IN VITRO INHIBITION ZONE TEST OF BINAHONG (*ANREDERA CORDIFOLIA*) TOWARDS *STAPHYLOCOCCUS AUREUS*, *ENTEROCOCCUS FAECALIS, ESCHERICHIA COLI*, AND *PSEUDOMONAS AERUGINOSA*

Amertha, IBPM, Soeliongan S, and Kountul C
Faculty of Medicine, Sam Ratulangi University, Manado-Indonesia

ABSTRACT

This is a true experimental research with post test-only control group design. The study was conducted to test the inhibitory zone of the Binahong leaf extract (*Anredera cordifolia*) against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Binahong leaf extract is prepared using maceration technique, by soaking it in a sealed jar for 24 hours with 95% methanol. Then subsequently filtered using a funnel with filter paper, and the filtrate is collected inside an erlenmeyer. The filtrate then concentrated using a rotavapor, this concentrated extract dissolved into aquadest with a concentration of 50 ppm, 100 ppm and 1000 ppm. By taking a few colonies with a sterile loop into a stock of *Staphylococcus aureus, Enterococcus faecalis, Esherichia coli, Pseudomonas aeruginosa* then scratch it into MH blood agar medium, and incubate it for 24 hours with a temperature of 37^0C. The next day, bacterial suspension was made in test tube, which already contains 0.9% NaCl. The suspension tturbidity is equivalent to 0.5 *Mc Farland*. Bacterial inhibition zone of binahong leaf extract (*Anredera cordifolia*) is tested using absorbance disc method or better known as the *Kirby-Bauer* method. First, pour 10 ml of agar medium (± 400C) into a cup (petridish) and then wait until it's cold. After the medium becomes solid, the suspension of bacteria *Staphylococcus aureus, Enterococcus faecalis, Esherichia coli*, and *Pseudomonas aeruginosa* are slowly smeared with sterile cotton sticks on the surface of the media. Soak the paper discs into binahong leaf extract (*Anredera cordifolia*) with concentrations of 50, 100, and 1000 ppm, for about 5 minutes, and placed it on the surface of the petridish, together with the positive control (*amoxicillin*) and negative control (*aquadest*). Then incubate it at 37^0C for 24 hours. The effectiveness of binahong leaf extract (*Anredera cordifolia*) inhibition zone, can be determined by measuring the diameter of clear zone around the paper using a sliding-term. Binahong leaf extract (*Anredera cordifolia*) zone of inhibition is negative, a very slight different is showed by the *amoxicillin* inhibition zone, for having a clear zone diameter of 28 mm for *Staphylococcus aureus* and *Esherichia coli*, and 21 mm for *Enterococcus faecalis*. This fact is probably caused by several things concerning the mechanism of action of a substance as an anti bacterial of the binahong leaf extract (*Anredera cordifolia*).

Keywords: inhibitory, zone, turbidity, sliding-term, antibacterial.

INTRODUCTION

Today the development of bacterial resistance to antibacterial drugs became a thorny issue in the medical world. Many evidences anti-bacterial resistance cases were reported to almost all over the world. The first case of antibacterial-resistence was reported in 1990, the cases occurred simultaneously in Australia, New Zealand and the United States.[1] In 1997, there was fatal cases reported in Minnesota and North Dakota, where four children died due to infection of CA-MRSA.[2,3]

Until today, it was known that Staphylococcus aureus is a bacterium with the highest resistance rate in the world. The case is reported almost simultaneously throughout the world. Other bacterium, nosocomial pathogen Enterococcus faecalis is in third place, this bacterium is reportedly resistant to antibiotics such as aminoglycosides, penicillin, tetracycline, klorampenikol, and vancomycin.[4] Although Escherichia coli is part of normal flora in the intestine, the bacteria strain 0157 was reported in Germany as a cause of food poisoning to hundreds of people.[5]

Bacterium that has high levels of resistance to extreme environments is Pseudomonas aeruginosa. The bacteria are able to live in the atmosphere that has low oxygen levels, and can live in the aircraft's fuel, so it is often detrimental to corrode airlines around the world. Pseudomonas aeruginosa have also resistance to many antimicrobial drugs, and will multiply rapidly when normal flora is pressed.[4]

Natural product or its simplicia has been proven effective to cure various diseases. Simplicia or extract of natural product is actually the basis of modern medicine manufacture or synthesis of. In some parts of the world such as India, China, including Indonesia traditional medicine is still an option for treatment of diseases. Many studies of natural medicinal plants for medication have been carried out. The results indicate

Correspondent: Amertha IBPM
Address: Faculty of Medicine Sam Ratulangi University, Manado-Indonesia

that the herb were effective, efficient, economical, and safe.[6-8] One of the traditional plants applied for medicine is binahong (*Anredera cordifolia*). Amertha found, that binahong (*Anredera cordifolia*) leaf extract proved more effective in healing burns on chicks compare to drug used clinically.[9]

Abou Zeid reported that the binahong leaf extract has some activities, i.e antihyperlipidemic, anti-inflammatory, analgesic, antipyretic, anti convulsant, and cytotoxic activities. Chemical content of the leaves are binahong phytol, alpha-pinene and 6,10,14-trimethyl pentadecanone. Other compounds identified are neophytadiene, methyl hexadecanoate, methyl-9,12,15-octadecatrienoate, and methyl octa deca-9 .12-dienoate, and flavone-C-glucosides.[10] Other researcher, Rochani reported that binahong leaf extract was found active towards fungus Candida albicans. This fungus induces candidiasis that can invade vagina, skin, nails, lungs, and gastrointestinal tract.[11]

In general, this study aims to determine whether binahong (Anredera cordifolia) leaf extract has an ability to inhibit bacterial growth of Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa.

METHODS

Research Design

This is a true experimental research with post-test only control group design. The study was conducted to determine whether binahong leaf extract inhibit the growth of bacteria Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa. Bacterial growth inhibition was determined by measuring inhibition zone of four colonies mentioned above.

Place and time study

The research was conducted at the Analytical Laboratory of the University of Udayana to make binahong leaf extract and the Laboratory of Microbiology Faculty of Medicine, Udayana University for bacterial inhibition zone test. This research was carried out for two weeks starting on December 15, 2011 through December 29, 2011.

Population and sample

Since bacteria were the subject of this research, the sample size was determined based on Mc Farland standard in which 10^8 bacteria were employed. The bacteria were cultered and suspended bacteria prepared within turbidity equal to 0.5 Mc Farland scale.[4]

Binahong leaf extract was then prepaired in varies concentration, i.e. 50 ppm, 100 ppm and 1000 ppm. This is in accordance with bioactivity test in which the drug is considered active when it's LC_{50} below 1000 ppm.

Research variables

Independent variable: binahong leaf extract concentration of 50, 100, and 1000 ppm. Dependent variable: number of colonies of Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa. Controlling variable: temperature, incubation time, culture media, measurement, tools and materials sterilization of bacteria applied in this study.

Procedure

Preparation of binahong leaf extract

Binahong fresh leaves were washed before treated and weighed. These leaves were then cut into small pieces to make it easier to dry. These pieces were dried in a wind dried, it needs 4 days to dryness. These dried leaves were blended to make powder and macerate with 95% methanol for overnight. This material was filtered and evaporates to gain crude extract of binahong leaves.

Preparation of media for bacterial growth

Media for bacterial growth was prepared by dissolving Mueller Hinton (MH) blood media agar in distilled water. This was carried out based on standard method for preparing media agar for growing bacteria. Around 5 mL of Blood of goats was added to the solution and then sterilized to protect contamination. These media were ready to use for growing bacteria used in this research.

Bacteria rejuvenation

Rejuvenation of bacteria was carried out to obtain pure isolates of Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa. This was carried out by rejuvenation of bacteria taken from the stock at Laboratory of Microbiology Udayana University. All bacteria, Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa were seeded in the MH agar media and incubated for 24 hours at 37^0C. In the next day, the colonies obtained were identified according to standard procedure adopted at Udayana university to ensure that the colonies were Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa.

Preparation of bacterial suspension

Bacterial suspension of Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa are made from colonies grown in MH agar media. A number of 1-2 colonies were seeded in 0.9% NaCl, by this procedure it will be obtain the turbidity of the suspension is equal to 0.5 Mc Farland scale. Sterile cotton stick was dipped in bacterial suspension and squeezed to reduce fluid

taken. This cotton stick was then applied to the MH media agar in a petridish as can be seen in Figure 1.

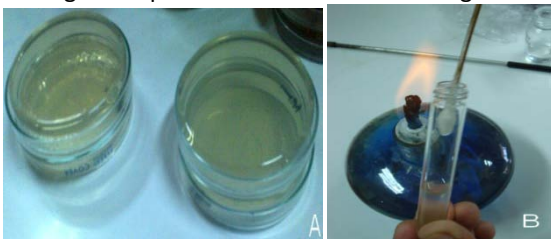

Figure 1
A. MH agar media for bacteria to test the inhibitory zone
B. Bacterial suspension was taken with sterile cotton sticks.

RESULTS

Mueller Hinton (MH) Media

MH media was prepared based on standard method for preparing media applied to determine bioactivity of natural product towards bacteria. The media prepared can be seen on Figure 2.

Figure 2
Mueller Minton Media

Inhibition growth zone test

Inhibition growth zone of binahong (*Anredera cordifolia*) leaf extract was determined by measuring clear zone diameter around paper disc after overnigth incubation. The inhibition growth zone experimental model was presented in Figure 3.

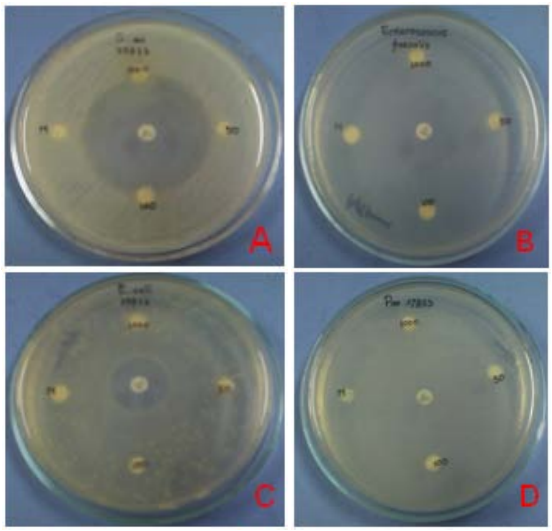

Figure 3
Inhibition Growth Zone Test of Binahong Extract Towards A) Staphylococcus aureus, B) Enterococcus faecalis, C) Escherichia coli, and D) Pseudomonas aeruginosa

Data of inhibition growth of bacteria zone by binahong (*Anredera cordifolia*) leaf extract towards bacteria Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa were presented in Table 1.

Table 1
Data of Inhibition Growth Zone of Binahong (*Anredera cordifolia*) Leaf Extract.

Groups	Diameter Zona Hambatan (mm)			
	S. aureus	*E. coli*	*E. faecalis*	*P. aeruginosa*
P.1	28	28	21	negative
P.0	negative	negative	negative	negative
P.2	negative	negative	negative	negative
P.3	negative	negative	negative	negative
P.4	negative	negative	negative	negative

P.1= Amoxicillin, P.0 = Aquadest, P.2 = (50 ppm), P.3 = (100 ppm), P.4 = (1000 ppm).

DISCUSSIONS

In this study, Mueller Hinton (MH) agar media was selected because the media was recommended by the FDA and WHO to test anti-bacteria, especially aerobic bacteria and facultative anaerobic bacteria for food and clinical material. This media has also shown good and reproducible results. This agar media containing sulfonamides, trimethoprim, and low tetracycline inhibitor, and provide good growth pathogen.[27] Addition of goat's blood acts as a provider of nitrogen compounds, vitamins, carbon, sulfur, and amino acids.

Inhibition zone test of binahong leaf extracts was carried out against four types of bacteria, namely Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa. These bacteria were chosen to represent two types of bacteria, namely Staphylococcus aureus and Enterococcus faecalis from gram-positive group, and Escherichia coli and Pseudomonas aeruginosa from gram-negative group. It turned out that the inhibitory zone of binahong (*Anredera cordifolia*) leaf extract as shown in Table 1 and Figure 3 is negative towards all bacteria tested. However, it is different for amoxicillin in which 28 mm of diameter zone obtained for Staphylococcus aureus and Escherichia coli and 21 mm of diameter zone for *Enterococcus faecalis* were observed.

This fact is probably caused by several things concerning the mechanism of action of a substance as an anti-bacterial. An anti-bacterial compounds can act as an anti-bacterial through inhibition mechanism of folic acid synthesis and inhibition of protein synthesis in bacteria.[16, 17] Folic acid is essential for both bacterial and mammalian organisms that serves as a precursor to the formation of DNA or RNA. Role of a compound as an anti bacterial through inhibition of folic acid synthesis is closely related to how the compound forming macromolecules in the bacteria cells. There

are three types of reactions that play a role in the formation of macromolecules,[16] namely;

1. Group 1 reactions involve the use of glucose and some carbon sources to produce energy (ATP) and some simple compounds such as citric acid.
2. Group 2 reactions advantaging energy and precursor to form amino acids, nucleotides, phospholipids, carbohydrates and growth factor.
3. Group 3 reactions combining simple molecules produced in group 2 reactions to generate macro molecules such as proteins, RNA, DNA, polysaccharides and peptidoglikan.

Regardless of these three type reactions, therefore, binahong leaf extract could not showing their bacterial activity through these type reaction. It is probably its activity through another mechanism. Binahong leaf extract was found active to improve wound healing, therefore, this activity is probably through anti adhesion, not as an antibacterial or as a bactericide.[3]

CONCLUSIONS

From the results of this study, it can be concluded, that the leaf extract binahong (*Anredera cordifolia*) has no inhibitory activity to the growth bacteria, i.e. Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa.

FUTURE WORK

We found that, binahong leaf extract was not active to inhibit growth zone of four bacteria observed, i.e. Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa. On the other hand, this extract was found active to improve wound healing in animal studied. Therefore, it is important to find out how the mechanism of this wound healing.

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REFFERENCES

1. Raygada JL and Levine DP. "Managing CA-MRSA Infections: Current and Emerging Options". Infections in Medicine 26 (2). Jakarta: Consultant live; 2009.
2. Okuma K, Iwakawa K, Turnidge J, et al. "Dissemination of new methicillin-resistant Staphylococcus aureus clones in the community". J Clin Microbiol 40 (11): 4289–94. doi:10.1128/JCM.40.11.4289-4294.2002. PMC 139674. PMID 12409412; 2002.
3. Itzhak, O., David, L.H., and Nathan, S. Anti-Adhesion Therapy Of Bacterial Diseases: Prospects and Problems. FEMS Immunology and Medical Microbiology. 38: 181-191; 2003.
4. Jawetz, Melnick, Adelberg. Mikrobiologi Kedokteran. Jakarta: EGC; 2004.
5. William Neuman. *Number of Rare E. Coli Cases in U.S. Rose Last Year*. New York: New York Times; 2010.
6. Cheppy, S. Budi Daya Tanaman Obat Komersial. Jakarta: Gramedia; 2010.
7. Hembing. Hidup Sehat Cara Hembing. Jakarta: Gramedia; 2002.
8. Nala, N. Kebudayaan Kesehatan. Denpasar: Universitas Udayana; 2002.
9. Amertha, P. M. I. B. Daun Binahong Sebagai Obat Luka Bakar. Dipresentasikan pada Lomba AVICENA Competition Fakultas MIPA Farmasi Universitas Udayana; 2007.
10. Abou-Zeid, A. H. S., Soliman, F. M., Sleem, A. A., and Mitry, M. N. R. Phytochemical and bio-activity investigations of the aerial parts of Anredera cordifolia (Ten.) Steenis. Bulletin of the National Research Centre Cairo. Volume: 32, Issue: 1: p. 31-33; 2007.
11. Rochani, N. Uji Aktivitas Antijamur Ekstrak Daun Binahong (*Anredera cordifolia*) terhadap *Candida albicans* serta Skrening Fitokimianya. Skripsi. Fakultas Farmasi Universitas Muhamadiyah Surakarta; 2009.
12. Starr, F., Starr, K., and Loope, L. *Anredera cordifolia*, Madeira vine Bassellaceae. United States Geological Survey--Biological Resources Division Haleakala Field Station, Maui, Hawai; 2003.
13. Sholekhudin, M. Pien Tze Huang, Populer tapi Misterius. Majalah Intisari. No. 510. Th.XLIII; 2006.
14. Kobayasi, Y., Hasegawa, T., Sato, M., Suzuki, E., and Arakawa, M. Pneumonia due to the Chinese medicine pien tze huang. Nihon Kyobu Shikkan Gakkai Zasshi. 34(7): p. 810-5; 1996.
15. Lee, K. K., Kwong, W. H., Chau, F. T., Yew, D. T., and Chan, W. Y. Pien Tze Huang protects the liver against carbon tetrachloride-induced damage. Pharmacol Toxicol. Oct;91(4): p.185-92; 2002.
16. Rang, H. P., Dale, M. M., and Ritter, J. M. Pharmacology. 3rd ed. New York: Churchill Livingstone; 2003.
17. Ryan, K. J. *Sherris Medical Microbiology*, Ray CG (editors). 4th ed. New York: McGraw Hill; 2004.
18. Deirdre, C., Sameer, E., Owen,R., Brent, W., and Robert, L. Burn Wound Infections. Clinical Microbiology Reviews. 19(2): p. 403-434; 2006.

19. Albert, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. Molecular Biology Of The Cell. 3rd ed. USA: Garland Publishing, Inc; 2004.
20. Suzanne, C. S., and Brenda, G. B. Keperawatan Medikal Bedah. Edisi 8. Jakarta: EGC; 2002.
21. Anonim. Gram Positif dan Gram Negatif. Jakarta: Wikipedia; 2007.
22. Whitt, Dixie D.; Salyers, Abigail A. "14". Bacterial Pathogenesis: A Molecular Approach (2nd ed.). USA: ASM Press. ISBN 1-55581-171-X; 2002.
23. Curran JP, Al-Salihi FL. "Neonatal staphylococcal scalded skin syndrome: massive outbreak due to an unusual phage type". Pediatrics 66 (2): 285–90. PMID 6447271; 2002. http://www.cirp.org/library/complications/curran1/.
24. Gotama, I. B. I. , Sugiarto, S. , Nurhadi, M. ,Widiyastuti, Y. Wahyono, S. , Prapti, I. J. Inventaris Tanaman Obat Indonesia. Jilid V. Jakarta, Departemen Kesehatan Badan Penelitian dan Pengembangan Kesehatan; 1999.
25. Tshikalange, T. E., Meyer, J. J. M, and Hussein, A. A. Antimicrobial activity, toxicity and the isolation of a bioactive compound from plants used to treat sexually transmitted diseases. Journal of Ethnopharmacology. Volume 96, Issue 3: p. 515-519; 2005.
26. Dewi, F.K. Aktivitas Antibakteri Ekstrak Etanol Buah Mengkudu (*Morinda Citrifolia, Linnaeus)* Terhadap Bakteri Pembusuk Daging Segar, Univ. Sebelas Maret Surakarta, Jurusan Biologi MIPA; 2010.
27. Acumedia. Mueller Hinton Agar (7101). PI 7101, Rev 02, 11/14/04; 2004. Available from : http//:www.neogen.com. Diakses tgl. 16 Mei 2011.
28. Itzhak, O. Federation of European Microbiological Societies. Published by Elsevier B.V; 2003.