state 2 and 3 (Kaufmann et al., 2001). *Mycobacterium tuberculosis* first to be recognized by macrophages through its various receptors such as TLR, mannose receptor, complement

receptors and scavenger receptor (Doherty, 2004). After the phagocytosis *Mycobacterium tuberculosis* by macrophages, furthermore Mycobacterium already present in the phagosome of macrophages to kill with mechanism such as phagosome fusion with lysosomes that would lysis Mycobacterium by enzymes in the lysosomes, Reactive Oxygen Intermediate (ROI) and Reactive Nitrogen Intermediate (RNI) in which nearly the whole of the process will occur in an optimal state of macrophage activation. Optimal macrophage activation will cause lysis of *Mycobacterium tuberculosis* but if it is not in an optimal activation, *Mycobacterium tuberculosis* can survive because they have cell walls containing lipoarabinomannan can prevent fusion lysosomes with the phagosome, inhibit phagocytosis, formation of ROI / RNI or the occurrence of respiratory (oxidative ) burst or evade phagosome trap so its remains free in the cytoplasm and avoid further killings and its occur mainly in non-activated macrophages (Schorey et al., 2002; Flynn, 2004). For the optimal activation of macrophages requires a good signal released by immune cells in the natural immune system such as NK cells and immune cells of the adaptive immune cells such as T Helper. Macrophages infected with *Mycobacterium tuberculosis* will secrete IL-12, IL-15, IL-18 and other cytokines that can activate NK cells to produce IFN-γ and IL-22, while approximately 3-4 weeks occur Th1 cell activation to produce IFN-γ and Th17 cells to produce IL-22 (Cooper et al., 2009; Yao S et al., 2010). IL-22 is secreted by NK cells binds to IL-22R1 found on the surface of macrophages infected with *Mycobacterium tuberculosis* then activate the signaling pathway JAK / STAT and MAP kinase. IL-22 receptor complex activates the JAK kinase causes phosphorylation of IL-22R1, JAK1, TYK2 and STAT3. Phosphorylation of STAT3 translocation to the nucleus and activates transcription of genes causing cellular response of IL-22 as an increase in delivery of *Mycobacterium tuberculosis* to lysosomes through increased fusion fogolisosom. The secretion of IL-22 in addition to increasing phagolisosom fusion causes lysis of *Mycobacterium tuberculosis* can inhibit the growth of *Mycobacterium tuberculosis* in macrophages (Lejeune et al., 2002; Dhiman, 2009). Genes encoding IL-22 with gene ID: 50616, located on chromosome 12q15 (NC_000012.11) consisting of 5257 bp of DNA, has 5 exons and 4 introns, location of the start codon in the sequence of bases no. 54 and a stop codon in the sequence of bases no.4703 (Anonim 3, 2011). Domoutier et al. (2008) mapped the human gene IL-22 / IL-TIF contains 6 exons and 5 introns including non-coding exons short consisting of 22 bp downstream of the TATA box. Genes encoding IL-22 is also called ILTIF gene (IL-10 related T cell-derived inducible factor), first identified in mice on chromosome 10 which is a gene induced by IL-9 in T cells and mast cells (Domoutier et al. , 2000). Genes IL-22 and IL-22R was first cloned by Xie et al., 2000, with sequence analysis predicted that the N terminal 33 amino acids of IL-22 functions as a signal sequence for IL-22 translation. mRNA of IL-22 (NM ¬ _020525.4) consisted of 1147 bp mRNA, which is divided into five exons. IL-22 mRNA encoded a protein cytokine IL-22 (NP_065386.1) comprising 179 amino acids. Natural variation present in mRNA levels occurred in aa no 158 where S ◊ G (dbSNP: rs2227507), in addition to as many as 17 mutations such as SNPs reported to have occurred at the mRNA level of IL-22 and are associated with various infections and inflammatory diseases (Anonim 3, 2011).

## CONCLUSION

Genes encoding IL-22 is located on chromosome 12q15 DNA consists of 5257 bp, 1147 bp mRNA to be translated into IL-22 which is contain of 179 amino acid in length Polymorphisms of the gene encoding IL-22 was associated with several diseases including tuberculosis susceptibility. IL-22 in tuberculosis has a role in natural and adaptive immune responses. Association of IL-22 to its receptor on the surface of infected macrophages can lead to increased fusion fagolisosom resulting lysis of *Mycobacterium tuberculosis*.

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# HUMAN PAPILLOMA VIRUS (HPV) GENOTYPING FROM PARAFFIN BLOCK ARCHIVE OF PATIENT WITH CIN DIAGNOSE THAT STORED AT PATHOLOGY ANATOMY DEPARTMENT FACULTY OF MEDICINE UDAYANA UNIVERSITY/ SANGLAH HOSPITAL BALI

**Dwija, IBN.Putra[1] and I Gusti Ayu Sri Mahendra Dewi[2]**
*[1]Microbiology Department, Faculty of Medicine Udayana University.*
*[2]Pathology Anatomy Department, Faculty of Medicine Udayana University*

ABSTRACT

Human Papilloma Virus (HPV) is a DNA, double stranded virus that infect cutaneous, feet and hand. Up to now more than a hundred types of HPV has been typing, and one third of its infected the anogenital tissue. Cervical cancer is one of malignancy that caused by HPV. DNA of the HPV can be isolated from scraping of cervical epithelial and from tissue that embedded in paraffin block. The quality of specimen, method that used to embed, the method and time to keep the blocks in storage place will influence the quality of DNA of HPV. The aim of this research is to know the quality of the DNA and genotyping of the HPV from the archive of cervical tissue that has been embedded in paraffin block for more than 2 years in Pathology Anatomy Department Faculty of Medicine Udayana University/ Sanglah Hospital. Fifty blocks were slicing with 10 µm thickness, HPV's DNA Isolated using PK-1 buffer method, PCR were done by set of general primer (GP5/6+), the PCR product was send to the Macrogen Company for sequencing and sequence result were analysis by Chromas 1.5 version. This research was conducted at Pathology Department Leids Universitair Medische Centrum (LUMC) Leiden, Netherland during April-May, 2012.

From the 50 block of tissue, DNA with best quality was isolated from 12 of 50 (24%) blocks and we failed to isolated DNA from 38 (76%) of paraffin blocks. From the 12 DNA isolated, PCR Product was detected from 9 specimens. This all of 9 PCR products was send to the Macrogen for sequencing. According to Chromas 1.5 version analysis, 3 of 9 (33.3%) is HPV 16, 1 (11.11%) is HPV 18, 1 (11.11%) is HPV 31, 1 (11.11%) is HPV 58, 1 (11.11%) is HPV 11 and 2 (22.22%) other has no sequencing result. From this result can we concluded that HPV 16 is the most HPV type isolated from the cervical tissue of paraffin block archive and the quality of the paraffin block is not good enough.

***Keywords:*** HPV typing, Paraffin Block, CIN, Sanglah Hospital

## INTRODUCTION

Gynecological cancer is still a major problem in Indonesia right now. Cervical Cancer is the most common gynecological cancer in Indonesia, based on Pathologic report, in 2002 as much as 2.532 cases of cervical cancer were registries with five years survival rate was poor in the higher stages of malignancies (Aziz, 2009). *Human Papilloma Virus* (HPV) is the most common agent of cervical cancer. HPV is a double stranded virus that infects coetaneous, feet and hand, with total genome is more than 7000 base pair (bp). Until now more than 100 genotypes has been established, and one third of them is categories as a high risk group.

Some kind of specimens can be use to isolation of DNA of HPV. Isolation of the HPV DNA from the Formalin Fixed, Paraffin Embedded (FFPE) tissue is one of the interests method in last few years to detect and genotyping of HPV from cervical specimen, head and neck cancer (Schlecht *et al*, 2011., Steinau, *et al*., 2011). Stored FFPE specimen has some of advantages, such as possible to be use as retrospective research and epidemiological study. HPV DNA that stored in a long time in FFPE still have a good quality, therefore, DNA fragmentation, DNA-protein cross linked because of the presence of formaldehyde, and the paraffin its self can be a source of negative effect in yield of DNA and PCR amplification (Steinau *et al*.,2011). Standard method that used to embedded the tissue and storage the FFPE block has been established, but different geographic condition such as temperature, humidity, and quality of the paraffin may be influence the quality of the FFPE block and DNA as well.

Detection of the high risk HPV (HR-HPV) is most important role to reduce the incidence of cervical cancer by active vaccination. Cervical cancer stills the most common cancer in developing countries, as found in Indonesia. Type specific HR-HPV is fluctuation in Indonesia during study (Vet *et al*, 2008; Boer *et al*., 2006).

As we know there is no data about the isolation of the HPV DNA and Genotyping. From the FFPE block archive in Pathology Anatomy Department Faculty of Medicine Udayana University/ Sanglah Hospital. The aim of this study is to determine the genotyping of HPV that isolated from the archive of the paraffin block that stored at the Pathology Anatomy Department, Faculty of Medicine Udayana University.

## MATERIALS AND METHOD

This Research is a cross sectional descriptive analysis using 50 archive of paraffin block from the patient with CIN diagnose that storage at the Pathology Anatomy Department Faculty of Medicine Udayana University Bali. Research was done at Pathology Department Leids Universitair Medische Centrum (LUMC) Leiden, Netherland, during April-May, 2012.

### DNA Extraction

As much as 1-2 slices of paraffin block of 10 µm thick was collected with new and sterile 1.5 ml micro tube. DNA extraction was done using PK-1 buffer method. Two hundred and fifty micro litter PK-1 buffers and 15 micro liter of Proteinase K were added to the microtube, the tube incubated at 560C overnight, until all the paraffin dissolved. If not all paraffin dissolved after overnight incubation add other 15 µl fresh Proteinase K and extended the incubation at 560C for 4 hour. After overnight incubation at 560C, followed by incubate the micro tube at 990C for 10 minutes to inactivate the proteinase K. Briefly centrifuge the micro tube at 13.000 rpm for 10 minutes at 40C. The liquid phase under the paraffin layer were collected and stored at -20$^0$C for further analysis.

### HPV PCR

Polymerase Chain Reaction (PCR) was done using GP5/6+ primer pair (GP5+ : 5'-TTT GTT ACT GTG GTA GAT ACT AC-3', GP6+ : 5'-GAA AAA TAA ACT GTA AAT CAT ATT-3') (van den Brule *et all*.,2002), which is amplified the L1 genome of HPV. As much as 11,5 Biorad®-supermix, 1 µl of forward and reverse primer, and 6.5 µl of de-ionized of water and 3 micro liter of DNA template were added to the total 23 micro liter volume PCR reaction. For the quality of DNA β-globin PCR was also done using a specific primer pair.

### Sequencing

Ten micro litter of PCR product was send to the Macrogen Company, Netherland for sequencing. Sequencing result was analysis using a free Chromas 5.1 program.

### H&E Staining

To analysis the abnormalities of the cervical squamosa cell, H&E staining were done with the standard method. The pathologist will read the staining slide to determine the stages of malignancies.

## RESULTS AND DISCUSSION

Among fifty slices of FFPE tissue were staining with H&E Staining, to determine the stage of the CIN. Among them 5 (10%) is Non Keratinizing squamous cell carcinoma, 19 (38%) is CIN 1-Chronic cervicitis, 5 (10%) Chronic cervicitis, and 21 (42%) is CIN 1-2. With the range of age is 27-66 (mean 45 years old). The cell morphology of the cervical cell with atipia coilositic can seen in figure 1.

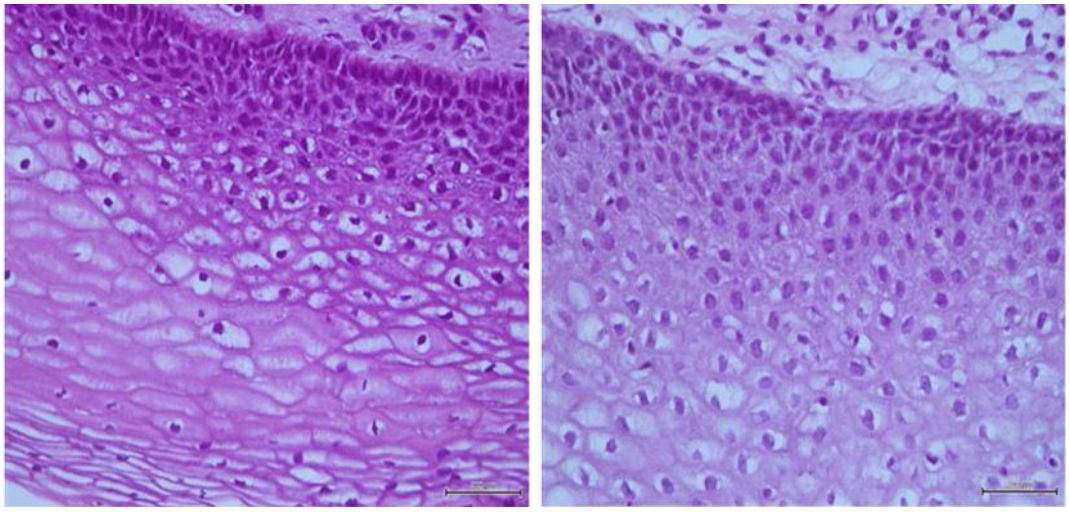

Figure 1. H & E Staining from the normal cervical cell and Cervical Intraepithelial Neoplasia (CIN) I with Koilocytic Atipia suspected with HPV infection.

On conventional histopathological examination, the HPV infection in cervical cells characterized by atipia koilositik, characterized by core atipia in cervical squamous cells, the core size is varied and enlarged to three times its normal size, hypercromasia on core chromatin, irregular nuclear membrane, the cavitations or halo around the nucleus of the cell cytoplasm and cell membrane thickening

DNA extraction with PK-1 buffer among fifty specimens has not good quality of DNA, that shown in the qPCR there is no band in the electrophoresis gel, to get good quality of DNA, we decide to purified the DNA with a robotic system, after purification, among 12 (24%) specimen has a good quality of DNA and still no DNA detected was in 38 (78%) specimen. PCR analysis we did from the 12 specimens but in electrophoresis the PCR product detected in only 9 specimens.

Based on the result of sequencing analysis of 9 specimen, 3 of 9 (33.3%) is HPV 16, 1 (11.11%) is HPV 18, 1 (11.11%) is HPV 31, 1 (11.11%) is HPV 58, 1 (11.11%) is HPV 11 and 2 (22.22%) other has no sequencing result.

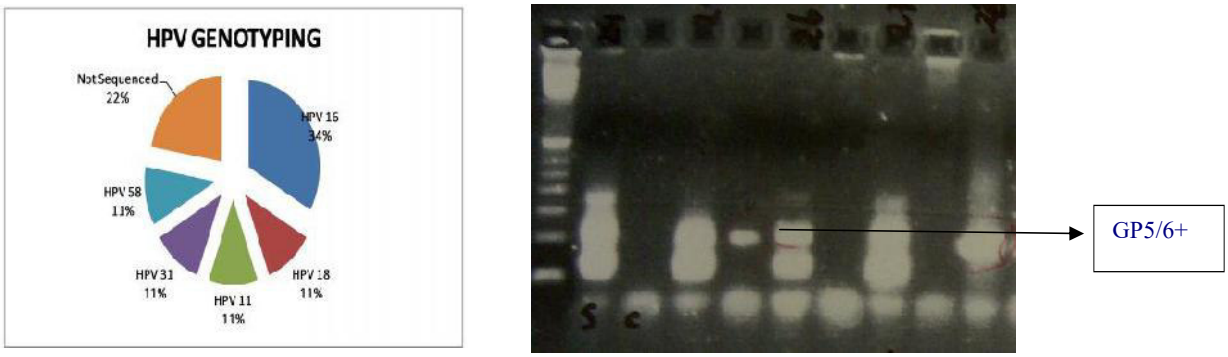


Figure 2. HPV type (left), PCR with GP5/6+ which amplify 155 bp of L1 gene of HPV (right)

Some of un-specific band was also found during electrophoresis, for cross check the type of the HPV, the PCR product was send to the Macrogen Company for Sequence with result as bellow.

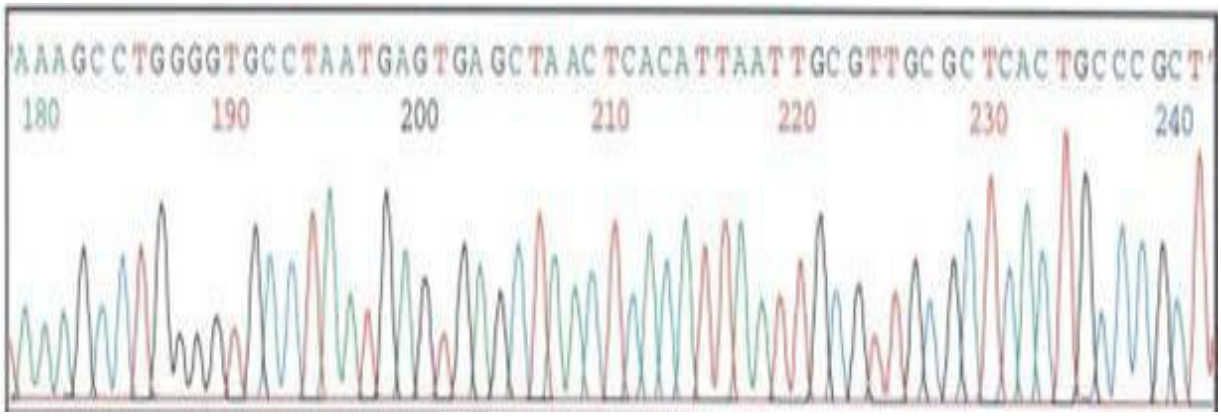


Figure 3. Sequence result of the PCR product of HPV Typing.

The recent finding of this research is extraction with PK-1 buffer has not good quality of the DNA. Some method and protocols of FFPE extraction has been published with a varying of success ( Gilbert *et all*.,2007; Man, *et all*., 2001). The quality of the DNA will determine the quality of the PCR, even PCR is needed a small amount of DNA. FFPE block can preserve the DNA for a long time period, extraction of DNA from FFPE specimens with applying the heat leaves melted paraffin and other some remain debris in the eluted DNA and influence the subsequent PCR reaction. DNA fragmentation, DNA-protein cross linked because of the presence of formaldehyde, and the paraffin its self can be a source of negative effect in yield of DNA (Stienau *et al.*, 2011; Santa and Schneider, 1991).

It was recognized that the quality and yield of DNA from FFPE specimen was low, even the extraction doing with perfect method, the best size of the DNA from FFPE for PCR amplifies is 450 bp (Hariri *et al*., 2012). Some of the primer pair for amplification of HPV L1 gene such as MY09/11, GP5+/6+, CPI/CPII which is amplifies different size of gene. We use the GP5+/6+ primer because this amplify the short fragment of DNA, about 155 bp, because of the possibility of fragmented DNA during storage, using primer with short fragment amplifies will improve the yield of PCR.

During this research HPV type 16 is the most prevalence type was found. Other research was also found the same result, de Boer *et al.* (2006) and has found HPV 16 is the most prevalence in Jakarta. Multiple HPV type infection was found predominantly in adenosquamous carcinomas which is type 16 and 18 was dominant (Schellekens, *et al*., 2004). In multi centre study in Indonesia, Vet *et al* (2008) found HPV 52 was the highest frequency, instead of HPV 16 and 18. Suggesting high risk HPV (HR-HPV) (type 16, 18 and 52) was same circulating in Indonesia.

As a conclusion, the quality of the DNA that extracted from the paraffin block archive is not good enough due to the quality of specimen during embedding or storage situation. HPV 16 is the most prevalence HPV type found, instead HPV-18,-31 and 52.

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# THE EFFECT OF AMARGO WOOD (*Quassia amara* Linn.) INFUSE ON THE MICE (*Mus musculus* L.) TESTICAL WEIGHT AND ITS RECOVERY

**Ni Gusti Ayu Manik Ermayanti**
*Biology Department, Faculty of Mathematic and Natural Sciences,*
*Udayana University, Bukit Jimbaran, Bali, Indonesia*

**ABSTRACT**

This research investigated the effect of amargo wood infuse on the mice testical weight and its recovery. Complete randomized factorial design was used in the experiment. Some 30 male mice were grouped into group treatments. Each group was further divided into 5 sub group dosage treatments, namely 0 (control), 0 (placebo), 1000, 2000, dan 4000 mg/kgBB. Infuse was given by oral gavage method at the volume of 1 mL each day for 35 days and the testical weight was observed. The analysis of variance (ANOVA) of the data showed that the amargo wood infuse significantly ($p<0.05$) affected the weight of mice testis (significant different between treatment I and II). For the observation I, an increase in the infusion dose caused a decrease in testical weight and for observation II infusion decreased the effect and showed an improvement of testical weight, although it is not fully recovered.

***Keywords:*** amargo wood, testical weight, fertility

# MOLECULAR ANALYSIS OF HBV PRE-S/S REGION OF HEPATITIS B VIRUS ISOLATED FROM MEN WHO HAVE SEX WITH MEN IN SURAKARTA

**Hudiyono[1,2], Angga Dwi Prasetyo[1], Afiono Agung Prasetyo[1,2,3]*, Yulia Sari[2,3,4], and Paramasari Dirgahayu[2,3,4]**
*[1]Department of Microbiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[2]Center of Study of Biotechnology and Biodiversity Research and Development, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[3]Biomedical Laboratory, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[4]Department of Parasitology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
**Correspondence Author E-mail: afieagp@yahoo.com; afie.agp.la@gmail.com*

**ABSTRACT**

Hepatitis B virus (HBV) is one of the most contagious hepatitis viruses. People infected with HBV could develop chronic hepatitis disease, with increasing morbidity and mortality rate. In order to build a molecular data base of HBV circulated in Men Who Have Sex With Men (MSM) in Surakarta, 143 MSM were involved in an molecular epidemiology study in October 2010-October 2011. The blood samples were collected from all participant study, aliquoted, and fractionated. Plasma was addressed for HBsAg assay. The nucleic acid was extracted from all HBsAg positive plasma samples, and subjected for the HBV genome detection to amplify the Pre-S/S region. The positive PCR products were sequenced and molecular analyzed. HBV/B3/adw2 was found predominant, followed by the HBV/C1. Deletion on pre-S1 start codon and substitution on D27E, S55A, P65T, T87P, T90A, L101V, A91T, and S109T were found, respectively. Results indicate the need for adequate management of HBV infection in the MSM community.

***Keywords***: HBV, Pre-S/S, MSM, Indonesia

# IDENTIFICATION OF *Vibrio cholera*'s *ctxA* GENE BY PCR METHODE FROM SEA WATER AROUND BALI ISLAND

**Komang Januartha Putra Pinatih, Made Agus Hendrayana***
*Clinical Microbiology Department, Udayana University, School of Medicine, Denpasar, Bali, Indonesia*
**Correspondence Author:agshendra@yahoo.com*

**ABSTRACT**

Sea water could be contaminated by pathogenic microorganisms. The biological contamination is associated with living microorganisms from the indigenous microflora of the marine environment, domestic, industry and livestock waste. One of the pathogenic bacteria that can contaminate sea water is *Vibrio cholera.* The main aim of this research was to identify *ctx*A gene from *V. cholerae* that isolated from sea water samples from 22 beaches tourism destination around Bali Island. Alkali peptone water and TCBS media were use on this study. We looked for the specific colonies on TCBS media. The human pathogenic *V.cholerae* was characterized by virulent genes. One of this gene is ctxA. The presence of virulent gene ctxA was determined by using PCR assay with specific primers ctxA-2. The positive results from *V.cholera* identification using TCBS media showed that 5 of 22 samples (22.7%) were *V.cholera* positive. From the PCR product electrophoresis results, none of five *V.cholera* positive samples (0.0%) was PCR positive. The results indicated that none of *V.cholerae* samples had ctxA gene.

***Keywords***: *Vibrio cholera, ctxA* gene, sea water

# TREATMENT OF TOPICAL ASTAXANTHIN GIVE A PROTECTIVE EFFECT ON COLLAGEN IN PREMATURE SKIN AGING INDUCED BY UV B IRRADIATION IN MICE (*Mus musculus*)

**Komang Ardi Wahyuningsih, AAGP Wiraguna, Wimpie Pangkahila**

**ABSTRACT**

Photoaging is an extrinsic skin aging caused by chronic ultraviolet (UV) irradiation. Characteristic of photoaging is wrinkle formation caused by reactive oxygen species (ROS) generate when skin exposed to UV light. It can be prevented by antioxidant, given topically or orally. One of the most powerfull antioxidant is astaxanthin. The aim of this study was to evaluate that UV B irradiation with total dose 840 mJ/cm$^2$ in 4 weeks could decrease the dermal collagen expression and to evaluate the protective effect of topical astaxanthin to the skin from UV B irradiation that characterised by the increase of dermal collagen expression in mice. Mice (*Mus musculus*) aged 2.5 month old were used in this study as subject since anatomically they are similar to the young adult human. This study was an experimental study using the research method of pre-test post-test control group design. Sample of these study was 27 mice that divided into 2 groups, which was pre-test group, 3 mice, and post-test group, 24 mice. The post-test group than divided into 3 groups, those were control group, UV B group, and UV B+Astaxanthin group, each group consist of 8 mice. The UV B and UV B+Astaxanthin group received UV B irradiation for 4 weeks with total dose 840 mJ/cm$^2$ and the UV B+Astaxanthin group applied with 0,02% topical astaxanthin before and after irradiation while the UV B group applied with base gel. Before the treatment, mice in the pre-test group was decapitated and histologic preparation of skin with sirius red staining were made for measuring the dermal collagen expression of the skin using digital analysis method. In the end of study period, the post-test group was decapitated and histologic preparation were made using the same method and than measuring the dermal collagen expression of the skin using the same method. The result showed, mean difference dermal collagen expression post-test and pre-test in control group was 0.051; in UV B group was -0.031, and in UV B+Astaxanthin group was 0.102. That were tested with Anova test and the result was significantlly different ($p<0.05$). Furthermore it were tested with post hoc test for multiple comparison between group, the result was significantlly different ($p<0.05$). In conclusion UV B irradiation with total dose 840 mJ/cm$^2$ in 4 weeks decrease dermal collagen expression and pure topical astaxanthin increase dermal collagen expression so it has a role of protective effect on dermal collagen expression on the skin of mice induced by UV B irradiation.

***Keywords***: photoaging, ultraviolet B, collagen, astaxanthin

# CIGARETTE SMOKE EXPOSURE DECREASE SPERM QUALITY OF MALE MICE (*Mus Musculus* L.)

**Ni Made Rai Suarni, A.A.S.A.Sukmaningsih, Iriani Setyawati**
*Biology Depertement, faculty of Mathematic and Natural Sciences, Udayana University,Bali*

**ABSTRACT**

The effects of cigarette smoke on several aspects of sperm quality of male mice *(Mus musculus* L.) were observed. Ten male mice, 10-11 weeks old were exposed by ten cigarette smoke, labeled Country every day for 36 days and ten male mice for control. Sperms were obtained from the caudal part of epididymis. The results showed that cigarette exposure decreased the sperm quality, i.e. decrease the amount of spermatozoa, increase of the immotile, dead and abnormal spermatozoa.

***Keywords****: cigarette smoke, caudal epididymys, sperm quality*

# EPIGENETIC APPROACH AS POTENTIAL ANTI-AGING THERAPY OF ANDROPAUSE IN THE FUTURE

**IGN Sri Wiryawan, Ni Made Linawati, I Wayan Sugiritama, IA Ika Wahyuniari, IGA Dewi Ratnayanti, and IGK Nyoman Arijana**
*Histology Department, Faculty of Medicine, Udayana University, Denpasar, Bali, Indonesia*
*Correspondence Author: nyomanarijana@yahoo.com*

**ABSTRACT**

Andropause is a condition in aging men with declined testosterone serum level, and several studies have shown declined testosterone level is not the result of reduced luteinizing hormone level, but effect of decreased testosterone production in aging Leydig cells. Further studies have shown declined component of testosterone synthesis in aging Leydig cells play a role, these include luteinizing hormone receptor, steroidogenic acute regulatory protein, translocator protein, and steroidogenic enzymes. The defects are related to reactive oxygen species-induced damage during steroidogenesis in mitochondria. However, the mechanism of down-regulated expression these proteins in aging Leydig cells is not well understood. Meanwhile in other age-related disease animal model, there are growing evidence that alteration epigenetic profile play a role in aging associated with reactive oxygen species-induced damage. Based on these growing evidences, this review discusses the logic and possibility that alteration epigenetic profile is occurred in aging Leydig cells. Hence, epigenetic approach will become potential target therapy of aging Leydig cells in andropause.

***Keywords***: epigenetic, aging, andropause, oxidative stress

# ANTIOXIDATIVE AND NEPHROPROTECTIVE ACTIVITY OF BALINESE PURPLE SWEET POTATO (*Ipomoea batatas* L.) IN DIABETIC RATS

**I Wayan Sumardika[1], I Made Jawi [1], Agung Nova Mahendra[1], and Made Linawati[2]**
*[1]Department of Pharmacology and [2]Histology Faculty of Medicine, Udayana University*
*Correspondence Author: sdikarox@yahoo.co.uk*

**ABSTRACT**

Anthocyanins from plants are potent antioxidants, so they may have beneficial effects against oxidative stress-related diseases, such as diabetic nephropathy in diabetic patient. Balinese purple sweet potato is an anthocyanins-rich food source. This study was aimed to investigate the effect of water extract of Balinese purple sweet potato on oxidative stress in diabetic rats. Subject of this study were 30 male adult rats divided into 3 groups with randomized post-test only control group design. Group 1 (Diabetic group) were given streptozotocin 60 mg/Kg BW. Group 2 (Treatment group) 3 days after streptozotocin were given 4 ml of water extract of Balinese purple sweet potato per day for 60 days. Group 3 (Control group) only given placebo as control group. Blood glucose, malondialdehyde (MDA), and total antioxidant level of all rats were measured after 50 days of treatment. The data were then analyzed by using Anova. The results showed a significant increase of blood glucose and malondialdehyde (MDA) level in diabetic group ($p < 0.05$), and decrease of total antioxidant level ($p < 0.05$). The increase of blood glucose and malondialdehyde (MDA) level, and the decrease of total antioxidant level in treatment group are significantly lower than in diabetic group ($p < 0.05$). From these findings, it can be concluded that water extract of Balinese sweet purple potato can decrease blood glucose and oxidative stress in rats with streptozotocin-induced diabetes mellitus.

***Keywords***: Balinese purple sweet potato, blood glucose, total antioxidant, diabetic rats

# DEVELOPMENT OF SPECIFIC PCR-RAPD METHOD FOR DETECTION OF *Lactobacillus* sp F2

**K.A. Nocianitri[1], Y. Ramona[2,3], and I N Sujaya[2,4 *)]**
*[1]Department of Food Sci. Tecnol., Faculty of Agricultural Technol, [2]Integrated Lab. Biosci. Biotechnol.; [3]Department of Biology, Faculty. of Sci., [4]Sch. of Public Health, Faculty of Medicine., Udayana University, Bali, Indonesia*

**ABSTRACT**

The objective of this reserach was to develop a molecular method that could be applied to differentiate *Lactobacillus* sp F2 with other closely realted Lactobacilli, Pediococci, and Weissella. The method was developed by searching an appropriate primers that produced a dignostic band specific for *Lactobacillus* sp F2 based upon random amplified of the genomic DNA using several primers, such as OP, M13F and M13R. It was that the OP1, OP3, and M13 produced a more divers bands when compared to other primers. Event though not all the primers used could produce a diagnostic band for *Lactobacillus* sp. F2, M13R was found to produce diagnostic bands for descriminating the *Lactobacillus* sp F2 from other related strains. Th size of the bands ranged from 1200-1300 bp (2 distinct bands) and a 700 bp band. Further experiment conducted by direct PCR of *Lactobacillus* sp. F2 colony isolated from agar plates produced similar results to those obtained using pure DNA. This suggested that the PCR RAPD using M13R primer specific for differentiation of *Lactobacillus* sp. F2 from other lactic acid bacteria used in this study.

***Keywords:*** *Lactobacillus*, PCR-RAPD

# MOLECULAR ANALYSIS OF HBV PRECORE/CORE REGION OF HEPATITIS B VIRUS ISOLATED FROM MEN WHO HAVE SEX WITH MEN IN SURAKARTA

**Yulia Sari[1,2,3], Hudiyono[3,4], Ibnu Yudistiro[4], Afiono Agung Prasetyo[2,3,4]*, and Paramasari Dirgahayu[1,2,3]**

*[1]Department of Parasitology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[2]Center of Study of Biotechnology and Biodiversity Research and Development, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[3]Biomedical Laboratory, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[4]Department of Microbiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[*]Correspondence Author: afieagp@yahoo.com; afie.agp.la@gmail.com*

**ABSTRACT**

Hepatitis B virus (HBV) is one of the most contagious hepatitis viruses. People infected with HBV could develop chronic hepatitis disease, with increasing morbidity and mortality rate. In order to build a molecular data base of HBV circulated in Men Who Have Sex With Men (MSM) in Surakarta, 143 MSM were involved in an molecular epidemiology study in October 2010-October 2011. The blood samples were collected from all participant study, aliquoted, and fractionated. Plasma samples were addressed for HBsAg assay. The nucleic acid was extracted from all HBsAg positive plasma samples, and subjected for the HBV genome detection to amplify the Precore/core region. The positive PCR products were sequenced and molecular analyzed. The results showed that HBV/B3 was found predominant, followed by the HBV/C1. Nucleotide variations were found in BCP region, precore region, and core region. It is concluded that the results indicated the need for adequate management of HBV infection in the MSM community.

***Keywords:*** HBV, Precore/core, MSM, Indonesia

# FUNGAL INFECTION IN HUMAN IMMUNODEFICIENCY VIRUS PATIENTS IN MOEWARDI GENERAL HOSPITAL SURAKARTA

**Ruben Dharmawan[1], Murkati[1], and Afiono Agung Prasetyo[2,3,4]***

*[1]Department of Parasitology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[2]Biomedical Laboratory, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[3]Center of Study of Biotechnology and Biodiversity Research and Development, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[4]Department of Microbiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[*]Correspondence Author: afieagp@yahoo.com; afie.agp.la@gmail.com*

**ABSTRACT**

Human immunodeficiency virus (HIV)-infected patients are at risk of opportunistic infection including that of caused by fungal infections. The opportunistic infection is increasing the morbidity and mortality of the HIV-infected patients. However, there is no report about the presentation of fungal infections in Indonesian HIV patients' blood has ever been published, for the best of our knowledge. In an ongoing molecular epidemiology study of blood borne virus, HIV patients in Moewardi General Hospital Surakarta were enrolled in the study in November 2011-December 2011. The blood samples were aliquoted, subjected for CD4 assay, and fractionated. The nucleic acid was extracted from all blood samples, and subjected for the molecular assays to detect the presentation of pan fungal genome, by amplifying part of yeast 5S RNA. The results showed that one hundred and one blood samples were retrieved from 101 patients. The PCR results and the CD4 results from all blood samples were analyzed. It was concluded that the results indicate the need for adequate management of HIV patients, especially in the presentation of fungal infections.

***Keywords:*** Fungal infections, HIV patients, Indonesia

# ANTIOXIDANT ENZYME ACTIVITIES IN VARIOUS TISSUE ON RAT UNDER HYPERGLYCEMIA CONDITION

**I Nyoman Suarsana[1*], Bambang Pontjo Priosoeryanto[2], Tutik Wresdiyati[2], and Maria Bintang[3]**
[1]*Laboratory of Biochemistry, Faculty of Veterinary Medicine, Udayana University*
*Jl. PB. Sudirman Denpasar, Bali*
[2]*Faculty of Veterinary Medicine, Bogor Agriculture Institute, Bogor*
[3] *Laboratory of Biochemistry, Faculty of Mathematics and Natural Sciences, Bogor*
**Correspondence Author: suarsana65@yahoo.com*

## ABSTRACT

Oxidative stress has been considered as an explanation for the tissue damage that accompanies chronic hyperglycemia. Chronic exposure to hyperglycemia can lead to generation of free radicals and decreased intracellular antioxidant causing cellular dysfunction. This study aimed to determine the levels of intracellular antioxidant in erythrocyte, liver, kidney, and spleen tissue of rat under hyperglycemia conditions. This study used 10 male rats Spraque Dawlly average body weight of 200 g. They were sub-divided into two groups; (1) a control group, without treatment, and (2) a hyperglycemia group which was treated by allloxan 120 mg/kg bw intraperitoneally. The treatment was conducted for 28 days. The rats were given commercial feed and water ad libitum. At the end of treatment all of rats were euthanasia with cethamine-HCl. Erythrocyte, liver, kidney, and spleen tissue were collected for analysis of intracellular antioxidant enzyme levels using a spectrophotometric method. The results showed that the liver tissue contained intracellular antioxidant enzymes, i.e. superoxide dismutase (SOD), glutahion peroxidase (GPx) and catalase higher than that found in erythrocyte, kidney, and spleen tissue. While erythrocyte tissue contained the least intracellular antioxidant enzymes. Hyperglycemia conditions caused intracellular antioxidant enzyme levels to decrease at all tissue. Under hyperglycemia condition, the most significant decrease of antioxidant enzymes in all tissues was SOD enzyme.

***Keywords**:* Antioxidant, superoxide dismutase, gluthation peroxidase, catalase, rat.

# ADMINISTRATION OF ORAL ALPHA-LIPOIC ACID INHIBITS THE REDUCTION OF SKIN DERMAL COLLAGEN EXPRESSION INDUCED BY UVB IRRADIATION IN BALB/C MICE

**Made Sudarjana, AAGP Wiraguna, Alex J Pangkahila, M. Swastika Adiguna**

**ABSTRACT**

Chronic exposure of human skin to UV radiation is known to damage structure and function of the skin. Photo aged skin displays prominent alteration in the cellular component and extracellular matrix of the connective tissues such as a marked decrease of interstitial collagen. It is well known that Alpha-Lipoic Acid (LA) has the function as an essential co-factor of the mitochondrial multi-enzyme complex and thus plays an important role in energy metabolism. Currently, it attracts the attention as a nutritional supplement because of its uniquely potent antioxidant property. This study investigated the influence of administration oral alpha lipoic acid that gives protective effect in skin dermal collagen on balb/c mice induced by narrow band UVB irradiation. This study was an experimental study using the research method of pre-test post-test control group design. Sample of these study was 45 balb/c mice that divided into two groups, which was pre-test group consist of nine mice and 36 mice as a treatment group. The treatment group then divided in to three group, those were control group, (UVB+LA 0.87 mg) group and (UVB+LA 1.74) group. Each group consists of 12 balb/c mice. The control group received aquabidest and UVB irradiation. The (UVB+LA 0.87 mg) group received UVB irradiation and 0.87 mg doses of oral LA. The (UVB+LA 1.74 mg) group received 1.74 mg oral dose and UVB irradiation. All group received UVB irradiation for four weeks with the total dose 840 mJ/cm$^2$. Before the treatment mice in the pre-test group was terminated and histologic preparation of skin with Sirius Red staining were made for measuring the dermal collagen expression of the skin using digital analysis method. In the end of study period all of the treatment group were terminated too and histologic preparation were made using the same method then measuring the collagen expression. The result showed, mean difference dermal collagen expression post-test in control group was 0.131; in (UVB+LA 0.87 mg) group was 0.487 and (UVB+LA 1.74 mg) was 0.301. That were analyze with Anova test and the result has significant difference ($p<0.05$). Further more it were tested with Post Hoc test for comparisons between group and the result was different significantly ($p<0.05$). In conclusion of this study, LA with the different dose inhibited the reduction of skin dermal collagen expression and that means *alpha-lipoic acid* has a role of protective effect on skin dermal collagen induced by UVB irradiation.

***Keywords***: *ultraviolet B, alpha-lipoic acid, collagen*

# DUPLEX PCR IS RAPID METHOD FOR DETECTION *Legionella pneumophila* and *Legionella sp* ON ENVIRONMENTAL WATER IN HOTELS AT BADUNG REGENCY - BALI

**Ni Made Adi Tarini, I Made Agus Hendrayana, Agus Eka Darwinata**
*Division of Clinical Microbiologist Faculty of Medicine Udayana University/Sanglah Hospital*

**ABSTRACT**

Legionellosis is a disease that attacks the respiratory system can lead to severe pneumonia. The discovery of cases of Legionellosis at the end of 2010 in Bali showed a delay in the early detection of this source of *Legionella* infection. To detect the transmission of *Legionella* from environmental sources, the gold standard is culture method. But the general culture methods have limitations that require a specific media and require a long time to grow, and these methods are not always able to grow *Legionella sp*. Therefore necessary to develop a method for detecting *Legionella* faster and more accurate as the duplex PCR method. The purpose of this study is Gaining rapid assay method for detection of *Legionella pneumophila* and *Legionella sp* in environmental water samples. A total of 9 samples from some hotels in Badung regency consisting of a hotel room tap water, tap water in the spa, the pool water, the water in the reservoir, the water showers, warm water in the kitchen and air conditioning. The nine samples was conducted culture and PCR duplex examination. Culture methods were performed, grown in BCYE media CM655 (Oxoid) were added SR110A growth supplement (Oxoid) containing ACES buffer, ferric pyrophosphate, L-cysteine HCL, α-ketoglutarate, and selective supplement SR111E (Oxoid) containing glycine, vancomycin , polymixin and Cycloheximide. Cultures were incubated in CO2 incubator (5-7% CO2) at 37 degrees Celsius and colony growth was observed after 48 -72 minutes, until the 14th day. In the PCR method, all samples of DNA isolation using QIAamp DNA mini kit (Qiagen □) and then performed PCR with two p pairs LspF-LspR for detection of *Legionella sp* with product PCR is 212 bp and LpnF-LpnR to detect *Legionella pneumophila* with product PCR is 124 bp (Templeton et al), the annealing temperature at 59°C. The results of the nine specimens in this study, no growth of colonies of *Legionella sp* on the culture method but the bacteria control *Legionella pneumophila ATCC 33125* grow well on the culture method, whereas the duplex PCR method was found 8 samples showed bands 212 bp (*Legionella sp*) and only one sample of the pool water at the Spa showed bands 124 bp (*Legionella pneumophila*). This proved that the difficulty of growing the bacteria *Legionella sp* despite using special media. Many factors affect *Legionella* culture media such as pH of , the amount of bacteria that sufficient (10 CFU / mL), the effectiveness of selective media supplements containing antibiotics and the uniqueness of the bacteria that live inside the cells of protozoa that cause difficult to be grown in vitro. No growth of bacteria in culture media can also be caused by bacteria actually died due to chlorination is done the hotel. Detection of the bacteria *Legionella pneumophila* and *Legionella sp* on samples with duplex-PCR method because that is required the DNA of bacteria, so that the dead bacteria can also be detected. Based on the above, the bacteria *Legionella sp* is actually there in the environment hotel, so the hotel are advised to keep doing chlorination as an attempt to kill bacteria and Duplex-PCR method is more sensitive and specific than culture and can detect *Legionella sp* rapidly and accurately.

***Keywords***: *Legionella sp, Legionella pneumophila, Duplex-PCR*

# ISOLATION AND CHARACTERIZATION LYTIC PHAGE OF *Salmonella* sp. FR38.

**Sri Budiarti[1], Iman Rusmana[2], Riri Sunarti[3], and Sang Ayu Putu Listya[4]**
*Department of Biology, Institut Pertanian Bogor, Indonesia*

**ABSTRACT**

Lytic phage was used as natural and non-toxic agent to reduce and control the human pathogenic bacteria. The phage has a great potential application to control the antibiotics resistant bacteria. Phage lytic *Salmonella* sp. FR 38 was isolated from waste water taken from Babakan, Darmaga. The phage can lyse antibiotics resistant *Salmonella* sp. isolated from the faeces of the diarrhoea patient in Sindang Barang, Bogor district hospital. The aim of this research was to isolated and characterized lytic phage *Salmonella* FR38, such as: morphology and analyze the stability of lytic phage of *Salmonella* sp. FR38, i.e. morphological structure and stability of lytic activity of phage on different conditions of pH, temperature, and buffers. Transmission electron microscopy analysis showed that phage FR38 had a hexagonal icosahedral head 73.3 ± 0.21 nm in diameter and non-contractile tail of 93.7 ± 0.21 nm in length and 17.3 ± 0.07 nm in diameter. Phage 38 could survive at various pH conditions. Thermal stability and buffer for storage indicated that a temperature above 60°C was deleterious for survival of the phage. The best performance buffer for storage of phage was buffer A at pH 7 and temperature of 27°C. Phage stored in buffer at low temperatures (4°C) showed that the number of plaque was not different to that of at room temperature.

***Keywords***: Lytic phage, *Salmonella* sp., Isolation

# COINFECTION OF *Toxoplasma gondii* AMONG HIV/HCV INFECTED AND UNINFECTED PEOPLE IN CORRECTIONAL FACILITY INDONESIA

**Paramasari Dirgahayu[1,2,3]*, Afiono Agung Prasetyo[3,4], Yulisari[1,2,3], Faqihudin Ahmad[3]**

*[1]Department of Parasitology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia. [2]Biomedical Laboratory, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia. [3]Center of Study of Biotechnology and Biodiversity Research and Development, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia. [4]Department of Microbiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia. [5]Division of Virology, Faculty of Medicine, Tottori University, 86 Nishi, Yonago 683-8503, Japan.*

## ABSTRACT

*Toxoplasma gondii* is an ubiquitous protozoan parasite and a major opportunistic pathogen in immunocompromised patients especially those co-infected with virus. It is known that MSM (*Men Who Have Sex with Men*) and IDUs (*Intravenous-Drug User)* develop drug addiction in the body and spirit, which, in turn, impairs cellular immunity and humoral immunity which may possible to be co-infected with *toxoplasma gondii*. Until recently, the data of Toxoplasma infection in person infected with HIV in the high risk communities in Indonesia is still very rare.

Our group performed a molecular epidemiology study of blood borne viruses by collecting the epidemiological data and blood specimens from the high risk communities in Central of Java Indonesia for 2 years. In total, 518 samples were collected. All samples already tested for HIV and hepatitis viruses including that of HCV (hepatitis C virus). From all samples, 4.8% (25/518) samples were anti HIV positive, and 60% (15/25) anti HIV samples were derived from the Injecting Drug Users (IDUs). In order to find out the Toxoplasma status in persons with anti-HIV positive in the communities described, we screened the anti HIV plasma aliquot stocks using AIM toxo IgM and IgG Elisa Kit to detect the IgM and IgG Toxo, respectively. Also, we screened the anti HCV positive plasma aliquot stocks derived from the IDUs.From 25 anti-HIV positive samples, 60% (15/25) were IgG Toxo positive. Interestingly, all of the anti HIV and IgG Toxo positive samples were derived from the IDUs. Moreover, 70.6% (24/34) of anti HCV positive samples derived from the IDUs were also positive for anti IgG Toxo. None of the IgM Toxo detected in all samples tested at present study.

In on going study, we found the high co-infection rate of Toxo with HIV or HCV. Also, it seems that the Toxo was transferred via injecting drug route in the high risk communities in Indonesia. Now we are going to test the aliquot plasma stock samples derived from the non IDUs.

***Keywords***: *Toxoplasma gondii,* HIV, HCV, drug abuser prisoner

# THE EXTRACTS OF CACAO BEANS (*Theobroma cacao* L.) INCREASE BLOOD SOD CONCENTRATION IN OXIDATIVE STRESS WHITE RAT (*Rattus norvegicus*)

**Ida Ayu Ika Wahyuniari[1], and Ida Ayu Dewi Wiryanthini[2]**
*[1]Department of Histology, [2]Department of Biochemistry, Faculty of Medicine*
*Udayana University, Jl PB Sudirman Denpasar 80232 Bali*
*Telp: 0361-8001045/08123614856, Email : ikawahyuniari@yahoo.com*

**ABSTRACT**

A study in adult *Wistar* white rats on the effect of extract of cacao beans on the SOD level in oxidative stress induce by psychosocial stress was carried out. As many as 20 adult *Wistar* white rats with oxidative stress induced by psychosocial stress in this study were divided into 4 groups with 6 rats in each group in *Pretest-Posttest Control Group Design* as Control group or placebo (P0), intervention group by 70 mg extracts of cacao beans (P1), 140 mg (P2), and 280 mg (P3) for 14 days. All groups were examined for blood SOD concentration before and after intervention. This study revealed increase SOD concentration in oxidative stress white rat induced by psychosocial stress after administration of cacao beans extract in group P1 (535.59 vs 554.24), P2 (540.68 vs 632.2) dan P3 (545.76 vs 696.61) with p = 0.000. This study showed that cacao beans extract can inhibit oxidative stress caused by psychosocial stress with increased SOD concentration.

***Keywords***: Psychosocial stress, oxidative stress, cacao beans extract, SOD

# APOPTIN EXPRESSION PLASMID FOR EXPRESSION OF APOPTIN IN MAMMALIAN CELLS

**Suyatmi[1], Sri Wulandari[2], and Afiono Agung Prasetyo [3,4,5]***

[1] *Department of Histology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta, Indonesia.*
[2] *Department of Physiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta, Indonesia.*
[3] *Department of Microbiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta, Indonesia.*
[4] *Biomedic Laboratory, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta, Indonesia.*
[5] *Center of Biotechnology and Biodiversity Research and Development, Jl. Ir. Sutami 36A, Surakarta 57126, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta, Indonesia.*
**Correspondence Author: afieagp@yahoo.com; afie.agp.la@gmail.com*

## ABSTRACT

To understanding more about the role of Chicken Anemia Virus Apoptin in apoptosis of mammalian transformed cells, a mammalian expression plasmid (p-Apo) was constructed. A complete open reading frame of the apoptin gene of CAV/WT was amplified by PCR. The PCR product was then purified from the agarose using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), subcloned into the respective sites of pHM6 vector (Roche Diagnostics, Mannheim, Germany). The HA (hemagglutinin) gene in the pHM6 expression plasmid was removed by direct mutagenesis. The molecular clone was transformed into E. coli, purified, sequenced and the nucleotide sequence was analyzed by CLC Main Workbench (CLC Bio). The clone thus obtained, p-Apo, was successfully constructed. The apoptin expression plasmid will express a wild-type apoptin in the mammalian cells driven by the CMV promoter of the pHM6 back bone plasmid, without tagged by HA.

***Keywords:*** apoptin, mammalian expression plasmid

# MYCOBACTERIUM TUBERCULOSIS IN HUMAN IMMUNODEFICIENCY VIRUS PATIENTS' BLOOD IN MOEWARDI GENERAL HOSPITAL SURAKARTA

**Marwoto[1], Leli Saptawati[1], and Afiono Agung Prasetyo[1,2,3]***
[1]Department of Microbiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.
*[2]Biomedical Laboratory, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
*[3]Center of Study of Biotechnology and Biodiversity Research and Development, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*
**Correspondence Author: afieagp@yahoo.com; afie.agp.la@gmail.com*

**ABSTRACT**

Human immunodeficiency virus (HIV)-infected patients are at risk of Mycobacterium tuberculosis infection. The Mycobacterium tuberculosis can cause pulmonary infection as well as extrapulmonary lesions (Lymph node, skin & soft tissue, joint or systemic disseminated infection) in immunocompromised hosts. However, there is no report about the presentation of Mycobacterium tuberculosis in Indonesian HIV patients' blood has ever been published, for the best of our knowledge. In an ongoing molecular epidemiology study of blood borne virus, HIV patients in Moewardi General Hospital Surakarta were enrolled in the study in November 2011- December 2011. The blood samples were aliquoted, subjected for CD4 assay, and fractionated. The nucleic acid was extracted from all blood samples, and subjected for the molecular assays to detect the presentation of Mycobacterium tuberculosis by three different multiplex PCRs. One hundred and one blood samples were retrieved from 101 patients. The PCR results and the CD4 results from all blood samples were analyzed. Results indicate the need for adequate management of HIV patients, especially in the presentation of *Mycobacterium tuberculosis*.

***Keywords***: *Mycobacterium tuberculosis*, HIV patients, Indonesia

# USE OF GLUCOSE DETERMINATION FOR *Candida albicans* β GLUCANS EXAMINATION

**Darukutni[1], Sri Haryati[1], Murkati[1], Yulia Sari[1,2,3], Ruben Dharmawan[1], and Afiono Agung Prasetyo[2,3,4]**

[1]*Department of Parasitology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*

[2]*Biomedical Laboratory, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*

[3]*Center of Study of Biotechnology and Biodiversity Research and Development, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*

[4]*Department of Microbiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.*

**ABSTRACT**

*Candida albicans* β glucans examination has been used for invasive fungal detection in human blood and approved by Food and Drug Administration. However, the method is rarely used in Indonesia and the cost is hardly affordable. Modification of the method using spectrophotometric glucose determination is hoped to provide simple and affordable *Candida albicans* β glucans measurement. β glucans as heterogenous molecules that constitute the major carbohydrates fractions of cell wall and readily detected in supernatans of *Candida albicans* cultures are hydrolyzed by β glucanase to form glucose. This glucose is measured using the Megazyme GOPOD-Format Procedure® at 510 nm. *Candida albicans* were identified and cultured from patients of Dr. Moewardi General Hospital, Surakarta in July, 2012. Results show that β glucans from *Candida albicans* is able to be measured in the amount of μgram samples.

***Keywords***: *Candida albicans*, β glucans, glucose, spectrophotometry.

# POSTER PRESENTATIONS: AGRICULTURE

# PRODUCTION OF CRUDE ENZYME CELLULASES FROM CASSAVA WASTE BY *Trichoderma viride*

**I Wayan Arnata[1], Dwi Setyaningsih[2], and Nur Richana[3]**
[1] Agroindustrial Technology, Udayana University, Bali, Indonesia
[2] Agroindustrial Technology, Bogor Agricultural University, Bogor, Indonesia
[3] Research Institute for Food Crops Biotechnology, Bogor, Indonesia
Correspondence author: yan_kadir@yahoo.com

**ABSTRACT**

Cellulases are a complex enzymes system, commercially produced by filamentous fungi and bacteria. It has wide applicability in food and beverage industry for effective saccharification process. In this study, the crude enzyme cellulase was produced from cassava waste by *Trichoderma viride*. The experiments was carried out in two phases. The first phase was the cultivation of *Trichoderma viride* to determine the stationary phase of maximum spore yield. The second phase was the determination of the produce time needed that gave maximum crude enzyme activity. Enzyme assay was conducted by measuring the FPase and CMCase activities. The results showed that the stationary phase of maximum spore yield occurred after 6 days of cultivation *Trichoderma viride* with maximum spore number of $1.6 \times 10^9$ spores $mL^{-1}$. Optimum activity of the crude enzyme obtained after 7 days of fermentation with the FPase activities of 4.77±0.72 $UmL^{-1}$ and the CMCase activities of 5.05± 0.42 $UmL^{-1}$.

***Keywords***: cassava waste, cellulase, *Trichoderma viride.*

## INTRODUCTION

Cellulose being an abundant and renewable resource, are the largest fraction of the plant cell wall of agricultural residues such as straw from wheat, corn, rice soy and cotton, sugarcane bagasse, and cassava waste. In Indonesia, cassava waste was called "onggok". Onggok is one of the agro-industrial by products that it was obtained from processing of cassava tubers for tapioca production. Onggok generally in solid formed and discarded to the environment without any treatment, so that it would coused problems to environmental polution, espesially in arounded of factory areas (Arnata et al., 2011).

The dry of onggok has a composition of starch 56-60%, cellulose 15-18%, hemicellulose 4-5%, lignin 2-3%, protein 1.5-2.0%, pentosans 2%, and reducing sugars 0.4-0.5% (Wongskeo et al., 2012), while, Susijahadi et al. (1997); Yuwono and Sutopo (2008) reported that onggok was contained high carbohydrates such as 10% crude fiber and 50-70 % starch. Utilization of "onggok" for material in bioprocesses product is one way to given value added in cassava tuber, in the other hand, it was helped to solve pollution problem. The "onggok" can be used as substrate in ethanol production (Susijahadi et al., 1997) and lactic acid production (Yuwono and Sutopo, 2008). Various product of starch derived such as maltose, maltodextrine and glucose syrup was also made from onggok. In the fermentation process, onggok was used as substrate for fermentation of citic acid and fumaric acid (Panday et al., 2000), amyloglucosidase enzyme (Arnata et al., 2011).

One of the alternative to give value edded onggok was used as a substrate for fermentation of cellulase enzyme. Cellulase is a complex enzyme system, commercially produced by filamentous fungi under solid-state and submerged cultivation. It has wide applicability in textile, food and beverage industry for effective saccharification process (Chellapandi and Jani, 2008). Commercial cellulase generally obtained from filamentous fungi (Stoilova *et al.* 2005). One of filamentous fungi that it known able to produce cellulose is *Trichoderma viride*. The production of cellulase is highly influenced by several factor i.a. component of the growth medium, especially carbon sources and time of fermentation process. In this study was conducted to determine the effect of time of fermentation process with *T. viride* as an inoculum and onggok as an inducer on crude enzyme cellulose.

## MATERIALS AND METHODS

### Raw material

The "onggok" (by-product tapioca factory) was obtained from Bogor West Java. It was sun dried and milled to uniform size (40 mesh). *T. viride* was obtained from microbiology laboratorium PAU IPB. Onggok was analysed to determine chemical compotition before it is used to fermentation process. Analysed that is moisture, fat, proteine, carbohydrate and crude fiber contents.

### Inoculum culture *Trichoderma viride*

The strains *T.viride* were used in this work. Stock cultures were maintained on potato dextrose agar (PDA) and cultivation for 5 days at 30°C. Properly sporulated cultured were used for inoculation.

### Cellulase Production

Fermentation process to cellulase production was conducted in batch system at 250 mL erlenmeyer flask with work volume 100 ml. The composition of medium for cellulase production by *T. viride* was 14 ml $(NH_4)_2SO_4$ 10%, 15 ml 1M $KH_2PO_4$, 3 ml of 10% urea, 3 ml $CaCl_2$ 10%, 3 ml $MgSO_4.7H_2O$ 10%, 1 ml of trace elements stock and 2 ml Tween 80, then made 1 liter. Trace elements composed of 495 ml of distilled water, 5 ml of concentrated HCl, 2.5 g $FeSO_4$, 0.89 g $MnCl_2.4H_2O$, 1.76 g $ZnSO_4.H_2O$, 1.25 g $Co(NO_3)_2.6H_2O$. For the production of cellulase added 5-10 g cassava wate/"onggok" and 0.5 to 1.0 g polipepton to 1 liter of medium. The production process is carried out at a temperature of 30°C and pH 4.0. The ability to produce cellulase enzyme was observed by measuring activity of FPase and CMCase every 24 hours for 7 days. Enzyme activity curves were made and used to see the achievement of maximum activity time *T. viride* to produce cellulase.

### Isolation of cellulase

Samples were collected from each periodic time of fermentation were added 1% tween 80 and agitated thoroughly on orbital shaker at 100 rpm for 30 min. The crude enzyme was filtered through Whatman # 42 filter paper. The filtrates were centrifuged at 10000 rpm for 30 min at 4°C to remove the suspended particles.

### Analysis methods

The crude enzyme cellulase was determined by Pothiraj et al. (2006) method. The celluase activity was assayed using filter paper (FPase) and carboxymethyl cellulase (CMCase). Reducing sugars released were determined by the dinitrosalicylic acid method (Miller, 1959). One unit of enzyme activity is defined as 1 µmol glucose released/min/ml of culture supernatant. The pH of substrate was determined by pH-meter.

## RESULTS AND DISCUSSION

The cultivation of *T.viride* was carried out for 7 days by calculating the number of spores were formed every day. At the beginning of inoculation are the average number of spores 7.08 x $10^7$/ml. At the end of the first day, the number of spores decreased significantly due to spore germination. The next day, began to form spores are white with an average number of 1.02 x $10^8$/ml. *T.viride* produces spores quickly from the first day until the third day. After the third day, *T.viride* started showing relatively slow phase of spore formation. The color change of the mycelium and spores from white to green was formed after cultivation for 6 days. The maximum number of spores with an average of 1.58 x $10^9$/ml spores occurred on the sixth day. Growth curve *T.viride* is presented in Figure 1.

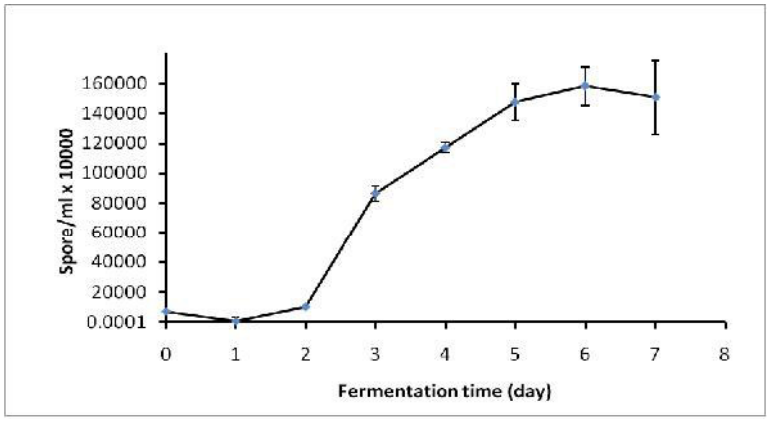


Figure 1. The growth curve of *T. viride* during fermentation process

In this study the production of cellulase enzyme using a modified Andreoti Media. Modifications done by replacing the pure cellulose as an induction of cellulase using cassava waste. Cassava can be used as an material inducement because it contains crude fiber is mainly fraction of cellulose that could be used as a carbon source for microbial growth. In addition, cellulose is also a compound of synthesis cellulase enzyme. Crude fiber content of cassava on average 6.58 ± 0.08% (w/w). The concentration of cassava waste used in this study was 10 g/L. According Richana et al. (2004), crude fiber content of cassava was 9.7%, consisting of 1.3% lignin, 5.8% xylan and cellulose 2.61%. Arnata et al. (2011) also reported that the contents of onggok were 8.65 ± 0.10 % moisture, 2.55 ± 0.14 % ash, 6.54 ± 0.02 % fat, 1.81 ± 0.03 % proteine, 2.69 ± 0.04 % crude fiber and 62.54 ± 0.00 % starch. Crude fiber content 69.98 % hemicellulose and 13,44 cellulose. Padonou et al. (2005) was reported that cassava flour content 0,56 % fat (wb), while according to Pothiraj and Eyini (2007) Cassava waste was found to have by dry weight 55.8% starch, 14.5% cellulose, 1.21% free reducing sugars and 3.13% protein.

In Figure 2 shows that at the initial time of fermentation was decreased CMCase activity until the fourth day. After that, CMCase enzyme activity tend to have increased and the maximal activity of 5.05 ± 0.42 U/ml was obtained after fermentation for 7 days or a week. It is also shown on the amount of FPase activity, which at the initial time of fermentation was decreased until the third day and on the next day was increased. Maximal activity of 4.77 ± 0.72 U / ml was also obtained after fermentation for 7 days.

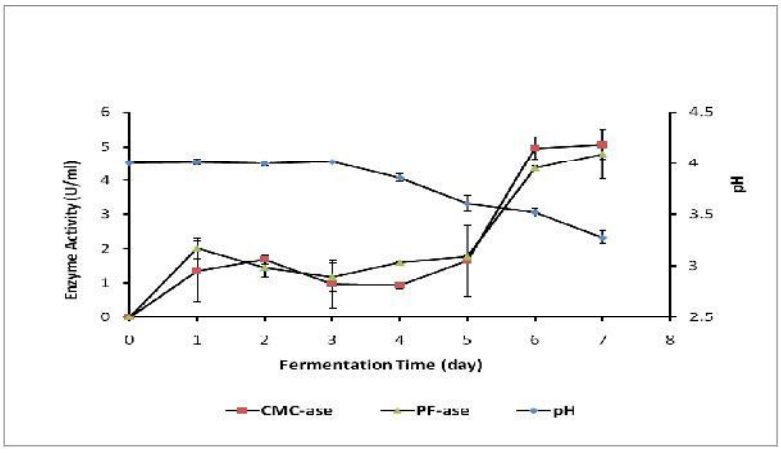


Figure 2. The change of pH and cellulase activity during fermentation process

The increased activity of the enzyme cellulase (CMCase and FPase) showed that *T. viride* have to degradation of the cellulose fraction contained in the substarat to produce glucose to be used for cell metabolism. Suhartono (1989) reported that the synthesis of extracellular enzymes in the greatest number,

normally occurred in the time before sporulation, ie, at the end of the exponential phase and early stationary phase. The condition is expected because in the transition of exponential phase followed by decrease in the number of carbon sources in the medium, so the synthesis of cellulase enzymes started to increase.

The increased activity of the enzyme in the fermentation process thought to be caused by the change of pH from pH 4.0 to 3.28 at the beginning of the seventh day. Enari (1983) mentioned that the optimal pH for growth of Trichoderma about 4.0, whereas for cellulase production approaching 3.0. During the production of the enzyme, the pH should be maintained in the range from 3.0 to 4.0 due to inactivation of the enzyme would occur if the pH is below 2.0. Decrease in pH that occurs in cellulase production is directly related to the consumption of carbohydrates contained in the cassava. The pattern of change in pH during fermentation for the production of cellulase enzymes is presented in Figure 2.

The stationary phase of maximum spore yield occurred after 6 days of cultivation *T. viride* with maximum spore number of 1.5 x $10^9$ spores m/L. Optimum activity of the crude enzyme obtained after 7 days of fermentation with the FP-ase activities of 4.77±0.72 U m/L and CMC-ase activities of 5.05±0.42 U m/L.

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# UREA-LIME MIXTURE AS A RUMEN SLOW-RELEASE UREA ON IN VITRO RUMEN FERMENTATION AND MICROBIAL PROTEIN SYNTHESIS

**I G. L. O. Cakra*, I B. Sudana, I G. Mahardika, and I B. G. Partama**
*Faculty of Animal Husbandry*
*Udayana University Denpasar Bali*
**orresponding author: lanangcakrafapet@yahoo.com*

**ABSTRACT**

The experiment was aimed to know the effect of urea-lime mixture as a rumen slow-release urea on in vitro rumen fermentation and microbial protein synthesis. The experiment used completely randomize design (CRD) with six ration as the treatment and three replication. The treatments were: 50% elephant grass + 50% concentrate containing 4% urea and 2% lime (R1), 50% elephant grass + 50% concentrate containing 4% urea and 3% lime (R2), 50% elephant grass + 50% concentrate containing 4% urea and 4% lime (R3), 50% elephant grass + 50% concentrate containing 5% urea and 2% lime (R4), 50% elephant grass + 50% concentrate containing 5% urea and 3% lime (R5), and 50% elephant grass + 50% concentrate containing 5% urea and 4% lime (R6). Variables observed were: pH, ammonia level, Vollatyle Fatty Acid (VFA), dry and organic matter digestible coefficient, and microbial protein synthesis. The result of the experiment showed that on four hours incubation, the acidity level (pH) in normal range (6.63–6.82) for all treatments, the lowest supernatan ammonia level is R1 treatment which indicated that 4% urea and 2% lime mixture is the best for inhibiting urea hydrolysis. The highest microbial protein synthesis is R1 treatment with 4% urea and 2% lime. It can be related to the highest of R1 treatment on dry and organic matter digestible coefficient, as well as supernatan VFA. It can be concluded that 4% urea and 2% lime in concentrate could use as a source of urea slow-release which give the lowest of ammonia, and the highest of VFA, dry and organic matter digestible coefficient, and microbial protein synthesis.

***Keywords*:** urea-lime mixture, in vitro fermentation, microbial protein synthesis

## INTRODUCTION

Supply the nitrogen protein and energy balance of feed in forming rumen microbe fermentation are aimed to maximize the utilization of degradable protein in rumen and to increase the utilization of feed protein efficiency. The rumen microbe requirement of protein (nitrogen) about 80% is obtained from ammonia group.

Supplementation of urea could use as source of nitrogen but urea is fast in release the nitrogen, and could fast to produce ammonia, so if the dosage plentiful will cause toxicity, eventhough death (Stanton and Whittier, 2006). Huntington *et al*., (2006) reported that urea could hydrolise fast in rumen and could reach the peak of ammonia produce in one hour after the urea offer. Slow release ammonia method from urea hydrolisis in rumen is more efficient and safe because could inhibit ammonia toxicity (Galo *et al.,* 2003). In invitro experiment, urea-calcium sulphat could decrease ammonia rumen concentration compare with urea (Cherdthong *et al*., 2010). (Cherdthong *et al*., 2010) reported that supplementation of urea-$CaSO_4$ in concentrate contain 70% cassava could increase rumen ecology and the forming of microbial protein in cattle.

Based on the above description, it is need an experiment to know the best level of urea-lime mixture in feed to slow release of ammonia forming in invitro method.

## MATERIALS AND METHODS

The experiment was conducted in Nutrition Laboratory at Faculty of Animal Husbandry Udayana University and Analitic Laboratory Udayana University. The experiment use completely radomized design (CRD) with facorial pattern 2×3 with 3 replication, for each combination replicate. The first factor is urea level (4 dan 5%), and the second factor is lime level (2, 3 dan 4%) in concentrate.The invitro experiment use Minson & Mc Leod (1972) method and modificated in 240 minutes. The experiment is examined of six ration consist of: 50% concentrate dan 50% king grass, with urea lime mixture level combination in concentrate (Table 1). Ration is arranged based on goat requirement fulfill standard 15 kg weight and body weight gain 75 g per head per day (Kearl 1982).

**Table 1.** The Composition and Nutrient Content of Concentrate Invitro

| Composition (%) | Concentrate[2)] | | | | | |
|---|---|---|---|---|---|---|
| | 1 (U4L2) | 2 (U4L3) | 3 (U4L4) | 4 (U5L2) | 5 (U5L3) | 6 (U5L4) |
| Pollard | 25.00 | 25.00 | 25.00 | 25.00 | 25.00 | 25.00 |
| Molases | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| Rice bran | 40.00 | 39.00 | 38.00 | 39.00 | 38.00 | 37.00 |
| Soybean peel | 25.00 | 25.00 | 25.00 | 25.00 | 25.00 | 25.00 |
| **Urea** | **4.00** | **4.00** | **4.00** | **5.00** | **5.00** | **5.00** |
| **Lime** | **2.00** | **3.00** | **4.00** | **2.00** | **3.00** | **4.00** |
| NaCl | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 |
| Mineral vitamin | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| TOTAL | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| Concentrate Nutrient [1)] (% Dry Matter) | | | | | | |
| Dry Matter | 88.35 | 89.68 | 89.67 | 89.68 | 89.67 | 89.66 |
| Crude Protein | 22.87 | 24.71 | 24.61 | 27.53 | 27.43 | 27.34 |
| Ether Extract | 2.60 | 2.27 | 2.25 | 2.27 | 2.25 | 2.22 |
| Crude Fibre | 27.43 | 15.18 | 14.99 | 15.18 | 14.99 | 14.80 |
| Total Digestible Nutrient | 59.46 | 58.91 | 58.35 | 58.91 | 58.35 | 57.79 |

Note:
[1)] Hari Hartadi *et al*. (1997)
[2)] Concentrate 1 (U4L2) concentrate containing 4% urea and 2% lime; Concentrate 2 (U4L3): concentrate containing 4% urea and 3% lime; Concentrate 3 (U4L4) concentrate containing 4% urea and 3% lime; Concentrate 4 (U5L2) concentrate containing 5% urea and 2% lime ; Concentrate 5 (U5L3) concentrate containing 5% urea and 3% lime ; Concentrate 6 (U5L4) concentrate containing 5% urea and 4% lime
Variables observed are: invitro dry matter and organic digestibility (Minson & Mc Leod Method, 1972 method), pH of supernatan liquid rumen, $NH_3$ concentration NH3 (Phenolhypochlorite Method, Solarzano L 1969), VFA concentration (Steam Destilation General Laboratory Procedure Method, 1966), and nitrogen microbial synthesis (ARC, 1984).

## RESULTS AND DISCUSSION

Result of the experiment showed that pH on all treatments in normal range (6,63-6,82) at 4 hours incubation. Analysis covariance showed that pH (acidity level) on R1 ration is significant ($P<0.05$) lower than the other ration, because VFA in ration R1 is high. VFA level in R1 ration is significant ($P<0.05$) higher (105.87 mM) than the other VFA ration (Table 2). Microbial activity in R1 ration is the best. VFA is a byproduct that's produced from rumen microbe activity (Van Soest, 1994). The high microbial rumen activity in R1 ration could see on the high of microbe protein synthesis (MPS), where as microbe protein synthesis on R1 ration (14,95 mg/kg DOMR (digestible organic matter rumen) is significant ($P<0.05$) higher than R2 and R5 (Table 2). High microbial protein synthesis (MPS) could see on rumen organic matter digestibility. ARC (1984), found that total microbial nitrogen was 32 g/kg DOMR . This is prove that if organic matter higher, so does the microbial protein synthesis (MPS). Higher the dry matter digestibility was due higher the organic matter digestibility because the organic matter is part of dry matter.

Table 2. The Effect of Urea Lime Balance to the pH, NH3, VFA, Dry Matter Digestible Coefficient (DMDC), Organic Matter Digestible Coefficient (OMDC) and MPS Invitro

| Ration | pH | NH3 | VFA | DMDC | OMDC | MPS |
|---|---|---|---|---|---|---|
| | | (mM) | (mM) | (%) | (% BK) | (mg/kg DOMR) |
| R1[1)] | 6.63[d2)] | 7.08[b] | 105.87[a] | 35.48[ab] | 38.84[a] | 14.9513[a] |
| R2 | 6.67[c] | 8.54[ab] | 90.01[b] | 31.01[c] | 33.44[b] | 12.8249[b] |
| R3 | 6.74[b] | 8.16[ab] | 69.74[c] | 36.83[a] | 38.52[a] | 14.8335[a] |
| R4 | 6.79[a] | 13.17[a] | 67.20[c] | 33.34[abc] | 36.39[ab] | 14.1141[a] |
| R5 | 6.80[a] | 10.59[ab] | 90.85[b] | 35.96[ab] | 33.42[b] | 12.8413[b] |
| R6 | 6.82[a] | 7.77[ab] | 74.82[c] | 32.57[bc] | 36.19[ab] | 13.8730[ab] |

Note:
[1)] R1= 50% elephan grass + 50% concentrate 1 (U4L2)
R2= 50% elephan grass + 50% concentrate 2 (U4L3)
R3= 50% elephan grass + 50% concentrate 3 (U4L4)
R4= 50% elephan grass + 50% concentrate 4 (U5L2)
R5= 50% elephan grass + 50% concentrate 5 (U5L3)
R6= 50% elephan grass + 50% concentrate 6 (U5L4)
[2)] Value with different alphabets in the same column means significant different ($P<0.05$)

The balance supply of nitrogen protein and energy of feed in rumen fermentation was aimed to increase the utilization of degradable protein for forming microbial cell. The 80% requirement of rumen microbial was get from ammonia groups (Leng dan Nolan 1984). The supplementation of urea could use as nitrogen source. Result of the research found that ammonia level of R1 ration (7.08mM) was significant ($P<0.05$) lower than R4 ration but not significant different ($P>0.05$) than R2, R3, R5, and R6 ration. This is show that mixture of 4% urea and 2% lime was the best in inhibiting urea hydrolisis.

Based on result of the experiment, it can be concluded that the mixture of 4% urea and 2% lime balance in concentrate could use as source of ammonia slow release because resulting the lowest ammonia, but the highest VFA level, organic matter digestible coefficient, dry matter digestible coefficient and microbial protein synthesis.

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# "PULSING" TECHNIQUE WITH ADDITION OF SUCROSE AND ANTIMICROBIAL TO MAINTAIN THE TEXTURE AND FRESHNESS OF CUT ROSES DURING STORAGE

**Ida Ayu Rina Pratiwi Pudja*, I Made Nada, Ida Bagus Putu Gunadnya**
*Department of Agricultural Engineering, Faculty of Agricultural Technology, Udayana University*
**Corresponding author: dayu_rina@yahoo.co.id*

**ABSTRACT**

Cut flower as a life crop needs water and nutrition to maintain its freshness. After cutting from its plant, cut flower still exhibits physiological processes and its life fully rely on the existing of water and nutrition in it. Loss of these two factors will decrease the endurance of cut-flower to remain to be fresh. The Aim of this research was to study influence of addition of sucrose and antimicrobial ($AgNO_3$) solution through "pulsing" technique on freshness of cut roses. Besides that, to know the best of concentration addition which can maintain its freshness. Treatment used in this research was the concentration of sucrose 20, 15, 10, and 5 g combined with antimicrobial substance of $AgNO_3$ with concentration of 5, 10, 15, and 20 ppm. Control was prepared without addition of sucrose and antimicrobial. Treatment was repeated twice and samples were stored for 9 days at room temperature (27±2°C). The results showed that rate of water loss of red roses decreased but in slower rate than control during storage. Cut red roses flower texture until 7 day still had value 4 (vigor or rigid and fairly brightness). Cut red roses flower texture was less vigor during storage, but it was still salable by combination treatment of concentration of sucrose 5 g and concentration of $AgNO_3$ 20 ppm. Its freshness until 7 day still got value 4 (fresh) that is > 50% fresh flower was treated with combination treatment of concentration of sucrose 20 g and 5 ppm of $AgNO_3$.

***Keywords***: Rose, sucrose, $AgNO_3$, cut flower

## INTRODUCTION

Cut flower needs water and nutrients to maintain its freshness when it is cut from its plant, because it will not be supported by water and nutrients supply from the root. Cut flower will rely on its existing water and nutrients, so these water and nutrients contents become limiting factor to keep its freshness. Unvapourable environmental factors such as temperature and humidity tend to accelerate wilting of the flower. So that it is necessary to supply water and nutrients as additional source of energy for the flower at a certain time. Giving flower freshener solution containing nutrients dissolved in water through flower stem can extend its freshness.

Basically role of refresher material is extending the freshness, increasing flower blooming size, increasing the number of flower buds, maintaining flower colour, and decelerating leaf yellowing. These characteristics of this material is important to floral agribusiness. Nutrients are supplied to flower immediately after harvest for a few hours by immersing it in water, before the flower wrapped and packed for delivery to the destination city. Providing refreshment by this method is called pulsing. Freshness solution generally contains nutrients and antimicrobials in certain doses and is useful to provide some nutrient and to eliminate microbial contamination of the flower.

As a major component of flower freshener is nutrients, which can be one of the various types of sugars such as glucose, sucrose or fructose. Other material is antimicrobial which can be determined based on their usage, because it is often very specific. Addition of citric acid is required to acidify the solution so that nutrients absorption is much easier and it can act as an antiseptic as well. In its preparation, components need to be prepared carefully by weighing accurately to get precise dose. This work is not difficult, but necessary accuracy. The most inexpensive components that can be used as a basic formula is sugar (as a source of carbohydrate, sucrose can also be used but it is rather expensive than sugar) and citric acid at doses up to 320 ppm or pH 3-4 for every liter of water (Prabawati, 2007).

Rose is one of the horticultural crops of interest and have been cultivated and grown in Indonesia because it has a high economic value (Anon., 2002). Rose production increased in 2001, but its production decreased until 2004. Red roses are the most popular flower and its total production reached 78,147,515 stalks in 2000, while in 2001 as many as 127 956 354 stalks. In 2003, production of rose flower fell to 55,708,137 in 2004 the production was 50,776,666 stalks (Murtiningsih and Satuhu, 2005). Cut roses

is one of the horticulture that highly susceptible to damage due to physiological, physical and chemical changes. The damage of the flower will be faster if the damage is preceded by physical and mechanical damage characterized by discoloration.

With the addition of antimicrobial substances and nutrients into refreshment solution the cut flower will be able to maintain its freshness in a longer time. Antimicrobial substances that exist in solution can keep the flower stalk from rapid decay and to prevent clots that can inhibit the absorption of water. Microbial activity in the flower stalk produces mucus that clog the stalk. In this study, nutrient used was sucrose and antimicrobial agents used was silver nitrate ($AgNO_3$). Silver nitrate is readily available and affordable for flower farmers. So, it is necessary to find the exact concentration of sucrose and silver nitrate ($AgNO_3$) in maintaining the freshness of cut roses.

Based on above explanation, a research has been done to study the effect of sucrose and antimicrobial agents addition through the technique of "pulsing" on the quality of fresh cut roses.

## MATERIAL AND METHODS

Cut red roses was obtain from Malang Rose Garden. The study consisted of two factors, namely: The first factor was the concentration of sucrose (S), which consisted of four levels: S1 = 20 g of sucrose; S2 = 15 g of sucrose; S3 = 10 g of sucrose; S4 = 5 g of sucrose

The second factor was an antimicrobial agent with $AgNO_3$ concentration (P), which consists of four levels: P1 = 5 ppm of $AgNO_3$; P2 = 10 ppm of $AgNO_3$; P3 = 15 ppm of $AgNO_3$; P4 = 20 ppm of $AgNO_3$ and K0 as a control, which prepared without the addition of sucrose and antimicrobial substances. From two factors mentioned above then it was obtained 16 combinations of treatments, each treatment had 2 replicates and stored for 9 days. Sensory assessment was conducted daily during the cut roses storage. The data obtained were analyzed descriptively. Implementation of the study was shown in Figure 1.

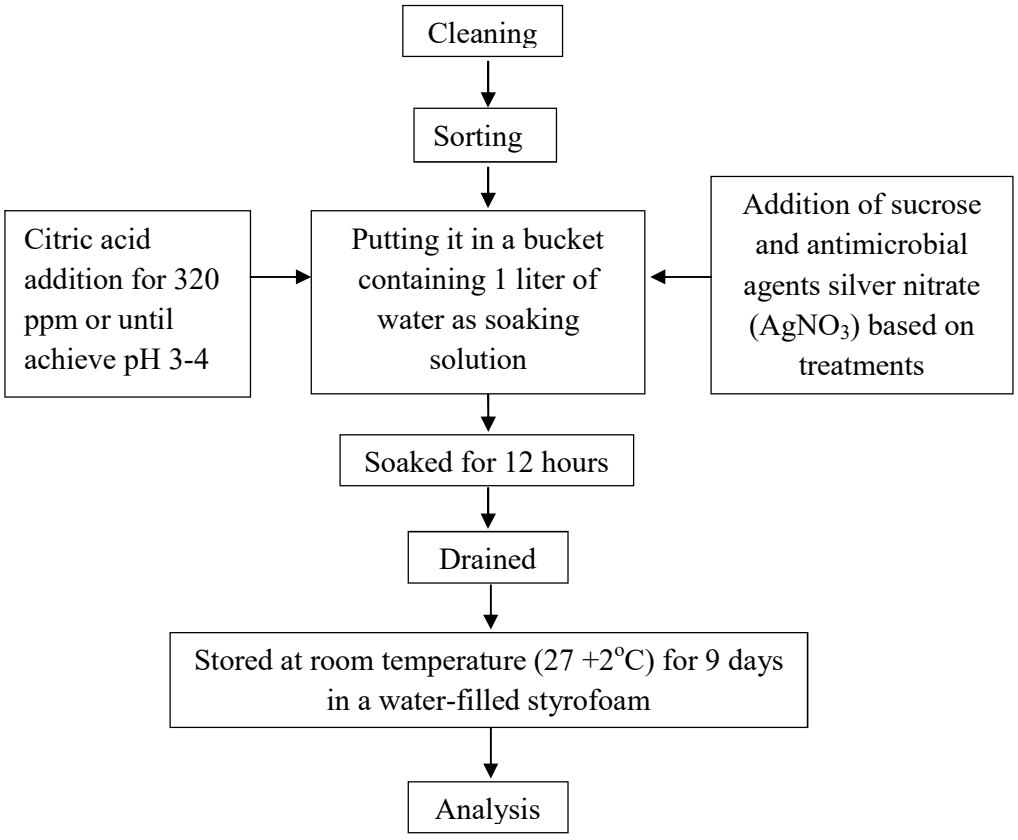


Figure 1. Flowchart of Implementation Research

Variables observed were texture and the level of freshness of cut roses subjectively. Criteria for the assessment of the texture of the cut roses was showed in Table 1. Table 2 displayed the criteria for assessment of the level of freshness of cut roses.

Table 1. Criteria and numerical scores scale test for texture

| Criteria | Numerical Scale |
|---|---|
| Strong, fresh and contained (in flower) | 5 |
| Strong and rather pale (less fresh) | 4 |
| Somewhat wilted | 3 |
| Wilted/tender (marketed limited) | 2 |
| Very wilted and can not be used | 1 |

Table 2. Criteria and numerical scores scale test for freshness

| Criteria | Description | Numerical Scale |
|---|---|---|
| Very fresh | Fresh flowers | 5 |
| Fresh | > 50% of fresh flowers | 4 |
| Somewhat refresh* | 1-5% interest rather wilted | 3 |
| Somewhat wilted** | wilted 5-10% of wilted flowers | 2 |
| Wilt | > 10% of wilted flowers (drying) | 1 |

*) cause in a price adjustment commercially
**) Percentage calculation was based on the amount of wilted flower.

## RESULTS AND DISCUSSION

### Texture of Cut Roses

Results of the sensory assessment on the texture of cut roses during storage are presented in Table 3. Table 3 showed that on day 0, day 1, and day 2 values of texture of the cut roses was 5, which means that the cut roses has strong texture, fresh and contained. On the 3rd day the freshness value of cut roses was still 5 (strong floral, fresh and contained), except S2P3 treatment (combined treatment 15 g of sucrose and 15 ppm of $AgNO_3$), S3P2 (combination treatment 10 g of sucrose and 10 ppm of $AgNO_3$), and K0 (control) had value 4 (rigid and somewhat pale, which less fresh flowers.) Until the 4th days of storage, textures values of cut roses was 5 and 4 that indicate that the cut roses are tough, fresh and contained, somewhat pale and less fresh but it can still be marketed limitedly. However cut roses without treatment (control) demonstrated slightly wilted (freshness value was 3).

### Freshness of Cut Roses

The results of the assessment of the freshness of cut roses during storage were presented in Table 4. The table showed that on day 0, day 1, and day 2 freshness values of the cut roses was 5, which means the cut roses are very fresh with vigor texture. On the 3rd day the value of freshness of the cut roses was still 5 (very fresh with vigor texture), except the treatment of S1P4 (combination treatment 20 g of sucrose and 20 ppm of $AgNO_3$), S2P3 (combination 15 g of sucrose concentration and 15 ppm of $AgNO_3$), S3P1 (combined treatment of 10 g sucrose and 5 ppm of $AgNO_3$), S4P3 (combination treatment 5 g of sucrose and 15 ppm of $AgNO_3$), while value of freshness of K0 (control) was 4 (fresh). Until the 4th day freshness levels of cut roses was 5 and 4, which mean the cut roses is between very fresh and fresh.

Table 3. Sensory value of cut roses texture during storage

| Treatment Combination | Storage time (day) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| S1P1 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 2 | 2 |
| S1P2 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 2 | 2 |
| S1P3 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 3 | 2 | 1 |
| S1P4 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 3 | 2 | 1 |
| S2P1 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 3 | 2 |
| S2P2 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 2 | 2 |
| S2P3 | 5 | 5 | 5 | 4 | 4 | 3 | 3 | 2 | 2 | 1 |
| S2P4 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 3 | 2 |
| S3P1 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 3 | 3 | 2 |
| S3P2 | 5 | 5 | 5 | 4 | 4 | 3 | 3 | 2 | 2 | 1 |
| S3P3 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 2 | 2 | 1 |
| S3P4 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 2 | 2 |
| S4P1 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 3 | 2 |
| S4P2 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 3 | 2 | 1 |
| S4P3 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 3 | 2 | 1 |
| S4P4 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 3 | 2 | 1 |
| K0 | 5 | 5 | 4 | 4 | 3 | 3 | 3 | 2 | 1 | 1 |

Table 4. Sensory value of freshness of cut roses during storage

| Treatment Combination | Storage time (day) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| S1P1 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 4 | 2 | 2 |
| S1P2 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 3 | 2 | 2 |
| S1P3 | 5 | 5 | 5 | 5 | 4 | 3 | 3 | 2 | 2 | 1 |
| S1P4 | 5 | 5 | 5 | 4 | 4 | 3 | 3 | 2 | 1 | 1 |
| S2P1 | 5 | 5 | 5 | 5 | 5 | 3 | 3 | 2 | 2 | 2 |
| S2P2 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 2 | 2 |
| S2P3 | 5 | 5 | 5 | 4 | 4 | 3 | 3 | 2 | 2 | 1 |
| S2P4 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 2 | 2 |
| S3P1 | 5 | 5 | 5 | 4 | 4 | 3 | 3 | 2 | 2 | 1 |
| S3P2 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 2 | 2 | 1 |
| S3P3 | 5 | 5 | 5 | 5 | 4 | 3 | 3 | 2 | 2 | 1 |
| S3P4 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 2 | 2 |
| S4P1 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 2 | 2 |
| S4P2 | 5 | 5 | 5 | 5 | 4 | 3 | 3 | 2 | 2 | 1 |
| S4P3 | 5 | 5 | 5 | 4 | 4 | 3 | 3 | 2 | 2 | 1 |
| S4P4 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 2 | 2 | 2 |
| K0 | 5 | 5 | 5 | 4 | 3 | 2 | 2 | 1 | 1 | 1 |

Table 4 showed that at day 7 to day 9 most of freshness values of cut roses was 3 (somewhat fresh), 2 (somewhat faded) to value 1 (wilted). These findings suggest that the red roses have experienced freshness changes where the value was 3. The value of 3 indicates that 1-5% flower rather wilted and cause price adjustment commercially. The value of 2 indicated 5-10% of flower wilt and cannot be marketed, and the value of 1 represents that 5 - 10% of flowers wilt and decay. On the 7th day the freshness value of cut

roses was still 4 (red), which indicates that more than 50% of the flower is in fresh state caused by S1P1 treatment (combination treatment 20 g of sucrose and 5 ppm of $AgNO_3$).

## CONCLUSION

1. Sensory texture value of cut red roses on the 7th day is still 4 (rigid and rather pale) showed that more than 50% of cut flower can be marketed freshly. The treatment that caused cut red roses had this freshness value was S4P1 (combined treatment of 5 g sucrose and 20 ppm of $AgNO_3$).
2. The level of freshness of red roses to 7th day was still 4 (red) suggest that more than 50% of cut flower was in fresh condition. Cut red roses with this condition were obtained from cut red roses treated with S1P1 treatment (combination of 20 g of sucrose and 5 ppm $AgNO_3$).

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# THE EFFECTS OF DIFFERENT ENERGY - PROTEIN RATION FOR CARCASS OF KAMPONG CHICKENS

**Gusti Ayu Mayani Kristina Dewi*, I Gede Mahardika, I Ketut Sumadi, I Made Suasta, and I Made Wirapartha**
*Faculty of Animal Science, Udayana University, Kampus Sudirman, Denpasar, Bali*
**Correspondence author: elly_unud@yahoo.com*

**ABSTRACT**

The objective of this research was to determine, the effects of different energy-protein ration for carcass of Kampong chickens. This research was conducted at 10 weeks available in Peguyangan District, Denpasar Regency, Bali Province. A number of forty eight (48) unsexed of DOC Kampong chicken were used in this experiment. A completely randomized design was used with 4 treatments and 4 replications of each have 3 birds. The treatments were a ration with metabolic energy 3100 kcal/kg and 22% protein (A) ; ration with metabolic energy 3000 kcal/kg and 20% protein (B); ration with metabolic energy 2900 kcal /kg and protein 18% (C); ration with metabolic energy 2800 kcal /kg and 16% protein (D). The variable studied were: final body weight, carcass weight, dressing percentage, carcass percentage, breast, thigh, wing and back, carcass composition (meat, bone, skin), and microbiology of fecal.

Results of this experiment showed that final body weight, carcass weight, dressing, carcass percentage, breast, thigh, wing and back, abdominal fat, carcass composition (skin), microbiology of fecal was non-significantly (P<0,05) among the treatments. But carcass composition (meat, bone) RA treatment was significant affect (P<0.05) than RB, RC and RD treatment. It was concluded that the effect of treatment with metabolic energy- protein 3100 kcal/kg and 22% protein (A), 3000 kcal/kg and 20% protein (B); 2900 kcal /kg and protein 18% (C); and 2800 kcal /kg and 16% protein (D) on the ration gave non-significant effect (P>0.05) to final body weight, carcass weight, dressing percentage, carcass percentage, breast, thigh, wing and back, abdominal fat, carcass composition (skin), microbiology of fecal among the treatments. But carcass composition (meat, bone) with A treatment was significant affect (P<0.05) than B, C and D of Kampong chickens.

***Keywords: Kampong chickens, energy, protein, carcass, abdominal fat***

## INTRODUCTION

Kampong chickens is an important asset to Indonesia people to the raise it as a saving, strategic commodity in market demand for the products (meat and egg) is high. The one problem encountered in developing the commodity is low productivity (Rusmana *et al*. 2010 and Dewi 2010). This low production potential may be attributed to lack of improved poultry breeds, the presence of predators, the poor feeding and cannot used a count of nutrition in ration for different age of the chickens (Sartika *et al.* 2008). Feed is a vital component of semi-intensive and intensive rearing of Kampong chickens. This is attributed to low availability potential local feed resources and fluctuating of feed price. Approximately, feed cost is 75% of total capital cost. Knowing the composition of the available ingredients, ratio energy – protein ration, the basic nutritional requirements it is possible to formulate a diet that promote optimum growth (meat).

According to Anderson and McAdam (2004) animal welfare is related to the subjective feelings of animal, which is difficult to quantify. The welfare of poultry can be considered in relation to housing environment management practices. Different kind of stress, disease and morbidity, body condition, reproduction, behavior and physiology are costly economic problems in Kampong chickens. Enhancing the immune response nutritional on the ration means might improve Kampong chickens health and reduce the cost of the production (van Winsen *et al.* 2002). However the use of ration from local animal or nabati feed for production can give beneficial effects for health to eliminate microbial pathogens in the manure which reduce the potential hazard to public health (Sumarsih *et al*. 2010).

The objective of the research to determine production carcass of Kampong chickens with different energy – protein ration.

## MATERIALS AND METHODS

### Birds and treatments

This research was done 10 weeks in Peguyangan District, Denpasar Regency, Bali Province. Forty eight (48) of unsex Kampong chickens used in the research allotted to 4 treatments with 4 replicates of 3 chickens each. The treatment were as follows: A, B, C and D. Consist of metabolic energy (ME) 3100 kcal/kg and protein 22% (A), ME 3000 kcal/kg and 20% protein (B); ME 2900 kcal/kg and 18% protein (C) and ME 2800 kcal/kg and 16% protein (D).

Ration were formulated with ingredients are presented in Table 1.

The cage used battery pens consist of 16 cages, were made up of the stainless wire and each pen (65 cm X 50 cm X 75 cm), tree birds were housed. The bottom of the floor was also made up plastic for collecting feces. Feed and water supplied were provided *adlibitum*.

The variable studied were: final body weight, carcass weight, dressing, carcass percentage, breast, thigh, wing and back, carcass composition (meat, bone, skin), abdominal fat, microbiology of fecal.

### Trait Measured

Kampong chicken was weighed the day before slaughter and birds were killed via vena jugularis and then depluned. The carcasses were weighed, the body cavity was open and the heart, liver, viscera and spleen were removed and weighed. The carcass and organs were expressed as percentage of live body weight. The breast meat (pectoralis major and pectoralis minor was removed by cutting laterally at the wing joint and dissecting the muscle from the carcass). Carcass quality was measured by determining slaughtered yield (USDA 1979). The abdominal was removed and weight to determine the percentage of abdominal fat. The fresh fecal sample was collected from each replicate between alternate weeks to determine the bacterial pathogen enumeration by (Fardiaz, 1989).

Data obtained were statistically analyzed using ANOVA and mean comparison was tested by Duncan's Multiple Range Test (Steal and Torrie 1993)

Table 1. Composition and Nutrition Content of the Ration

| Ingredient | A | B | C | D | Standard * |
|---|---|---|---|---|---|
| Yellow corn | **48.15** | **50.70** | **50.80** | **54.00** | |
| Soybean meal | **27.70** | **20.00** | **14.00** | **6.90** | |
| Coconut meal | **8.88** | **12.00** | **11.90** | **16.20** | |
| Fish meal | **7.95** | **7.40** | **6.59** | **5.60** | |
| Rice bran | **6.53** | **9.05** | **15.91** | **16.40** | |
| Coconut oil | **0.35** | **0.40** | **0.30** | **0.30** | |
| Premix | **0.25** | **0.25** | **0.30** | **0.40** | |
| Salt | **0.20** | **0.20** | **0.20** | **0.20** | |
| **Nutrient Composition** | | | | | |
| **ME (kcal/kg)**** | **3100** | **3000** | **2900** | **2800** | **2600 - 2800** |
| **Crude Protein (%)**** | **22** | **20** | **18** | **16** | **18 – 21** |
| **Crude fiber (%)**** | **4.73** | **5.02** | **5.33** | **5.63** | **7 - 9** |
| **Calcium (%)**** | **0.58** | **0.53** | **0.47** | **0.40** | **1 – 1.2** |
| **Phosphor (%) **** | **0.47** | **0.44** | **0.40** | **0.36** | **0.30** |
| **Arginine*** | **1.78** | **1.64** | **1.50** | **1.38** | **1.06** |
| **Systeine*** | **0.37** | **0.32** | **0.30** | **0.28** | **0.34** |
| **Lysine*** | **1.52** | **1.31** | **1.13** | **1.27** | **1.06** |
| **Methionine*** | **0.44** | **0.38** | **0.34** | **0.30** | **0.24** |

*Standard of Scott *et al*. (1982)
** Analysis of Laboratory of Nutrition, Animal Science, Udayana University (2010).

## RESULTS AND DISCUSSION

The results of this study are presented in Table 2. There was improvement in final body weight, carcass weight, dressing percentage, carcass percentage, breast, wing and, carcass composition (skin) non-significant effect (P>0.05) between ration with difference energy-protein level was increased from 2800 to 3100 Kcal/kg-protein 16 to 22% on the diets. The recommended is similar reports by Scott. *et al.* (1992) and Husmaini (2000). It is possible that performance of carcass Kampong chickens with high levels energy-protein better than lower dietary levels. According van Winsen *et al.* (2002) production is often suggested as an alternative need energy, protein and essential nutrients for maintenance, metabolic functions and growth (meat).

Yield commercial cuts (breast) of Kampong chickens with A treatment (3100 kcal/kg and 22% protein) were 23.17%, but treatment B, C, and D as follows 23.15%; 23.54%; 23.50% there were no significant (P>0.05) after the treatment (Table 2). Dietary all treatments did not significant affects on percentages of thighs, back, wings and characteristic physic carcass (skin) (Table 2).

Table 2. The Effect of Treatment for Production and Microbiology Fecal of Kampong Chickens

| Parameters | Treatment [1] | | | |
|---|---|---|---|---|
| | A | B | C | D |
| Final Body Weight | 605.75[a] | 595.75[a] | 591.00[a] | 591.25[a] [2] |
| Dressing % | 77.99[a] | 78.00[a] | 78.10 [a] | 78.19[a] |
| Carcass Percentage | 68.64[a] | 68.64[a] | 67.08[a] | 66.93[a] |
| Breast (%) | 32.93[a] | 33.15[a] | 31.80[a] | 31.88[a] |
| Thighs % | 23.17[a] | 23.15[a] | 23.54[a] | 23.50[a] |
| Back (%) | 30.50[a] | 30.30[a] | 31.06[a] | 31.32[a] |
| Wings (%) | 13.40[a] | 13.40 [a] | 13.60[a] | 13.30[a] |
| *Physical Characteristic of Carcass* | | | | |
| Meat % | 40.98[b] | 40.00 [a] | 40.10[a] | 40.20[a] |
| Skin % | 33.55[a] | 32.31[a] | 33.61[a] | 33.10[a] |
| Bone % | 19.47[b] | 20.69[a] | 20.89[a] | 22.70[a] |
| Abdominal fat (%) | 3.1[a] | 3.09[a] | 2.90[a] | 2.80[a] |
| *Microbiology* | | | | |
| Fecal Enterobactericeae (mcu/g) | 1.12x10$^4$ [a] | 1.13 x10$^4$ [a] | 1.25 x10$^4$ [a] | 1.15 x10$^4$ [a] |
| Fungi (mcu/g) | ND | ND | ND | ND |

1. A: Ration with 3100 Kcal ME/kg and 22% protein; B: 3000 Kcal ME/kg and 20% protein
   C: 2900 Kcal ME/kg and 18% protein ; D: 2800 Kcal ME/kg and 16% protein
2. Means with different Superskrip in the same row differ significantly (P<0.05)
3. ND: Not Determined (not identified from the sample)

The characteristic physic carcass (meat and bone) percentage of Kampong chicken were significant effect (P<0.05) among the treatment (Table 2). Table 2 showed that meat percentage of A treatment was higher than B, C and D. This means that level 3100 Kcal ME/kg and 22% protein able to increased meat percentage than others treatment. It is possible that increase of protein intake, retention and metabolic energy in rations used for body composition (Dewi and Sinlae 2004; Dewi 2010). This findings was agreed with Young *et al*. (2001) who reported that protein content of meat is influenced by nutrition, age, sex, and duration of post mortem aging on percentage yield, parts of broiler carcasses, and carcass quality of Kampong chicken (Zuidhof 2004). Table 2 shows the A treatment significantly reduced the number of bone percentage than B, C and D treatment. Because on the A treatment, energy –protein ration was adequate for optimum meat and less for bone percentage. According Watkin (1993) many factors influencing of bone in rapidly growing meat type poultry are genetics, nutrition (Ca, P mineral), implications of preslaughter for carcass, and total of meat.

Kampong chickens yielded eviscerated carcasses of between organs, abdominal fat were expressed as percentage of live body weight. The result showed that abdominal fat percentage in four treatments were decreased non significantly (P 0>0.05).

The total enterobacteriaceae excreted by the birds on the fecal, C treatment were no significantly (P>0.05) higher counts than A, B, and D. The rations with deferent level energy – protein helps to develop and stabilize a competent microflora in the gastrointestinal tract that should successfully avoid the proliferation of pathogens in reduction of fecal enterobacteriaceae. Similar results in reduction of fecal enterobacteriaceae have been obtained by (Sulistiyanto *et al.* 2011). The latter finding is surprising that records a fecal fungi was not found/ determined on all treatment in this research.

## CONCLUSION

The effect of treatment with metabolic energy- protein 3100 kcal/kg and 22% protein (A), 3000 kcal/kg and 20% protein (B); 2900 kcal /kg and protein 18% (C); and 2800 kcal /kg and 16% protein (D) on the ration gave non-significant effect (P>0.05) to final body weight, carcass weight, dressing percentage, carcass percentage, breast, thigh, wing and back, abdominal fat, carcass composition (skin), microbiology of fecal among the treatments. But carcass composition (meat, bone) with A treatment was significant affect (P<0.05) than B, C and D of Kampong chickens.

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# COMPOSTING ACCELERATION OF KITCHEN WASTE USING MICROBIAL INOCULANT

**Ni Made Indri Hapsari Arihantana and Ni Luh Yulianti**
*Faculty of Agriculture Technology, Udayana University, Kampus Bukit Jimbaran, Bali.*
*Correspondence author: mdindri@yahoo.com*

**ABSTRACT**

Organic waste such as leftover foods from kitchen, vegetables, leaf, and other spoiled foods can be utilized into composting organic material and also decrease the risk of environmental hazardous. Naturally, decomposition process takes longer time, human power and generate annoying health problems. Referring to these reasons, a new technology is required. The addition of microorganism as a decomposer will accelerate the process if they have a favourable condition. The research was conducted to produce an inoculant from rumen of cows, goats, chicken manure and also to analize kitchen waste (left over foods) by using different inoculants. Firstly moisture content, total microorganism, fungi, and pH of the inoculants were measured. The second step was to determine pH and ash content of kitchen waste. Moisture content of cow, goat rumen, and EM4 was 90% respectively, while chicken manure remained 60%. All inoculants have a neutral pH, total microorganism 1.1 x $10^{11}$ cfu/ml and total fungi, 3.4 x $10^{4}$ cfu/ml, while chicken manure contains 1.1 x $10^{11}$ cfu/ml microorganism and 1.0 x $10^{6}$ cfu/ml fungi. The.average pH of the kitchen waste was 7.0 and ash content was 2.3%. The highest ash content was produced from the kitchen waste treated with inoculants from chicken manure.

***Keywords*: *cow rumen, goat rumen, chicken manure, inoculant, kitchen waste***

## INTRODUCTION

Organic waste such as leftover foods from kitchen, vegetables, leaf, and other spoiled foods can be utilized into composting organic material and also decreased the risk of environmental hazardous. Compost is an organic waste which have decomposed by interaction of microbe. Naturally, decomposition process takes more time, human power and generated annoying also health problem. Refers to those reason, the need of technology in composting is required. Additional of microbe decomposer can help composting acceleration process if appropriate and favorable conditions are available.

Kitchen waste which can be composted consists of cellulose. Therefore, if the materials were inoculated with microorganism, the composting of kitchen waste is expected to run faster and produced better quality compost. Mala (1994) reported that inoculation of *Trichoderma harzianum Rifai Aggr*. (cellulolytic microorganism) on rice straw composting, composting able to accelerate to 19 days to reach the ratio of C/N 20 as the criteria of mature compost.

Manure is one of the materials that can be used as inoculants. Manure or rumen contents of cattle (ruminants) is a type of slaughter house waste generated continuously at a large amount, which ranges from 10-20% of animal live weight. It is known that rumen microorganism could accelerate the composting process and also improve the quality of compost. Rumen fluid contains protozoa and bacteria digesting cellulose, hemicellulose, starch, sugar, protein, lactate-eating bacteria and methane-forming bacteria (Hungate, 1966). Each ruminants contain various rumen microorganism, that influences composting and compost quality produced. The aim of this research is to obtain rumen microorganism from cows, goats, and chicken manure to be used as inoculants, and also to analize macro nutrients of kitchen waste.

## MATERIALS AND METHODS

Fresh cow rumen was obtained from Sanggaran slaughter house at Denpasar, fresh goat rumen from goat abattoir in Haji Rifai Wangaya Denpasar. Chicken manure was obtained from a poultry farm in the village of Kuum, Marga, Tabanan regency. Kitchen organic waste was obtained from several residential homes in Nuansa Hijau Utama Ubung, Denpasar. Media for inoculants are Nutrient Agar (oxoid) and 0.1% peptone water. The carrier material used is filtered compost. EM4 is used as a positive control for composting. The research consists of two phases, the first phase is the manufacture of inoculants and composting. The

study lasted for 9 months. The first phase includes: counting the initial population and observing the parameter of total microorganism, total fungi and pH. The second phase includes the implementation of composting that consists of sorting, addition of inoculant, planting litter bags and reversal process. The parameters observed are: an initial analysis of nutrient kitchen waste content and pH value. At this stage, the data were analyzed descriptively

## RESULTS AND DISCUSSION

The first stage measured levels of initial water of cow, goat rumen and chicken manure, by burning the material at 105°C for 24 hours. The measurements used is toluene on water content of rumen as reported by Sofyan, 1983. The water content of the rumen of cows and goats generally has an average moisture content ranging between 70-80% (pkplk-PLB). The results showd moisture content ranged around 90% for both types of rumen. The moisture content of the chicken manure ranged between 30-40%, while moisture content of the chicken manure ranged around 60%. Compared to the inoculants that are sold at market, EM4 has a moisture content of 90% which is quite similar with moisture contents of rumens of cow and goats (Figure 1).

pH of rumen usually ranges from 5.5 to 5.6, the research reported that rumen of cows and goats pH ranges around 6.5, chicken manure ranges around 7 and EM4 has a pH of 5.4. All materials were categorized as neutral pH (Figure 1).

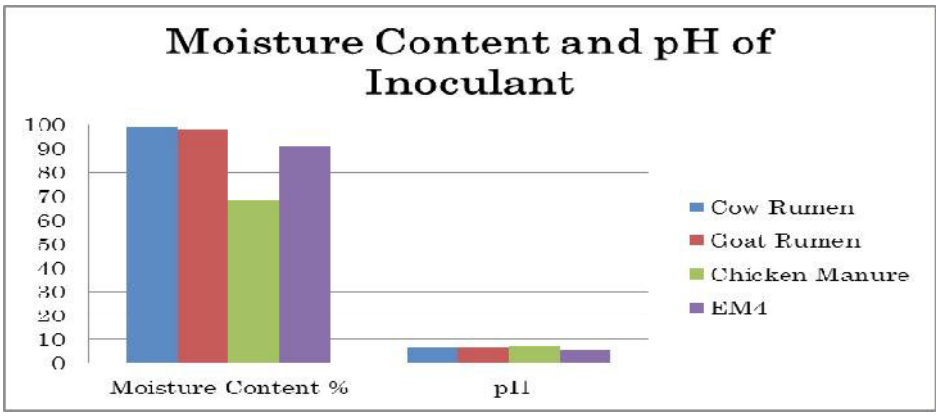


Figure 1. Moisture Content and pH Inoculant

Rumen fluid contains bacteria and protozoa. The concentration of bacteria is around $10^9$/ml rumen contents, while protozoa varies about $10^5$-$10^6$/ml rumen contents (Tillman *et al.*, 1991). Some types of bacteria / microbes present in the rumen contents are (a) bacterial / microbial lipolytic, (b) bacteria / microbes forming acid, (c) bacterial / microbial amylolytic, (d) bacterial / microbial cellulolytic, (e) bacteria / microbes proteolytic (Tillman *et al.*, 1991). In the goat rumen microbial population are divided into three main groups of bacteria, protozoa, and fungi (Tillman *et al.*, 1991). Types of bacteria found in the rumen of goat have the same function with the bacterial species present in the solution of EM4, quite higher in complexity.

The high TPC indicates the high quality of microorganism inoculants produced. In this study, the results of TPC show in Figure 2. The concentration of the bacteria found in the rumen of cows is 9.8 x $10^9$ cfu/ml. TPC on rumen of goats is 2.6 x $10^9$ cfu/ml and the chicken manure is 1.1 x$10^{11}$ cfu/ml. The EM4 inoculant is 10.4 x $10^9$ cfu/ml. Total fungi produced by rumen of cows is 0.5 x $10^4$ cfu/ml, 0.8 x $10^3$ cfu/ml for rumen of goats and chicken manure 3.6 x $10^4$ cfu/ml. The total EM4 mold inoculant produced is 3.1 x$10^3$ cfu/ml.

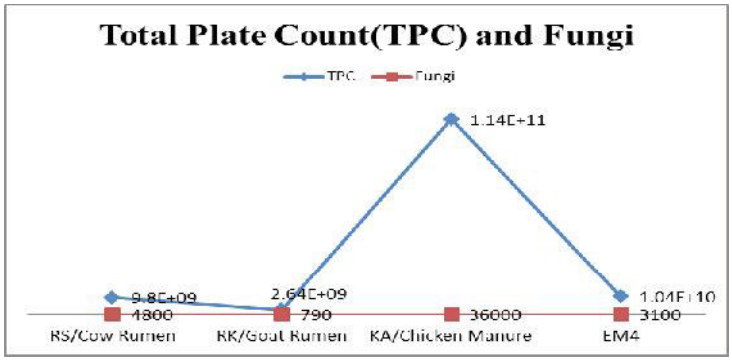


Figure 2. Result of Total Plate Count (TPC) and Fungi of Inoculant

Generally, kitchen wastes consist of a number of bacteria / microbes that are ready to revamp the organic materials contained. After the phase separation of organic waste, inoculant applied into each sample. In this study, the TPC results indicate, the concentration of bacteria found in kitchen garbage treated with rumen of cows inoculant is 1.7 x$10^{10}$ cfu/ml. TPC on rumen of goats inoculant is 1.2 x $10^{10}$ cfu/ml and the chicken manure inoculant is 1.1 x$10^{11}$ cfu/ml. The EM4 inoculant is 4.2 x $10^{10}$ cfu/ml. Total fungi produced by rumen of cows is 7.9 x $10^{3}$ cfu/ml, 1.0 x $10^{5}$ cfu/ml for rumen of goats and chicken manure 1.0 x$10^{6}$ cfu/ml. The total EM4 mold inoculant produced is 3.0 x $10^{5}$ cfu/ml.

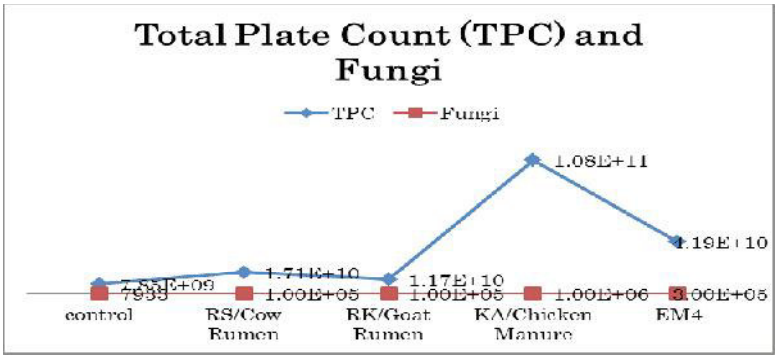


Figure 3. Result of Total Plate Count (TPC) and Fungi of Kitchen Waste Compost

The composting process occurs in a wide range of pH. The optimum pH for the composting process ranges from 6.5 to 7.5. The standard quality of pH is defined according to the World Bank, International, PT Pusri and Market is 7.5. The results of compost derived from kitchen waste treated with different inoculants showed an average pH of 7.

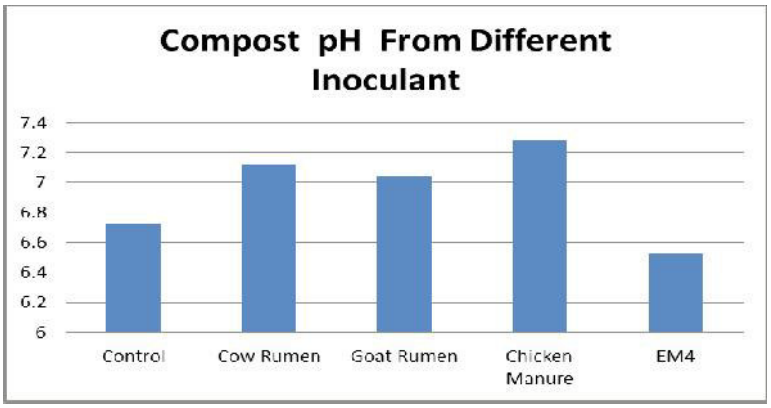


Figure 4. Kitchen Waste Compost pH from Different Iinoculant

Figure 5 shows the mineral of kitchen waste compost. Inorganic ash content is a component that remains after the material is heated at a temperature of 600°C. Generally the ash content varies depending on the inoculants used. Average of ash content of compost in five treatments was 2.3%. Ash content of compost was 2.2%, 2.19%, 2.44%, 2.26% by using rumen of cows and goats inoculant, chicken manure, EM4 respectively. In composting process, ash increased due to degradation of organic compounds into inorganic compounds (Sriharti and Salim, 2007).

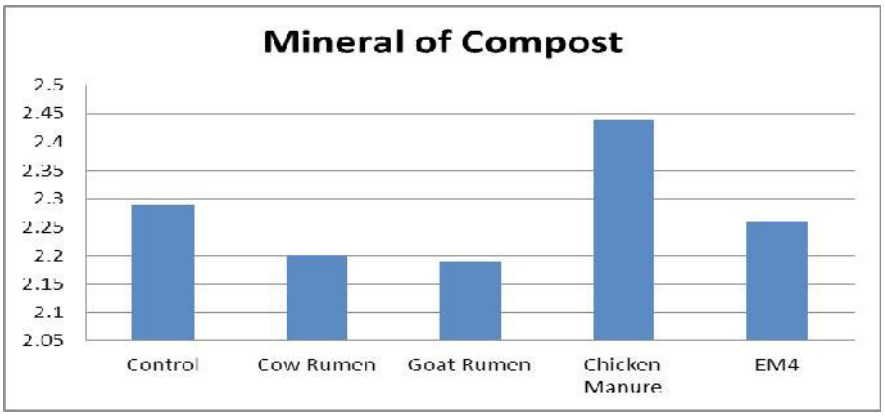


Figure 5. Mineral of Kitchen Waste Compost

## CONCLUSION

1. The average moisture content of rumen of cows, goats and EM4 ranges from 90%, while the water content of chicken manure ranges from 60%. pH inoculant is categorized st neutral pH.
2. TPC results showed that the highest concentration of bacteria found in chicken manure inoculant around 1.1 x $10^{11}$ cfu/ml while the total fungi produced by chicken manure is 3.4 x $10^{4}$ cfu/ml.
3. In compost, TPC results showed that the highest concentration of bacteria found in household waste inoculants treated with chicken manure is 1.1 x $10^{11}$ cfu/ml while the total fungi produced is 1.0 x $10^{6}$ cfu/ml.
4. The average pH of kitchen waste inoculant with different treatment is 7 while the ash content of domestic waste inoculants with different treatment is 2.3%. The kitchen waste inoculants treated with chicken manure has a low ash or high mineral of 2.44%.

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# DESIGN OF THE FLUIDIZED BED DRYER FOR CHILI SEED

**Ni Luh Yulianti, Gede Arda, Putu Surya Wirawan, Made Anom S. Wijaya**
*Jurusan Teknik Pertanian, Fakultas Teknologi Pertanian Universitas Udayana*
*yulianti_ardana@yahoo.co.id*

**ABSTRACT**

The aims of this research are to design a small scale fluidized bed dryer for chili seed and to assess its performance to meet the demand of chili farmers to produce 1 kg chili seed in each process. The research was conducted through 3 steps, those were: manufacturing of dryer component, assembly and assessment of the dryer's performance. Designing of the dryer was emphasized on drying chamber volume, drying air velocity demand, and heat exchanger unit. The measured parameters were weight and moisture content of chili seed, drying air temperature and drying rate. Drying chamber volume was 3,456.62 $cm^3$ with its diameter and height are 12 cm and 31 cm respectively. Dryer was operated at drying air velocity of 79.99 $m^3$/hr and needed 421 watts of energy. Drying air temperature of 40°C is obtained 5 minutes atfer dryer was turned-on. The result showed that designed fluidized-bed-dryer's drying performance was good in drying for chili seed.

***Keywords***: Chili seed, fluidized bed dryer

## INTRODUCTION

Chili is one of the agricultural commodities produced and consumed in indonesia. Besides, dried-chili also exported to some countries as seed. The dried-chili seed grows significantly from 88.29 tons in 2003 to 854.32 tons in 2004 (agrsiculture statistics, 2005). Therefore, it is needed the proper process to provide dried-chili seed. Good process of dried-chili seed production is demanded in order to produce not only the good quality dried-chili seed but also longer shelf life. Accordig to Indonesia national standard for chili, it is required to reduce the moisture content of chili seed to 10% before it is stored (SNI, 2004).

Farmers, so far, dries their chili seed under the sun because it is easi to conduct and need no cost. However, this method give the chili seed disadvantage, that is, production process greatly depends on the weather. Therefore, it is needed proper method which do not depend on the weather and have lower possibility of lost chili seed.

One of the drying method which can apply to reduce the disadvantages of using sun drying method to dry chili seed is using the artificial drying process. One of the articficial drying process which is suit for chili seed is fluidized bed dryer. This type of dryer can dry the seed which its size range from 20 micron to 10 mm faster. Therefore, the aims of this research are to design the small-scale-fluidized-bed-type dryer for chili seed and to assesst the functional performance of the dryer on chili seed drying process. Small scale means the designed dryer can dry 1 kg of chili seed per batch.

## MATERIALS AND METHODS

### Materials And Equipments

Equipments needed in this research are metal cutter, electrical drill**,** welding machines, lathes, nuts, bolts, scales, thermometer, anemometer, moisture tester, wrenches, and other tools needed in the manufacture of tools, data loger , scales, knives container. The materials used are, blower fan, the heater, angle iron to order equipment, pipe roll, aluminum pipe, holo steel, cable, wet chili beans, thermostat, filter gauze, wire welding, grinding leaves

### Research Phases

This research is conducted trough 3 phases, those are to design of the dryer, to asemble and to assesst the functional performance. The complete research phases are showed in Figure 1.

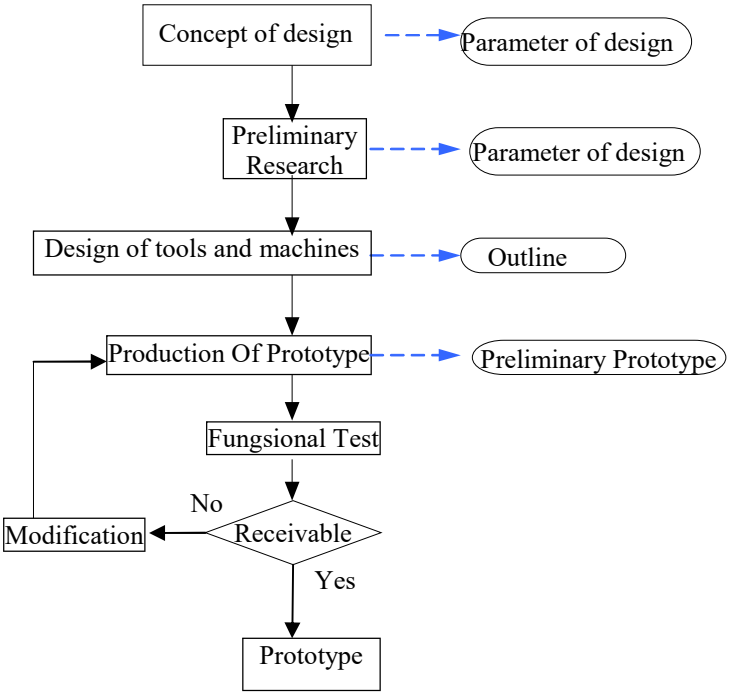


Figure 1 research phases

## DESIGN PARAMETERS

Related parameters can be devide into three groups, those are:

1. Initial moisture content, Density and size of the chili seed.
2. User: easy to use, easy to maintain, easy to sell, and benefit the user.
3. Environment: humidity, temperature, availability of power.

## DESIGN SELECTION

Temperature controlling is the most crucial parameter in designing good dryer, therefore, thermostat is used to control the temperature. Dryer consists of several unit: 1). Drying chamber unit. Its functions as place for chili seed being dried. This chamber has a window for users to observe the chili seed drying process. 2). Blower Unit which functions as drying air supplyer to the heat exchanger. 3). Heat Exchanger unit which functions as heater for drying air so as increase temperature of drying air.

a. Drying chamber volume calculation

The formula for calculating drying chamber volume calculation is:

$$V = \frac{W}{\rho_{chili}} \quad \text{.....(1)}$$

To ensure that fluidization proces of chili seed is enough, it demand enough space that is twice larger than V (Pery, Robert,H., 1984). If D is the tube diameter, the height of tube will:

$$\frac{1}{4}\pi D^2 L = 2V \quad \text{.....(2)}$$

b. Blower velocity

Inlet and outlet temperature of drying air and humidity are needed to calculate the velocity of blower. If m kg chili, $m_1$ initial moisture content and $m_2$ final moisture content, the amount of water evaporated from chili will:

$$E = \frac{E(m_1 - m_2}{1 - m_2} \quad \text{.....(3)}$$

Therefore, evaporation rate :

$$G = \frac{E}{T} \quad \text{.....(4)}$$

And, the drying air rate :

$$v = \frac{G}{(h_c - h_a)} x\ vs \quad \text{.....(5)}$$

c. Drying air rate calculation

The amount of heat needed to increase the temperature of drying air can be calculated using Formula (6) (Taib, *et al.* 1988)

$$Q = \frac{v}{vs} x(h_c - h_a) \quad \text{.....(6)}$$

## OBSERVED PARAMETERS

The observed paramater which are included in this research are:

1. Weight and moisture content of chili seed.
2. Temperature at base, middle and outlet of the drying chamber.

## RESULTS AND DISCUSSION

**Design Result Of Fluidized Bed Dryer**

Initial research has found that moisture content, density and diameter of chilli seed are 44% (wet basis), 578.6 kg/m$^3$ and 1-1.5 mm respectively. Mujundar (1995) suggested that granulated shape materials which range from 20 micron – 10 mm in diameter should processed using fludized bed tipe dryer.

## DRYING CHAMBER

Drying chamber is one of the dryer unit where chilli seeds are dried. Observing window is made at drying chamber wall with 25 cm x 2 cm x 0.5 cm glas attached in it. This unit is made from galvanized pipe 12 cm in diameter which has several advantages compare to other type of materials: those are strong enough against high pressure or load work on it, is not easy begin to rust. The galvanized pipe's properties suit for agricultural comodities though its cost higher than others type of pipe (Anon, 2010).

Using Formula 1 and 2, with pipe diameter is 12 cm, it will result in height of the drying chamber unit is at least 31 cm and its volume is 3.456.62 cm$^3$. The smaller chamber is made to facilitate loading process of chilli seed before and after drying process. In order to ensure that fluidization process run properly, a piece of metal mesh is used as the smaler-drying- chamber base.

## BLOWER UNIT

This unit blows drying air into the drying chamber during drying process. Calculation of drying air rate is base on the psychrometric chart in which all the parameters of the environment are measured. To reduce chili seed moisture content from 44% to 10% moisture content, it is needed to remove 0.37 kg water per 1 kilogram chili seed and 79.77 $m^3$ air/hour. Therefore, it need the blower which able to blow drying air 79.77 $m^3$ air/hour.

### Heat exchanger unit

Based on calculation it is needed 295 Watt electrical power to raise draying air temperature from room temperature to about 40 $^oC$. Assuming that effeciency of heat exchanger unit is 70%, therefore it is needed unit which provide at least 500 Watt of electrical power. Designed-fluidized-bed-type dryer is shown in Figure 2.

Figure 2 Designed-fluidized-bed-type dryer

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### Loaded Functional Assessment

The dryer is designed for working at drying air temperature of 40$^oC$. drying air blown by blower pass the heat exchanger which produce 600 watt of heat. Heat exchanger temperature is controlled by a thermostat, therefore it will ensure the temperature of drying air passing the chili seed does not exceed 40$^oC$ + 1$^oC$.

Chile seed which are being dried are fed into the drying chamber when temperature of drying chamber reaches 40$^oC$ + 1$^oC$. It takes about 5 minutes after the dryer is turned on. Measurementof drying process temperatures are conducted periodically; that is every 15 minute. Temperature probes are located at 3 places those are at the base, middle and the outlet of drying chamber.

Table1. Temperature at the base, middle and the outlet of drying chamber

| time (Minute) | temperature ($^oC$) | | |
|---|---|---|---|
| | $T_{base}$ | $T_{middle}$ | $T_{outlet}$ |
| 0 | 26.90 | 27.80 | 29.00 |
| 15 | 27.60 | 30.10 | 31.50 |
| 30 | 29.70 | 29.26 | 32.10 |
| 45 | 34.00 | 29.80 | 32.70 |
| 60 | 37.70 | 32.20 | 34.60 |
| 75 | 38.90 | 34.10 | 35.20 |
| 90 | 38.90 | 36.10 | 36.30 |
| 105 | 39.10 | 39.10 | 37.60 |
| 120 | 39.20 | 39.90 | 38.70 |
| 135 | 39.20 | 39.70 | 39.10 |

Table 1 shows there are slight differences of temperature at initial drying process. Temperatures of drying chamber drop sharply when chili seed feed in to drying chamber. Temperature at base of the drying chamber is the lowest though it is the nearest part of the drying chamber to the heat exchanger because at initial drying process wet chili seed keep the base cooler than other parts of drying chamber. Wet chili seed absorb the heat from the drying chamber that cool down the drying chamber. High moisture content of chili seed absorb heat and then the absorbed heat evaporates some of the water from chili seed. Taib *et al*. (1985) said that free water on the chili seed surface easier to evaporate than water which is bonded physically or chemically.

Drying air and chili seed temperature rise gradually and reach about 40°C after 105 minutes at the base and the middle of drying chamber. The outlet temperature takes longer time to raise its temperature and reaches about 40°C in 30 minutes later. This means that differences between drying air temperature and chili seed temperature getting lower as process time increases, therefore, heat transfer rate decline gradually. The heat transfer rate consider zero at any point of the process when there is no apparent the temperature difference between drying air and chili seed (Taib *et al*., 1988).

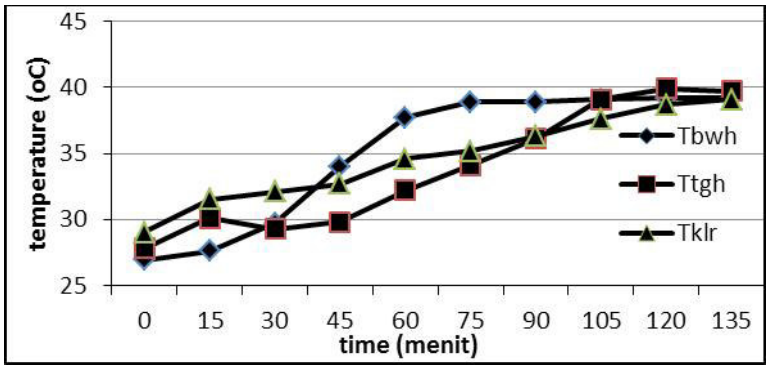


Figure 3. Temperature development in drying chamber

This research have finished the prototype of the fluidized bed dryer for chili seed. Functional assessment shows that this prototype of the fluidized bed dryer for chili seed perform the good result on chili drying process. Drying process is obtained in 139 minutes after machine are turned on. This research recommends error analysis in order to investigate variable which contribute the largest error and to minimize that variable.

We recommend to apply this prototype to dry others kinds of seed.

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# THE IMPLEMENTETION OF *Saccharomyces Spp.N-2* CULTURE ISOLATED FROM TRADITIONAL YEAST CULTURE FOR DECREASING ABNOMINAL FAT AND SERUM CHOLESTEROL CONTENTS OF BALI DUCKLING

**I. G. N. G. Bidura[1*], D. P. M. A. Candrawati[1], I. B. Gaga Partama[1], I. A. Putri Utami[1], E. Puspany[1], D. A. Warmadewi[1], N.K. Mardewi[2], and I. G. A. Istri Aryani[1]**

*[1]Faculty of Animal Science, Udayana University, Jl. PB. Soedirman Denpasar-Bali, Indonesia Phone/fax. 0361-235231*

*[2]Faculty of Agricultural Science, Warmadewa University, Denpasar-Bali*

**Corresponding Author: bidura_unud@yahoo.com*

**ABSTRACT**

There are three isolates of *Saccharomyces spp (Sc.*N-2; N-7; and G-9*)* were isolated from traditionalyeast culture product samples in the first experiment. All of isolate *Saccharomuces spp* showed resistant grew on both in different temperature ($10^0$-$45^0$C) and acid conditions (2,5-4,5), but only one isolate (*Sc.*N-2) were the potensial as a probiotics sources (resistant on both acid and bile salt, and can diskonjugated cholesterol). Therefore, this research was carried out to study the implementation of isolate *Saccharomyces spp. N-2* culture in diets for decreasing serum cholesterol contents and abdominal fat of male Bali duckling up to eight weeks old. One hundred day-old-duck (DOD) of male local Bali drake (*Anas sp*) were assigned to 4 treatments in a completely randomized design. Each treatment has five replications with five birds per replication. The treatments were (i) basal diets without *Saccharomyces spp. N-2* culture as control; (ii) basal diet containing 0.10% *Saccharomyces spp. N-2* culture; (iii) basal diet containing 0.20% *Saccharomyces spp. N-2* culture and (iv) basal diet containing 0.30% *Saccharomyces spp. N-2* culture supplemented, respectively. Experimental diets and drinking water were provided *ad libitum* during the entire experimental period (for a 8-week periods). Body weight and feed intake were recorded weekly. The study showed that supplemented of 0.10%-0.30% *Saccharomyces spp. N-2* culture in the basal diets there were no significant differences (P>0.05) on feed consumption. However, birds consumed 0.20%-0.30% *Saccharomyces spp. N-2* culture in the basal diets had higher final live weight, live weight gain (LWG)s and more efficient in using feed compared to those control fed and 0.1% *Saccharomyces spp. N-2* culture in the basal diets (P<0.05). Increasing the amount of *Saccharomyces spp. N-2* culture in the basal diets could decrease significantly different P<0.05) on total cholesterol content in blood serum and abdominal fat of the birds. It was concluded that supplemented of 0.20%-0.30% *Saccharomyces spp. N-2* culture in the basal diets could increase growth performance of male Bali duckling. Moreover it decreased both the serum cholesterol contents and abdominal fat of male Bali duckling.

***Keywords*:** *Saccharomyces spp.N-2.*, abdominal-fat, cholesterol, Bali duckling

## INTRODUCTION

*Aspergillus oryseae (AO)* and *yeast*, particularly *Saccharomyces cerevisiae*, have been used as probiotics by Piao *et al*. (1999). Both *Aspergillus sp.* and *Saccharomyces* belong to the *Ascomycoyina* subdivision and have many industrial applications in the brewing, distilling, and baking industries (Han *et al*., 2001).

Yeast culture product localy can improved nutrients digestibility and growth performance of Bali drake (Bidura *et al*., 2009) and can increase protein and metabolizable energy of rice bran (Bidura *et al.,* 2011). In contrast, Piao *et al*. (l999) reported no significant improvement in weight gain, feed intake and feed efficiency with 0.10% yeast culture. Feeding live yeast to broiler breeder reduced colonization of salmonella in their ceca and improved phosphorus utilization in growing chickens (Kumprechtova *et al.,* 2000).

There are many types of probiotic preparations in the market. Many studies have been conducted to test the afficacy of such preparations on animal growth and performance. Several studies with broiler have indicated that probiotics preparations improve live weight gain and feed conversion ratio (Wu *et al*., 2005; Jin *et al.,* 1997). Previous experiments showed that the inclusion of microorganisms in the diets improved feed conversion efficiency and reduced fat accumulation of broiler chicks (Bidura *et al*., 2008).

The use of *Saccharomyces spp* culture as probiotic source in poultry production becomes an area of great interest. Gut microfloral enzymes are beneficial to the nutrition of the host because they increase the digestion of nutrients, especially in the lower intestine. Previous experiments showed that the inclusion of microorganisms in the diets improved feed conversion efficiency and digestibility (Chen *et al*., 2005). The supplementation of *Saccharomyces cerevisiae* in ration can improve the dry substance digesting, protein

digesting, and phosphorus (Piao *et al*., 1999). Wu *et al.* (2005) showed that supplemented of *Aspergillus xylanase* in diets were increased growth performans and decreased seum cholesterol content of broiler.

Non starch polysaccharides (NSP)s can not be degraded in the digestive systems of the birds due to lacking of enzymes for the NSPs degradation in their digestive systems (Choct, 2002). NSP can be broken down by microflora permanently, colonizing in the gastrointestinal tract, and their breakdown mainly occurs in the hindgut of all non ruminants by microbial fermentation (Wang *et al*., 2003). These enzymes are effective in degradating the complex compounds such as b-glucans and arabinoxylans (Dubey, 2006).

Chen *et al.* (2005) reported that dietary supplementation of complex probiotic increased the ADG and decreased fecal NH3-N concentration, slightly improved digestibility of nutrients, however, blood characteristics and fecal VFA concentrations were not effected. Inconsistent reports about the effect of probiotics may be due to several aspects such as strains of bacteria, dose level, diet composition, feeding strategy, feed form, and interaction with other dietary feed additives. Sutarpa *et al.* (2011) reported that inclusion of lactic acid bacteria in the drinking water were increased LWG, rate of passage, but decreasing serum cholesterol contents.

This experiment was carried out to study the implementation of isolate *Saccharomyces spp. N-2* culture in basal diets for decreasing abdominal fat and serum cholesterol contens of male Bali duckling (*Anas sp*) up to eight weeks old.

## MATERIALS AND METHODS

### Animals and experimental design

One hundred day-old-duck (DOD) of male local Bali drake (*Anas sp*) were assigned to 4 treatments in a completely randomized design. Each treatment has five replications with five birds per replication. The average body weight had no significant differences among the four groups at the beginning of the experiment.

There were fourth dietary treatments; each treatment consisted of six replicates containing three birds per replicate (cage). A corn-soybean meal based diet (Table 1), was used for the control treatment (A). The B, and D diets were prepared by including *Saccharomyces spp.N-2 isolate* culture at the levels 0.10% to 0.30%; respectively. The average body weight of the four treatment groups were not significantly difference at the beginning of the experiment.

### Diet and drinking water

The diet which is used in this research is compiled by the calculation of Scott *et al.* (l982). All diets compiled by iso-energy (2900 kcal ME/kg) and iso-protein (CP:18%). All diets in this experiment met or exceeded NRC (l998) recommendations for all nutrients regardless of treatment (Table 1.). Through all the experimental period, birds were allowed *ad libitum* acces to feed and water. The diet is given in the mash form, consist of some substances such as yellow corn, rice bran, fish meal, palm oil, pollard, cocoa-pod, and mineral-mix. The composition of ration compiler substances and nutrient which is used in diets can be seen in Table 1 Diets and drinking water were provided *ad libitum* during the eight weeks experimental period. Body weight and feed intake were recorded weekly.

### *Saccharomyces spp.N-2* culture

*Saccharomyces spp.N-2 isolate* in this study was isolated from "Ragi tape" (a locally product known as"Na Kok Liong", ensiled in number 26895). The isolate of *Saccharomyces spp.N-2* which has been approved from bile salt and poultry digestive tract *in vitro* test could assimilate cholesterol for probiotics agency. The study was carried out at the Bioscience Laboratory of Udayana University, Bali, Indonesia. Approximately in 0.20% culture of *Saccharomyces spp.N-2,* there are $2 \times 10^7$ spores of *Saccharomyces spp.N-2.*

Table 1. Formula and chemical composition of treatment diets of growing male Bali duckling up to eight weeks old

| Diets | | Level of *Saccharomyces spp.N-2* culture in diets | | | |
|---|---|---|---|---|---|
| | | 0.0% | 0.10% | 0.20% | 0.30% |
| Ingredients (%): | | | | | |
| Yellow corn | | 51.95 | 51.95 | 51.95 | 51.95 |
| Rice bran | | 10.00 | 10.00 | 10.00 | 10.00 |
| Coconut meal | | 12.45 | 12.45 | 12.45 | 12.45 |
| Soybean meal | | 13.60 | 13.60 | 13.60 | 13.60 |
| Pollard | | 2.60 | 2.50 | 2.40 | 2.30 |
| Fish meal | | 8.00 | 8.00 | 8.00 | 8.00 |
| Palm oil | | 0.50 | 0.50 | 0.50 | 0.50 |
| NaCl | | 0.40 | 0.40 | 0.40 | 0.40 |
| Mineral-mix | | 0.50 | 0.50 | 0.50 | 0.50 |
| *Saccharomyces spp.N-2* culture | | 0.00 | 0.10 | 0.20 | 0.30 |
| Total | | 100 | 100 | 100 | 100 |
| Chemical composition *): | | | | | |
| Metabolizable energy | (kkal/kg) | 2902 | 2902 | 2902 | 2900 |
| Crude protein | ( % ) | 18,0 | 18,0 | 18,0 | 18,0 |
| Crude Fiber | ( % ) | 4,85 | 4,85 | 4,85 | 5,23 |
| Eter Extract | ( % ) | 6,76 | 6,76 | 6,76 | 9,53 |
| Calsium | ( % ) | 1,08 | 1,08 | 1,08 | 1,11 |
| P-available | ( % ) | 0,63 | 0,63 | 0,63 | 0,65 |
| Argynine | ( % ) | 1,52 | 1,52 | 1,52 | 1,53 |
| Lysine | ( % ) | 1,31 | 1,31 | 1,31 | 1,39 |
| Metionin+sistein | ( % ) | 0,79 | 0,79 | 0,79 | 0,82 |
| Tryptophan | ( % ) | 0,19 | 0,19 | 0,19 | 0,18 |

Note :
*) Based on calculation according to Scott *et al.* (l982)

**Measurements**

Feed intake was determined by measuring feed residues on weekly basis since the beginning of the experiment. Feed conversion was calculated by dividing feed intake by body weight gains. For analysis of total serum cholesterol, two ml of blood was taken from the *jugular vein* of each duckling and centrifuge at 3000 rpm for 20 minutes. The blood serum of cholesterol content analysed according to Plummer (1977).

**Statistical analysis**

Performances of male Bali duckling and cholesterol contents in their serum were analyzed using one-way analysis of variance (Steel and Torrie, 1989).

## RESULTS

At the end of the experiment (at eight weeks of age), the final body weight and live weight gain (LWG) of ducklings fed supplemented by *Saccharomyces spp.N-2* culture were significantly higher than control ($P<0.05$), but feed consumption between the four treatment groups were not significantly different ($P>0.05$). Consequently, their feed conversion ratios were significantly different, whereas the fed supplemented by *Saccharomyces spp.N-2* culture group more efficient in using feed compared to the control groups.

Table 2. Effect of *Saccharomyces spp.N-2* culture level in diets on serum cholesterol content and abdominal-fat of male Bali duckling

| Variabel | Level of *Saccharomyces spp.N-2* culture in diets | | | | SEM |
|---|---|---|---|---|---|
| | 0.0% | 0.10% | 0.20% | 0.30% | |
| Final body weight (g) | 1027.3[b] | 1047.9[b] | 1206.5[a] | 1217.6[a] | 36.801 |
| LWG (g/d/birds) | 17.35[b] | 17.66[b] | 20.52[a] | 20.73[a] | 0.975 |
| Feed consumption (g/d/birds) | 99.24[a] | 100.13[a] | 84.75[a] | 84.58[a] | 3.094 |
| FCR (feed/gains) | 5.72[a] | 5.67[a] | 4.13[b] | 4.08[b] | 0.371 |
| Abdominal-fat (g/100g body weight) | 2.69[a] | 2.62[a] | 2.35[b] | 2.29[b] | 0.052 |
| Serum cholesterol (mg/dl) | 174.05[a] | 168.52[a] | 143.85[b] | 146.71[c] | 5.064 |

Note:
- Means with different superscripts within raws are significantly different (P<0.05)

Level of 0.30% *Saccharomyces spp.N-2* culture was significantly affected the efficiency in using feed, whereas the birds fed with 0.0%-0.10% *Saccharomyces spp.N-2* culture were less efficient in using feed compared to birds fed with 0.0%-0.10% *Saccharomyces spp.N-2* culture in their diets. Supplemented of *Saccharomyces spp.N-2* culture at 0.2%-0.3% levels in diets were decreased significantly different (P<0,05) compare both than control and 0.10% *Saccharomyces spp.N-2* culture in diets. The total cholesterol content in the serum and abdominal fat of the *Saccharomyces spp.N-2* culture supplemented in diets group duckling were significantly lower compared to control diets. The Final body weight, live weight gains (LWG), feed consumption (FC), feed conversion ratio (FCR), abdominal-fat, and serum cholesterol content of all treatments are given in Table 2.

Table 2 shows that the supplementation of *Saccharomuces spp.N-2* culture in rations caused numerical increases both in the final body weight and LWGs. This case can be attributed to the positive effects of *Saccharomuces spp.N-2* culture as a probiotics agent in gastro intestinal tract. Chen *et al.* (2005) reported that dietary supplementation of complex probiotics were increased LWG and slightly improved digestibility of nutrients. This study is consistant with some studies which indicated that supplemented of yeast in the diets affected bird performance positively (Bidura *et al*. 2009). This case can be attributed to the positive effects of fermented feed product on phytates and protein. Wu *et a*l. (2005) reported that supplementation of *Aspergillus xlanase* can improve the performance of broiler fed the wheat-based diet.

Yi *et al.* (l996) reported that supplementation of microbial in diets improved N retention in broiler chickens and *in vitro* digestibility of vegetable protein. Also, Chen *et al.* (2005) reported that addition of 0.20% complex probiotic (*L. acidophilus* and *S. cerivisae*) in basal diets increased digestiblity of dry matter (DM) and crude protein (CP). Hong *et al*. (2004) reported that fermentation of feed using *Trichoderma reesei* increased digestibility of its DM and CP (Jaelani *et al*., 2008).

Addition of *Saccharomuces spp.* culture to the diet had better digestibility because *S.c* culture in the gastro intestinal tract can to be part of probiotic sources. *Saccharomyces cerevisiae* as part of probiotic increased retention of minerals such as calcium, phosphorus, and manganese (Piao *et al.,* l999) and could improve protein and energy retention in the gastro intestinal tract of the birds. Cho *et al. (*2007) reported that supplementation of microbe in diet could improve the bioavailability of dietary. These fungi are effective in degradating the complex compounds such as b-glucans and arabinoxylans (Dubey, 2006).

Jaelani *et al*. (2008) reported that fermentation of palm kernel meal using *Trichoderma-reesei* increase metabolizable energy and crude protein contents of palm kernel meal. Park *et al*. (l994) suggested that body weight gain and gain/feed ratio were significantly improved by the addition of 0.10% *yeast culture* in diets of broiler.

The NSP is known increasing the gut viscosity, reduce nutrient absorption in the intestine and affect indirectly the growth and performance of bird (Rames *et al*., 2006). Many studies have clearly demonstrated

that the addition of probiotics culture or enzymes to diets rich in NSP resulted in a significant reduction of the intestinal viscosity, enhances energy and protein utilization and improved the performance of birds (Kocher *et al.,* 2000) while Tang *et al.* (2007) reported that live body weight of broiler increased by the increase of dietary energy. Piao *et al*. (l999) also reported that the utilization of 0.10% *yeast* (*Saccharomyces cerevisiae*) in diets increased body weight gain, feed efficiency and absorption of nutrient in broiler, whilst N and P excretion decreased in its manure. Bidura *et al.* (2008) founded in their previous study that birds which were offered fermented diets showed improvement of drake's final body weight, LWG dan feed efficiency. Feed conversion ratio and LWG in bird offered 0.20%-0.30% of *Saccharomuces spp.N-2* culture in diets group are higher than other groups. Chen *et al*. (2005) reported that dietary supplementation of complex probiotic increased the LWG and slightly improved digestibility of nutrients.

Zhu *et al.* (2003) reported that feeding with high fiber ration resulted in a lowered rate of lipogenesis and increased the capacity of utilizing acetyl-CoA in pigs. Non starch polysaccharide (NSP) is carbohydrate component of CF and predominant substrates for anaerobic fermentation. NSP can be broken down to a certain degree by microflora, permanently colonizing in the gastrointestinal tract and their breakdown in all nonruminant, mainly occurs in the hindgut by microbial fermentation (Wang *et al*., 2003).

This condition caused cholesterol in diets was unabsorbed and excreted to feces. Pectin can chelate bile salt and cholesterol to throw away in excreta (Dubey, 2006). Numerous studies have been undertaken to obtain scientific evidences for beneficial effect of probiotic. Such beneficial effects are considered to include the protection from pathogen, enhancement of the immune system, antimutagenic and anticarinogenic effects and the reduction of serum cholesterol (Saavedra, 2001). Sutarpa *et al.* (2011) reported that inclusion of lactic acid bacteria in the drinking water were increased *rate of passage* times digesta in the digestive tract, but decreasing serum cholesterol contents. The increase rate of passage digesta in the digestive tract couses decrease of fat as an energy source to produce acetic acid (Acetyl Co-A). It was concluded that supplemented of 0.20%-0.30% *Saccharomyces spp. N-2* culture in the basal diets could increase growth performance of male Bali duckling. Moreover it decreased both the serum cholesterol contents and abdominal fat of male Bali duckling.

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# THE EVALUATION OF CORTISONE AND PAPAYA (*Carica papaya*) LEAF MEAL SUPPLEMENTATION ON DUCK PERFORMANCE

**I Gusti Agung Arta Putra, Tjokorda Gede Belawa Yadnya, and Anak Agung Ayu Sri Trisnadewi**
*Faculty of Animal Husbandry, Udayana University, Denpasar*
*Correspondence author: artaputra@gmail.com*

**ABSTRACT**

The objectives of the experiment was to study the effect of cortisone and papaya (*Carica papaya*) leaf meal supplementation on the performance of growing ducks. The experiment was conducted by using a completely randomized design (CRD). The treatment were arranged in a 3x3 factorial arrangement of cortisone and papaya leaf meal supplementation. The doses of cortisone were 0, 12.5 and 25 mg per head for the K1, K2 and K3 treatments, respectively. Dietary papaya leaf meal concentration were 0, 5.0 and 10.0% for the A, B and C treatments, respectively. There were nine treatments combination (AK1, AK2, AK3, BK1, BK2, BK3, CK1, CK2, CK3) and four replicates for each treatment. Each replication consisted of four ducks. The parameters of carcass characteristics (weight, percentage, physical composition of carcass), and meat quality (meat colour, pH, water content, Water Holding Capacity/WHC, cooking loss and texture) were observed in this experiment. The result showed that cortisone and papaya (*Carica papaya*) leaf meal supplementation improve the carcass and meat quality of growing duck.

***Keywords***: *cortisone, papaya leaf meal, carcass, meat, duck*

## INTRODUCTION

Utilization of agricultural waste and by-products for animal feed is needed to minimize cost production of traditional farm in Indonesia. These are well known as source of energy or protein and classified into energy rich by-product (Tilman *et al*, 2009; Mendu *et al.* 2012). On the other hand, they contain so many crude fiber, but give a good effect and can bind the body fat especially subcutaneous fat (Belawa, 2002). Alternative feedstuff like papaya leaf could also be used because it contains energy, protein and papain. Papain functions as a catalytic enzyme such as proteinase and could hydrolyze synthetic peptide. The using of papaya leaf meal is good for tough meat, and can be applied in powder form and sowed on surface of meat or injected to the duck (Hatono 1981). The treatment need to apply to the culled duck which have fatty meat and high content of cholesterol. Papaya leaf meal could use as a feedstuff of ration. Its catalytic enzyme degrade protein and increase meat tenderness.

The performance of duck not only affected by feed but also hormonal regulation. Hormone is an organic substance which excreted by endocrine gland to the blood circulation and forwarded to the organ target. Testosterone, growth, tyrosine, estrogen and cortisone were often used as a growth promoter. Guyton (1986) stated that cortisone is important to increase the permeability of cell wall to the glucose, protein and fatty acid. Moreover, it accelerates substances metabolism in the cell. On the other hand, Wirtha (2002) found that injecting cortisone to the broilers did not affect significantly to the body weight, carcass weight, abdominal fat weight, and feed conversion ratio. In addition, Yupardhi (1998) studied the biologycal effect of steroid hormone to the cattle and found that steroid hormone did not affect to the efficiency and the tenderness of meat.

Based on above description and because of no information of using cortisone and ration supplemented by papaya (*Carica* papaya) leaf meal to the growing ducks, therefore combination of cortisone and papaya leaf meal, and its effect to carcass and meat quality need to be explored.

## MATERIALS AND METHODS

### Animals

Ducks were obtained from farmer in Tabanan regency and UD Mertasari, Guwang . The experiment was conducted in Guwang, Food technology laboratory, Faculty of Animal Husbandry and Analytical Laboratory, Udayana University

### .Rations

The ration was formulated according to Scott *et al*. (1982) and consists of yellow corn, rice bran, coconut meal, fish meal, soybean, papaya leaf meal, premix, and NaCl. Ration composition was described in Table 1.

Table 1. The Ration Composition of Growing Ducks

| Composition (%) | Treatments | | |
|---|---|---|---|
| | A | B | C |
| Yellow corn | 55.36 | 55.36 | 55.36 |
| Fish meal | 8.12 | 8.12 | 8.12 |
| Ricebran | 14.14 | 7.98 | 7.01 |
| Coconut meal | 9.31 | 6.67 | 4.25 |
| Soybean | 11.97 | 11.97 | 11.97 |
| Papaya leaf meal | | 5 | 10 |
| Premix | 0.5 | 0.4 | 0.36 |
| NaCl | 0.52 | 0.42 | 0.4 |
| Total | 99.92 | 95.92 | 97.47 |

**Experimental Design**

The experiment was conducted by using a completely randomized design (CRD). The treatment were arranged in a 3x3 factorial arrangement of cortisone and papaya leaf meal supplementation. The doses of cortisone were 0, 12.5 and 25 mg per head for the K1, K2 and K3 treatments, respectively. Dietary papaya leaf meal concentration were 0, 5.0 and 10.0% for the A, B and C treatments, respectively. There were nine treatments combination (AK1, AK2, AK3, BK1, BK2, BK3, CK1, CK2, CK3) and four replicates for each treatment. Each replication consisted of four ducks. At the end of observation, one duck of each treatment was used for meat quality analysis.

**Parameters**

The parameters of carcass characteristics (weight, percentage, physical composition of carcass), and meat quality (meat colour, pH, water content, Water Holding Capacity /WHC, cooking loss and texture) were observed in this experiment..

**Statistical Analysis:**

The data were analyzed using analysis of variance, if there are significant different between treatments ($P<0.05$), further analysis of Duncan's test was applied (Steel and Torrie, 1989).

## RESULTS AND DISCUSSION

As shown in Table 2, slaughter weight of ducks consuming ration containing papaya leaf meal and cortisone is better than those without papaya leaf meal and cortisone. The existence of papaya leaf as a source of papain could hydrolyze protein to be amino acids which accumulated in tissue and changed into meat protein. Papaya leaf also contain protein, vitamin and mineral to accelerate the metabolism. Besides supporting nutrient absorbtion into body tissue, cortisone also increase glucogenesis in liver and muscle tissue (Wirtha, 2002). This experiment proves that papaya leaf meal and cortisone combination make slaughter weight carcass heavier than that without treatment combination.

Carcass percentage on treatment AK1 is 37.03. It shows that papaya leaf meal and cortisone can increase significantly the meat production. It proves that the blood protein is higher than other treatment, so the absorbtion of protein will increase. The vitamin, protein and mineral content of papaya leaf could increase metabolism especially protein tissue formation in meat (Tillman *et al*., 1986). The duck in treatment AK1 yielded subcuttaneous fat and skin percentage of 31.77%, but the offering of cortisone and papaya

leaf meal combination could decrease carcass fat percentage to 23.96% (CK3). Papaya leaf as source of fiber could bind the fat which excrete through feces, and distribute fat from adipose tissue to the blood circulation then decrease the accumulation of fat into adipose tissue, and therefore, the fat accumulation in under the skin will decrease (Guyton, 1986). Belawa (2004) found that ration containing different source of fiber and supplemented by papaya leaf decrease the carcass fat of ducks.

Abdominal fat consists of mesenterial fat, pad fat, and ventricular fat. Generally papaya leaf meal supplementation and cortisone decrease abdominal fat (Table 2), except on BK3 treatment because mesenterial and ventricular fat are higher than AK1 treament, so abdominal fat on BK3 higher than the other treatment.

Table 2. The Effect Cortisone and Papaya (*Carica* papaya) Leaf Meal Supplementation on Growing Ducks Performance

| Variables | Treatments | | | | | | | | | SEM |
|---|---|---|---|---|---|---|---|---|---|---|
| | AK1 | AK2 | AK3 | BK1 | BK2 | BK3 | CK1 | CK2 | CK3 | |
| Slaughter weight | 1111.25c | 1156,25bc | 1187.5ab | 1183.75ab | 1197.5ab | 1212.5ab | 1181.3ab | 1212.5ab | 1255c | 16.52 |
| Carcass weight | 752.5d | 768,7cd | 783.5c | 781.25c | 797.5c | 816.3bc | 786.25c | 822.5b | 845a | 5.71 |
| Carcass percentage | 66.71a | 66.5a | 65.89a | 65.99a | 66.59a | 67.1a | 66.45a | 67.83a | 67.33a | 1.35 |
| Physical composition carcass | | | | | | | | | | |
| - Meat | 37.03c | 40.80b | 40.9b | 39.32bc | 40.23b | 40.89b | 44.68a | 46.52a | 46.99a | 0.42 |
| - Bone | 30.89a | 27.78a | 31.11a | 31.15a | 30.53a | 32.09a | 29.83a | 28.71a | 29.03a | 1.21 |
| - Fat (including skin) | 31.77a | 31.41a | 27.99b | 29.84b | 29.24b | 27.02b | 25.48c | 24.77c | 23.96c | 0.43 |
| - Abdominal fat | 2.06b | 1.56f | 1.34g | 2.02c | 1.67c | 2.14a | 1.69e | 1.81d | 1.99c | |

The effect of cortisone and papaya leaf meal did not differ significantly on meat colour, pH and water consentration (Table 3). Meat colour depend on myoglobin concentration and influenced by its molecule type, chemical status, and other physical component of meat. Furthermore, Liu *et al.* (2003) reported that meat colour depend on hue of red, blue, and green and colour intensity. The three combination value determine the meat colour. The non significant different effect on meat colour between the treatments due to ration consumption did not differ significantly, so the concentration of myoglobin did not differ and so did the meat colour.

Water concentration did not differ significantly because papaya leaf meal contains high fiber and then water consumption will be balanced with its excretion through feces. Water concentration is almost the same with water content in meat. It means that $H^+$ ion that yielded by water hydrolisis is the same as pH of meat (ph = - log $H^+$). Sazili *et al.* (2005) stated that water concentration is affected by muscular fat and feedstuff of ration consumed by duck.

Water holding capasity (WHC) increase significantly, because the increasing of blood protein level affected WHC positively. Water holding capasity will effect on less or more nutrient binding in meat. Navid *et al.* (2009) stated that cooking loss depend on water holding capasity and ration consumption.

The offering of papaya leaf meal and cortisone increase the meat texture (Table 3). It probably caused by the existence of papain in papaya leaf could hydrolise protein to amino acid and form refined particle and yield refined meat fiber and meat texture be. Navid *et al*. (2009) also found that papaya leaf meal can improve meat quality in terms of meat tenderness, juiciness and enhanced redness of the uncooked breast fillet.

Table 3. The Effect Cortisone and Papaya (*Carica* papaya) Leaf Meal Supplementation on Meat Quality of Growing Ducks

| Variables | Treatments | | | | | | | | | SEM |
|---|---|---|---|---|---|---|---|---|---|---|
| | AK1 | AK2 | AK3 | BK1 | BK2 | BK3 | CK1 | CK2 | CK3 | |
| Colour | 5.83a | 5.50a | 5.60a | 5.67a | 5.72a | 5.68a | 5.80a | 5.82a | 5.84a | 0.71 |
| pH | 5.36a | 5.56a | 5.66a | 5.62a | 5.61a | 5.59a | 5.58a | 5.62a | 5.66a | 0.08 |
| Water concentration | 72.23a | 73.70a | 73.51a | 73.62a | 73.68a | 74.00a | 74.20a | 74.50a | 74.60a | 0.49 |
| Water Holding Capasity (WHC) | 61.04b | 62.10a | 63.50a | 64.40a | 64.40a | 64.50a | 64.80a | 65.70a | 65.80a | 0.86 |
| Cooking loss | 37.9a | 37.8a | 37.7a | 36.5ab | 36.6ab | 36.7ab | 35.5bc | 34.8bc | 34.51c | 0.44 |
| Texture | 5.8c | 6.55bc | 66.0abc | 6.7abc | 6.8abc | 6.8abc | 7.0ab | 7,2ab | 7.3a | 0.16 |

Based on this experiment, it can be concluded that cortisone and papaya leaf meal in ration could increase the slaughter weigth, carcass percentage, water holding capasity, and meat texture but decrease the subcuttan fat percentage and cooking loss of meat.

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# FIRST REPORT ON OCCURRENCE OF GRAPEVINE LEAF ROLL-ASSOCIATED VIRUS TYPE 3 ON GRAPEVINE IN INDONESIA

**I Gede Rai Maya Temaja*, I Putu Sudiarta, and Dewa Nyoman Nyana**
*Faculty of Agriculture, Udayana University, Denpasar*
**Corresponding author: tderai@yahoo.com*

**ABSTRACT**

In a survey of vineyard fields in Buleleng, Bali, Indonesia, some grapevine cultivars were found showing grapevine leaf roll disease symptomps, i.e.interveinal reddening or yelowing and fall leaves; down rolling of leaves; phloem disruption; and the fruit maturation is delayed. Molecular method was then used to determine the coat protein (CP) sequence. Analysis of leaf samples from diseased plants by reverse transcription-polymerase chain reaction (RT-PCR) using a pair of primer specific to Grapevine Leaf Roll-associated Virus type 3 (GLRaV-3) successfully amplified an expected 477 bp DNA fragment. The sequences of the PCR product confirmed that the grapevine plants were infected by GLRaV-3. This is the first report on occurrence of GLRaV-3 on grapevine plants in Indonesia.

***Keywords***: *Grapevine Leaf Roll-associated Virus type 3* (GLRaV-3)*,* grapevine (*Vitis vinifera*)*,* reverse transcription-polymerase chain reaction.

# POTENTIAL OF PLANT EXTRACTS AS GROWTH REGULATORS: THE INFLUENCE OF PLANT EXTRACT TO GROWTH OF CUTTING OF POTATO SEEDING

**Fachirah Ulfa[1,2*], Enny Lisan Sengin[1], Baharuddin[2], and Syatrianti Andi Syaiful[1]**
[1]Department of Agronomy, Faculty of Agriculture, Hasanuddin University, Makassar, Indonesia.
[2]Research Centre for Biotechnology, Hasanuddin University.
*Corresponding author: fachirah.ulfa@yahoo.com

**ABSTRACT**

An experiment has been undertaken at screenhouse of Central Potato Seed in Malino, South of Sulawesi (1600 m dpl). It aimed to select the best plant extract, as growth regulator of potato seedling, namely: water as a control, corn, onion, coconut water, beans, banana, and bean sprouts. Each part of plant is blended with water and then fermented for 15 days. The solution was centrifuged at 8000 rpm for ten minutes. Seedlings used were in-vitro nodule cutting of potato cv.*Kalosi*. Result revealed that corn extract gave the best treatment on potato seedling based on parameters of plant high, number of leaves, and length of root.

***Keywords***: plant extract, growth regulator, potato seedling.

# DETECTION OF POTATO LEAF ROLL VIRUS (PLRV), POTATO VIRUS Y (PVY) AND POTATO VIRUS X (PVX) ON FIVE POTATO VARIETIES BY USING OF DAS-ELISA AND RT-PCR METHODS

**Tutik Kuswinanti*, Baharuddin, Gusmiati and Masira S.**
*Research Center for Biotechnology, Hasanuddin University, Makassar, Indonesia*
**Corresponding author: koeswinanti@yahoo.com*

**ABSTRACT**

Potato is a staple food crop that widely grown around the world. Virus infection is main factor that affects great loss of the potato production. Potato virus X(PVX), potato virus Y(PVY),and potato leaf roll virus(PLRV) are top three viruses that result in decreased yield of potato in Indonesia. Therefore, the rapid methods of DAS-ELISA was studied to test tuber samples of five potato varieties; Granola, Atlantik, Raja, Super John, Kalosi, and Masalle. Two simple, rapid, sensitive, reliable detection techniques of three viruses are reported in the paper: they are DAS-ELISA and RT-PCR. Detection of all three viruses on positive control samples could be carried out with ELISA technique without purification of RNA virus. Neither PLRV nor PVX were detected on five potato varieties test samples, but PVY was detected only in Kalosi test sample. This method is simpler and rapider than RT-PCR technique, and is more suitable for the detection of a great number of potato samples. The sensitivity of ELISA is as same as that of RT-PCR.

***Keywords***: PLRV, PVY, PVX, DAS-ELISA, RT-PCR

# THE EFFECT OF WASTE FROM PURPLE SWEET POTATO SYRUP PRODUCTION ON THE PERFORMANCE, LIPID BLOOD CONTENT, AND TOTAL BACTERIA OF THE DIGESTIVE TRACT OF KAMPONG CHICKENS

**Sutarpa, I N.S.[1], Y. Ramona[2], I N.S. Miwada[1], I M. Suasta[1], M. Dewantari[1], and P. Tegik[1]**
*[1]Faculty of Veterinary Udayana University*
*[2]Integrated Laboratory for Biosciences and Biotechnology, Udayana University*

**ABSTRACT**

This research was conducted for 12 weeks in order to investigate the effect of waste from purple sweet potato syrup production on the performance, lipid blood content, and total bacteria of the digestive tract of kampong chickens. Complete randomized design with four treatments (0, 10, 20, and 30% w/w of waste from syrup production) and 5 replications per treatment was applied in the experiment. Each treatment consisted of one week old of 10 kampong chickens. During this 12 week experiment, all chickens were fed with 2850 kcal/kg food supplemented with 15.5% protein and water was given ***ad libitum*.** The results showed that addition of waste from purple sweet potato syrup production in the food of kampong chickens at the ratio of 30% w/w did not significantly ($p>0.05$) increase food consumption, food consumption ratio (FCR), and chicken body weight. Inclusion of this waste at the ratio of 20-30% w/w significantly ($p<0.05$) decreased LDL of blood plasma, total Coliform, *E. coli*, and Salmonella in the digestive tract of kampong chickens, but it significantly increased ($p<0.05$) the beneficial lipid (HDL) of blood plasma and beneficial lactic acid bacteria in the digestive tract of the chickens.

***Keywords:*** waste, kampong chicken, purple sweet potato.

# SCREENING AND CHARACTERIZATION OF MICROBIAL ANTAGONISTS FROM RHIZOSPHERE OF PASSION FRUITS (*PASSIFLORA* SPP) AS BIOCONTROL AGENTS OF FUSARIUM WILT

**Hilda Karim[1], Kuswinanti Tutik[2], Ade Rosmana[2] and Burhanuddin Rasyid[2]**
*[1] Department of Biology, Faculty of Math. and Natural Science*
*State University of Makassar.Jl. Daeng Tata Raya, Makassar 90224*
*[2]Faculty of Agriculture, Hasanuddin University of Makassar*
*hildakarim@yahoo.co.id*

**ABSTRACT**

Fusarium wilt caused by *Fusarium oxysporum* f.sp *passiflorae* is the most important disease on Passion fruit that cause yield losses up to 50-100%. The disease is difficult to control because the pathogen infected plants sistemically and can survive up to 5 years in the soil. The objective of this research was to select potentially antagonistic microbes in suppressing the growth of *Fusarium oxysporum* f.sp *passiflorae* (*Fop*) *in vitro* and *in vivo*. Antagonists were isolated from the rhizosphere of purple passion fruit (*Passiflora edulis* form e*dulis*) and sweet passion fruit (*Passiflora* sp) from Gowa and Makassar. To obtain best isolates, their ability to inhibit the growth of *Fop* and their production of cellulase, chitinase, pectinase as well as toxine compound were tested *in vitro*. The results showed that, from totally 40 fungal isolates and 25 bacterial isolates, 15 fungal isolates and 8 bacterial isolates gave an exellent growth inhibition to *Fop*. Highest percentage of growth inhibition was obtained by fungal isolate F14 (79.46%) and bacterial isolate B5 (72.05%). The highest cellulase, chitinase and pectinase enzyme production were observed on fungal isolate F10, followed by isolates F7 F13, F14 and F11. Only two bacterial isolates B5 and B6, showed highest cellulase, chitinase and pectinase enzyme production. The presence of toxin evaluated by Thin Layer Chromatography were only detected on fungal isolates F13, F3, F5, F1, and F8. HCN compound from bacterial isolates were obtained from isolates B1, B4 and B6.

***Keywords:*** Fusarium wilt, antagonist microbes, biological control

# THE PARASITISM LEVEL OF PARASITOID LARVAE *Diadegma semiclausum* Hellen (HYMENOPTERA:ICHNEUMONIDAE) ON *Plutella xylostella* (LEPIDOPTERA: PLUTELLIDAE) IN CANDIKUNING VILLAGE, TABANAN REGENCY, BALI, INDONESIA

**Ketut Ayu Yuliadhi and Trisna Agung Phabiola**
*Department of Agroecotechnology Faculty of Agriculture Udayana University*

**ABSTRACT**

The parasitism level of parasitoid larvae, *Diadegma semiclausum* Hellen (Hymenoptera:Ichneumonidae) on *Plutella xylostella* (Lepidoptera: Plutellidae) is studied on cabbage plants in Candikuning Village, Tabanan Regency – Bali. The purpose of this study was to determine the level of parasitism of *Diadegma semiclausum* on *Plutella xylostella*. *Diadegma semiclausum* is a kind of parasitoid larvae from cabbage caterpillars *Plutella xylostella* L., which can be found on cabbage farming in Candikuning Village, Tabanan Regency – Bali. The field observation done in May through July 2012 on 1 month, 2 months, and 3 months old cabbage plants had shown that the parasitism level of *D. semiclausum* were 23.08%, 69.39%, and 67.5 %.

***Keywords:*** *Diadegma semiclausum*, *Plutella xylostella*, cabbage

# MONITORING OF IMAGO OF *Spodoptera litura* AND *Helicoverpa armigera* USING SEX PHEROMONE TRAP

**Gusti Ngurah Gede Dharma Putra[1], Ketut Sumiartha[1], I Putu Dharma R. Srinivasan[2] and I Putu Sudiarta[1*]**
[1]*Faculty of Agriculture Udayana University*
[2]*AVRDC-The World Vegetable Center*
[*]*Corresponding author: putu.ueda@yahoo.com*

**ABSTRACT**

The utilization of sex pheromone to control insect pest is one of advance technology. The sex pheromone is one method to control insect pests for support food security. In other to know the ability of sex pheromone to trap insect the monitoring of insect using sex pheromone is needed. The lure of sex pheromone, glue, and box trap were imported from AVRDC-The World Vegetable Center. Sex pheromone trap is putted on the vegetable area in Bali, the locations including Sandan, Kembang Merta, Candi Kuning (Tabanan Regency) and Pancasari (Buleleng Regency). On each location trap was placed with two replications. Data collection was conducted every week, on 3rd week observation the imago/moth population is completely reduce. Therefore the trap was replaced for the new trap. That mean the observation was conducted 3 times per experiment (Observation 1,2,3). In this case the experiments were done 2 times (Experiment 1 and 2). The identification of imago was base on the morphological method. The imago was accounted for data collection. The decrease of population of *Spodoptera litura* and *Helicoverpa armigera* after 3 week observation is possible caused by the ability of pheromone active ingredient is also decrease. The distribution of imago *S. litura* and *H. armigira* are same for all locations. However the total population of *S. litura* and *H. armigera* is different. The population of *S. litura* was very high in all locations around 51-107. However the population of *H. armigera* was generally low except in Sandan Tabanan (70). In Pacasari the population of *H. armigera* was lowest around 14-15. The differentiation of imago *S. litura* and *H. armigera* were possible influenced by the host plant, altitude and also climate conditions.

***Keywords***: sex pheromone, *Spodotera litura*, *Helicoverpa armigira*

# THE MISTING PERIOD APPLICATION ON POSTHARVEST QUALITY OF OYSTER MUSHROOMS (*Pleurotus ostreatus*) iN LOW LAND CULTIVATION

**I Made Sukewijaya[1], Ni Nyoman Ari Mayadewi[1], Gede Menaka Adnyana[1], and Ni Made Wiwik Yuliartini[2]**
*[1]Study Program of Agroecotechnology, Faculty of Agriculture Udayana University, Denpasar, Bali - Indonesia*
*[2]Volunteer of Integrated Farming System, Agriculture Services of Bali Province, Denpasar, Bali - Indonesia*

**ABSTRACT**

Oyster mushrooms grow well in the plains are located between 400-800 m asl., while in low land with relative humidity of 50% of and temperature of 31°C generally. This condition is not optimum for oyster mushrooms growth. One way to keep the humidity and temperature to match the original habitat of the oyster mushroom is done by misting technique and by using storage locker. A completely randomized design (CRD) was used in this experiment with one treatment factor, namely the misting period, and it was repeated 10 times. The misting treatments were applied on storage locker all day (24 hours). Misting period consists of four levels, namely: without misting (K0), misting for 15 minutes and paused every 15 minutes (K1), misting for 30 minutes and paused every 15 minutes (K2), and misting for 45 minutes and paused every 15 minutes (K3). Postharvest handling was conducted by 2 storage techniques i.e. (1) on ambient temperature (30°C) and (2) by refrigerator (-1°C). Organoleoptic test was done to evaluate the color, aroma, texture, total performance, and shelf life. Shelf life could be extended by storing it on refrigerator. Oyster mushroom was stored in ambient temperature at treatment K0, K1, K2, and K3 were 3, 2, 2, dan 2 days respectively, whereas on refrigerator were 8, 7, 6, 6 and 6 days respectively.

***Keywords***: misting period, oyster mushrooms (*pleurotus ostreatus*), postharvest quality, low land

# THE VARIETY TRIAL OF RESISTANT CHILI PEPPERS TO ANTHRACNOSE

**I Dewa Made Putra Wiratama[1], Ketut Sumiartha[1], Made Sukewijaya[2], Made Supartha Utama[2], Joko Mariyono[3], Greg Luther[3] and I Putu Sudiarta[1*]**
*[1]Faculty of Agriculture Udayana University*
*[2]The Center for Research and Development on Horticultural Crops - Udayana University*
*[3]AVRDC-The World Vegetable Center*
*[*]Corresponding author: putu.ueda@yahoo.com*

**ABSTRACT**

The anthracnose has been recognized as one of the most divesting disease wherever chili pepper (*Capsicum* spp.). Anthracnose is cereus problem particularly in wet season causing by *Colletotrichum* spp. To control the disease the farmers use chemical pesticides however very difficult to reduce it. One of possible way is using plant resistant. Plant resistant can be prepared by the breeding of chilli mother plant. To evaluate the line after breeding the field trial is needed. Evaluation of chili pepper genotypes showed that some germplasms are very promising for development of cultivars with resistance or tolerance to the disease. Improvement of chili pepper varieties having resistance to the diseases and high productivity accompanied by better cultivation system is considered as the best approach to limit yield loss. The trials were conducted in Abang Batu Dinding, Kintamani Bangli. The purpose of the study in order to know the resistance of lines of chili pepper to anthracnose.The experiment was designed in Randomized Complete Block Design (RCBD) with 20 numbers of entries (16 AVRDC, 3 IVEGRI, and 1 local check varieties). Trial planting will be conducted by tree (3) replication and with independent randomization of each replication. The field resistance testing of chili line against anthracnose was conducted in wet season. The total number of plant per plot was 20. The result show the some lines were attacked by *Colletotrichum* spp. however the others were detected free of Anthracnose. The one line 1003 was found most sensitive by Anthracnose. On the other hand the highest of marketable yield was found of Tanjung line, with the average of yield 669.88 gr.

***Keywords:*** Chili, Anthracnose, resistance

# CONFIRMATION CVPD INFECTION TOWARD ORANGE PLANTS VARIETIES WITH PCR TECHNIQUE AND TOTAL PROTEIN PROFILE

**Made Sritamin**
*Faculty of Agriculture, Udayana University*

The confirmation of CVPD (*Citrus Vein Phloem Degeneration)* infection toward many varieties of orange plants can be detected with PCR technique that showed by 1160 bp DNA ribbon as the result of amplification with PCR by using specific primer for CVPD (*Citrus Vein Phloem Degeneration)*. At the result of total protein electrophoresis of the leaves varieties with SDS-PAGE method there are specific protein ribbon at 16 kda molecule weight which are not found at the uninfected orange leave. The existence of those protein is one of the symptom exist in plant infected by CVPD (*Citrus Vein Phloem Degeneration.* It can be concluded that PCR technique is a suitable technique to detect CVPD (*Citrus Vein Phloem Degeneration)* disease.

***Keywords:*** CVPD, DNA, PCR, Specific Protein, SDS-PAGE

# SOIL IMPROVEMENT EFFECTS OF FOUR GREEN MANURE CROPS IN GREENHOUSE CULTIVATION

**Yu Jin Jung [1], Kyung Hee Han[1], Hee Wan Kang [3], Ill Sup Nou[2], and Kwon Kyoo Kang[1]**
*[1]Department of Horticulture, Hankyong National University, Ansung 456-749, Korea*
*[2]Department of Horticulture, Sunchon National University, Suncheon 540-742, Korea*
*[3]Graduate School of Bio. & Information Technology, Hankyong National University, Ansung 456-749, Korea*

## ABSTRACT

Four different green manure crops, hairy vetch, Italian ryegrass, crimson clover, orchard grass were cultivated to test the differences in their effects on soil improvement. After plowing to incorporate the green manure crops, tomato plants were grown as a succeeding crop to investigate the effects of green manure on the soil physical properties and plant growths. The experimental results showed that green manure could decrease the soil hardness. Italian ryegrass and hairy vetch as the green manure were especially effective in decreasing the soil hardness. Orchard grass and Italian ryegrass provided more void in soil matrix, resulting increased soil moisture contents. After two months, bulk density was decreased in the plots of Italian ryegrass and orchard grass. However, there were no significant differences between green manure plots and control after the cultivation of the succeeding crop. The results indicated that the growing Italian ryegrass and hairy vetch as green manure helped growing tomato plants. Orchard grass also improved the soil characteristics, comparable to Italian ryegrass in spite of their relatively small biomass.

***Keywords:*** Green manure, Green manure crop, Soil improvement, Soil physical properties, Succeeding crop

# EFFECT OF GREEN MANURE CROP EXTRACTS ON POTATO COMMON SCAB (*Streptomyces scabiei*)

**Jong In Park[1], Hee Jeong Jung[1], Hee Wan Kang[2], Kwon Kyoo Kang[3], and Ill Sup Nou[1]**
*[1] Department of Horticulture, Sunchon National University, Suncheon 540-742, Korea*
*[2]Graduate School of Bio. & Information Technology, Hankyong National University, Ansung 456-749, Korea*
*[3]Department of Horticulture, Hankyong National University, Ansung 456-749, Korea*

**ABSTRACT**

Green manure crop is usually used for the enrichment of organic matter in the soil. But these crops also have the potentiality to control plant pathogen. In this study we investigated the possible role of green manure crop extracts in antimicrobial activity against potato common scab (*Streptomyces scabiei*). Green manure crops were extracted with hexane, ether, ethylacetate, methanol and water. The DPPH radical scavenging activity of ether, methanol and water was higher than hexane and ethylacetate fraction. Methanol, ethylacetate, ether and water fractions showed antimicrobial activity against *Streptomyces scabiei* from the concentration of 0.25 mg/mL. Methanol fraction of Sorghum showed the highest antimicrobial activity against this microorganism. This result suggests the Sorghum as good green manure crop with antimicrobial activity against potato common scab (*Streptomyces scabiei*).

# THE INFLUENCE OF CURING TIME AND EXTRACTION TIMES ON THE YIELD AND CHARACTERISTICS ESSENTIAL OIL OF SANDALWOOD FRANGIPANI FLOWERS (*Plumeria alba)*

**I Made Adi Parimartha[1], Ni Made Wartini[2*], I Wayan Arnata[2]**
*[1]Faculty of Agricultural Technology, Udayana University*
**Corresponding author: wartini_unud@yahoo.co.id*

**ABSTRACT**

The aims of this study were to 1) determine the influence of curing time, extraction time and their interaction on yield and characteristics of essential oil of sandalwood frangipani flower 2) find out the right extraction time and curing time to produce the highest yield and the best characteristics essential oil of sandalwood frangipani flower and 3) determine kind and the composition of compounds in the essential oil of sandalwood frangipani flower. This research used randomized block design with factorial pattern. The first factor (*curing* time) consists of 3 level namely fresh material, *curing* 2 and 4 days and the second factor (extraction time) consists of 3 level namely 2, 3, and 4 hours. Each treatment was done twice. The best treatment was measured with effectiveness test. The results showed that the curing time and extraction time had significant effect on the yield and characteristics of essential oil of sandalwood frangipani flower. The *curing* 2 days and extraction time of 4 hours is an appropriate treatment to produce essential oil of sandalwood frangipani flower with the highest yield and the best characteristics, with a yield of 1.17 %, the aroma preference of 5.8 (between like and really like) and aroma strength 7.1. The essential oil of sandalwood frangipani flower containing 48 kinds of compounds and 9 unidentified compounds Constituent compounds essential oil of sandalwood frangipani flowers consists of group alkane (20.15%), ester (31.91%), ketone (1.07%), alkene (0.83%), ether (1.08%), alcohol (23.62%), amide (1.24%), and un identified compounds (20.09%).

***Keywords***: sandalwood frangipani flower, curing, extraction time, essential oil, *Plumeria alba.*

# MEDIA COMPOSITION IMPROVED THE GROWTH OF NEW LEAVES IN ANTHURIUM PLANTS

**Made Ria Defiani**
*Biology Department, Natural and Basic Sciences Faculty, Udayana University, Indonesia*
*Defiani_ria@yahoo.com*

**ABSTRACT**

Wave of Love Plant (*Anthurium* sp.) is an ornamental plant that was famous years ago. It has vigorous and dark green colour leaves.Its price was veryexpensive and depend on the number of leaves.The plant growth was influenced by media to maintain the emergence of new leaves.Sometimes the flower produced seeds for its propagation.The seedlings required moist media for maintain viability of the seeds.Some media composition were observed to increase the growth of Anthurium plants.Mixed media A consists of soil:crushed fern:compost (1:1:1) offered better growth for new leaves than media B (soil:compost = 1:1) only.The seeds growth faster (5 days) in media A.Both media improved the emergence of seedlings (90-95%) in 10 days.Porous media was better than thick media for growing of roots, new leaves and healthy seedlings.

***Keywords:*** Anthurium, crushed fern, compost

# AUTHOR INDEX

# PROCEEDINGS

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