

Extract Ashitaba (*Angelica Keiskei*) Improving The Immune Response IL-2, Ifn- Γ balb/C Mice Vaccinated With Rabies Vaccine

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Abstract. Ashitaba plant (*Angelica keiskei*) is native to Japan that has been developed in Indonesia, has many benefits, as a vegetable and as immunomodulator. This study aims to determine the ethanol extract effect of Ashitaba leaves (*Angelica keiskei*) to increase immune response IL-2, IFN- γ Balb/C mice were vaccinated with rabies vaccine. This study used a completely simple randomized design. Treatment consisted of six points are without Ashitaba (control), giving a dose of 100 Ashitaba; 200; 300; 400, and 500 mg/kg orally for 21 days. Each treatment was repeated four times, so there are 24 units for researching. On the 28th day, do the vaccination with rabies vaccine to all groups of mice. On 42th day, the spleen was taken for viewing cultured lymphocytes producing cells. Variables observed are the levels of IL-2 and IFN levels of the lymphocyte. The results showed that the extract of Ashitaba can increase levels of IL-2 were significantly ($p < 0.05$). Respectively, the average levels of interleukin-2 after treatment Ashitaba extract dose 0; 100; 200; 300; 400, and 500 mg/kg, are 1,700 pg, 3,919 pg, 5,218 pg, 8,875 pg, 15,563 pg. Gammainterferon also increased as increase as given by dose of Ashitaba extract. Statistically, it showed significant difference ($p < 0.05$), except between 400 and 500 doses was not significant ($p > 0.05$). The average control levels of interferon gamma are 13,534 pg, 100 mg/kgbb dose (15,222 pg), dose 200mg/kgbb (15,745 pg), dose 300mg/kgbb (16,749 pg), dose 400mg/kgbb (17,116 pg) and dose 500mg/kgbb (17,278 pg). The conclusion, Ashitaba ethanol extract can improve the immune response of IL-2 and IFN- γ mice vaccinated with rabies vaccine.

Keywords: Ashitaba, Interleukin-2, Interferon- γ , Rabies

I. INTRODUCTION

Ashitaba (*Angelica keiskei*), is one type of immunomodulator that can improve immunity in animal experiments [6]. The use of Ashitaba as an immunomodulator being developed especially for bacterial infections used as a complementary therapy antibiotic [13]. In addition, Ashitaba is a natural immunomodulator of types of plants that grow well in Indonesia, Japan and other parts country, so this needs to be developed and enhanced. Researchers wanted to test the benefits of Ashitaba in infections caused by viruses.

Ashitaba (*Angelica keiskei*), have multiple benefits, such as antioxidants and potent cancer treatment as evidenced by research [9]. [6]. These results were confirmed by research Kimura and Baba [4]. Active compounds that inhibit the tumor is xanthoangelol, which inhibits DNA synthesis in tumor cells. Xanthoangelol also proven efficacy in treating neuroblastoma or nerve cancer and leukemia. [12] proved to be Xanthoangelol apoptosis after incubation for four hours in which a solution of caspase-3 is a protein in leukemic cells and becomes active after being neublastoma Xanthoangelol.

The conclusion of research on the benefits of Ashitaba as an immunomodulator in Balb/C mice that Ashitaba extract is also good for cancer therapy with demonstrated anticarcinogenic and antimutagenic activity in vitro study [4]. In vitro study of extract of Ashitaba are known to have effects in non-specific immune response by increasing macrophage chemotaxis and phagocytosis, neutrophil chemotaxis, cytotoxicity of natural killer cells (NK), and complement activation. Specific immune response against Ashitaba extract, it has the effect of increasing the proliferation of T lymphocytes, increase secretion of TNF- α , IFN- γ , IL-10 [9].

II. RESEARCH METHODS

Research Design

This study is a laboratory experiment using a randomized completely simple design (Post Test Fully Randomized Design). The study uses 24 units of study and were divided into six treatment are without Ashitaba (control), giving Ashitaba 100; 200; 300; 400, and 500 mg/kg bb. Route of administration and concentration Ashitaba as a method performed by Jayatirtha and Mirsha [3] as

well as Parle and Vasudevan [7]. Each treatment was repeated four times. After 21 days of extract of Ashitaba, mice vaccinated with rabies vaccine.

Sample Research

Samples were taken at random systematic and calculated based on the formula Ferderer[7]. Mice were aged 10 to 12 weeks weighing 25 to 30 g, adapted to the environment for two weeks, then given the ethanol extract of Ashitaba in accordance with the treatment respectively for 21 days, on day 22, vaccinated with rabies vaccine intraperitoneal, observed at all times on the health of the mice.

Research Variables

As the independent variable is the provision of Ashitaba in various doses (without Ashitaba, given Ashitaba 100, 200, 300, 400 and 500 mg/kgbb/day) for 21 days. As the dependent variable is an immune response that is: the number of IL-2, IFN- γ mice, were observed 4 weeks after inoculation of rabies vaccine. Controlled variables are age, weight, sex, food, beverages, types of mice, cages, feeding time, and the environment.

Preparation extract of Ashitaba

Ashitaba (*Angelica keiskei*) obtained from Sembalun Village, East Lombok, West Nusa Tenggara, Indonesia. Ashitaba leaves were collected and washed with water, wind dried. After drying destroyed (shredded) and then mashed with the blender, after it weighed 1000 grams. Ashitaba powder then soaked in 5000 ml of ethanol and stirred with a magnetic stirrer for one hour, then allowed to stand for one day at room temperature. Furthermore, filtered with Whatman paper No. 42 in order to obtain the filtrate-1. Dregs obtained, so that the extraction re-obtained filtrate 2. Filtrate-1 and-2 mixed filtrate was then evaporated with a rotary evaporator.

Giving Ashitaba

Giving oral Ashitaba done. A total of 4 mice of the first treatment was given 0.5 ml of sterile distilled water. While in 4 mice from the treatment to the 2nd, 3rd, 4th, 5th and 6th administered ethanol extract of Ashitaba respectively 100, 200, 300, 400 and 500 mg/kgbb/day. All treatments are carried out for 21 days.

Inoculation of rabies vaccine

Inoculation of rabies vaccine done three weeks after administration of Ashitaba. A total of 0.01 ml intraperitoneally inoculated with rabies vaccine.

Measurement of the production of IL-2 and interferon- γ

Examination of Interleukin-2 and Interferon gamma is done with the same technique that is ELISPOT (R & D systems). ELISPOT an ELISA method that can be used to detect cytokine or antibody-producing cells. Lymphocytes isolated from spleen advance. Lymphocytes then cultured in vitro with density 4×10^6 cells/ml into microplate Elisa had previously been attached to specific antibodies. Cells then were stimulated using phytohemagglutinin (PHA) or specific peptide and incubated at 37° C for 18 to 40 hours in an incubator containing CO₂. Post-stimulation microplate washed and biotin-labeled conjugate is added and incubated at room temperature. After washing, streptavidin-alkaline phosphatase substrate was added and incubated in the dark until the spots arise. The reaction was stopped by washing the road by using distilled water and dried microplate. Readings done by counting spots on the dissection microscope.

Examination Procedures

The number of IL-2 and interferon- γ in the plasma and lymphocyte culture supernatant fluids were determined by sandwich ELISA using IL-2/IFN ELISA development kit (R & D systems, USA). Into each micro-ELISA plate wells was added 10 μ l suspension mouse anti-human IL-2/IFN- γ monoclonal antibodies in PBS and left for 18 hours at room temperature. After being washed 3 times in PBS, each pitting blocked with bovine serum albumin solution 1 % in PBS and allowed to stand at room temperature for 1 hour. After being washed 3 times with PBS, into each of the wells was added 100 μ l of plasma/lymphocyte culture supernatant fluid. As a reference, also added IL-2/IFN- γ proteins whose concentration has been known to air and diluted multiples of 2 starting from a concentration of 600 pg/100 μ l up to concentrations of 4 pg/100 μ l. After enhancer protein sample and standard, microplates were incubated for two hours at room temperature and then washed with PBS three times as above. Into each of the wells are then added 100 μ l rabbit anti-human IL-2/IFN- γ labeled with biotin and incubated for two hours at room temperature. A total of 100 μ l avidin horse radish peroxidase (Avidin - HRP) in 0.1 % BSA in PBS was added into each of the wells, incubated for 20 min at room temperature, and washed with PBS as above. A total of 100 μ l of TMB substrate was then added to each micro- wells plate and left at room temperature for 20 minutes. The level of concentration of the substrate is then read by a spectrophotometer at 405 nm wavelength. IL-

2/IFN- γ amount determined by plotting the density of color (absorbance values) against a standard protein concentration is plotted on paper log3. IL-2/IFN- γ concentration in each sample was then determined by logistic regression with 4 parameter logistic with reference to a standard protein graphs.

Data Analysis

The data were tested normality and homogeneity with Levene's test if data is homogeneous then the variant analysis at a significance level of 5% and if there is a real difference then followed by Post Hoc test was used LSD.

Treatment differences were analyzed using Analysis of Variance at 5% significance level. If treatment showed a significant difference then further tested with LSD at a significance level of 5%. All assisted data analysis using SPSS version 17.00.

III. RESULTS AND ANALYSIS

Levels of Interleukin-2 after Treatment Ashitaba Extract

Interleukin-2 produced by lymphocytes observed by calculating spot (lymphocytes) that appeared to use the ELISPOT on day 42 (end of treatment). The mean levels of Interleukin-2 after treatment Ashitaba extract is presented in Figure 1.

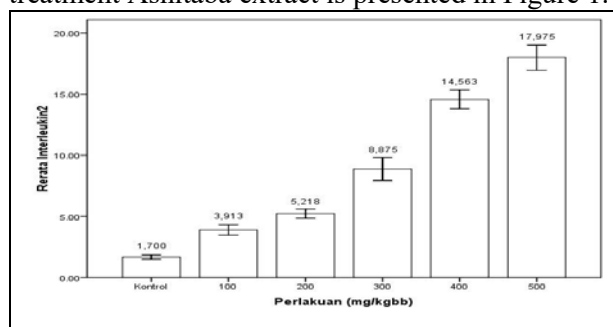


Figure 1. The mean Interleukin-2 After Treatment Ashitaba Extract

From Fig.1 shows that the average of Interleukin-2 after treatment Ashitaba extract dose 100mg/kgbb, 200mg/kgbb, 300mg/kgbb, 400mg/kgbb and 500mg/kgbb showed an increase with increasing dose given.

Levels of interleukin-2 secreted by lymphocytes was observed by counting the spots with the ELISPOT test on day 42 (end of treatment). Seen on the treatment dose interleukin-2 levels 500mg/kgbb highest (17.975pg). Statistical analysis showed that Ashitaba extract is able to increase the levels of IL-2 were significantly ($p < 0.05$), with the highest levels found in 500mg/kgbb dose, which was significantly

higher ($p < 0.05$) at a dose of 400 mg/kgbb (15.563 pg), 300mg/kgbb (8.87 5pg), 200mg/kgbb (5.218 pg), 100mg/kgbb (3,913 pg) and with control (1,700 pg). This is due to the Ashitaba plant is rich in vitamins, amino mineral as an active substance identifier that can be referred to as a multi-purpose plant. According to [2]. Ashitaba contains chlorophyll which is high enough so that it can increase blood production and balance body functions. The active substances contained in the chalcone beneficial to increase the production of red blood cells, increasing the body's defenses against infectious diseases. Production of IL-2 by lymphocytes increased with increasing doses of Ashitaba extract is given. These results show that Ashitaba extract is able to induce cellular immunity in experimental animals. Interleukin-2 is produced by T-helper lymphocytes type 1 (T-helper/Th-1). When the body is exposed to the rabies virus antigens contained in the vaccine, the body will process these antigens. Antigens are processed and transported to the cell surface via MHC molecules presented to the Th-1 which in turn produce IL-2, which triggers the multiplication of T cells including cytotoxic T cells (CD8+) [1].

The results showed significant differences between treatment groups, this study proves that Ashitaba extract is able to increase the levels of IL-2 as a result of the stimulation caused by rabies virus vaccine and Ashitaba able to stimulate macrophages to increase their activity, so as to be more responsive to antigens that enter the body. Then the rabies virus is also capable of giving a signal captured by macrophages to migrate and perform phagocytosis. The macrophage cells will secrete mediators such as IL-1, IL-2, IL-6, which stimulates macrophages to respond and approach other sources of stimulation. Production of IL-2 in this chain will increase the levels of IL-2 in the circulation system [10].

Increased levels of IL-2 production is due to the activation of macrophages and Ashitaba extract due to the stimulation by antigen vaccine rabies virus. Activation of macrophages in the absence of stimuli infection will not result in increased levels of IL-2. Interleukin-2 is a glycoprotein with a molecular weight ranging from 14 to 17 kD. This cytokine is mainly produced by CD4+ cells when activated by antigens, [8]. The main function of IL 2 is the influence of CD4+ and CD8+ that produced them (autocrine) or on neighboring cells (paracrine). Effect caused by IL 2 is spurring the development of T cells from the G1 phase to the phase of synthesis, stimulates the synthesis of IL-2 next stage by T cells within 24 hours after activated will stimulate the formation

of other cytokines, namely interferon gamma and limfotoksin, as well as increasing the synthesis of P55 is a receptor IL-2 alone, increases the growth and function of NK cells sitolitik. Because its function is closely associated with the immune system of quantity synthesis of IL-2 will determine the immune response (Munazir, et al., 2002) The mechanism of the increased activity of NK cells and T cells by the addition of IL-2 is to increase the number of receptors p70 p75. IL-2 receptor consists of two kinds of protein is 55 kDa polypeptide (P55) and polypeptides which ranged between 7075 kDa (p70p75). CD4+ cells were not activated p70p75 has little, and does not contain P55. When the cell is activated p75 and p70 led to an increase in P55 expression. Affinity p70 p75 higher than P55. In the absence of P55 cells that stimulate p70 p75 alone can stimulate cell proliferation and a half of the maximum ability when stimulated with a high concentration of IL-2, whereas cells without p70p75 cells an stimulate only P55 not spur to stimulate cell proliferation. When these two receptors form a complex that can be stimulated by low concentrations of IL-2 [2].

IL 2 receptor found on NK cells and T cells are not the same, both in humans and in mice . Receptor IL 2 on NK cells such as P70 P75, P55 so there were no need for activation of NK cells by IL 2 always high [2]. Fact is used as the basis for activating NK cells ex vivo to generate LAK cells [2]

Levels of Interferon Gamma (IFN- γ) After Treatment Ashitaba Extract

Interferon gamma produced by lymphocytes after observation by using the ELISPOT method presented in Fig. 2.

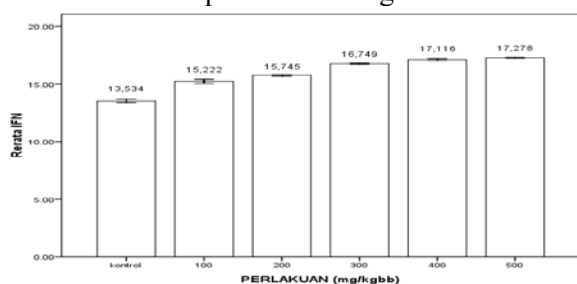


Figure 2. Mean Gamma Interferon After Treatment Ashitaba Extract (The data is transformed to ln)

From Fig. 2 shows that the average Gamma Interferon after Ashitaba extract treatment doses 100mg/kgbb, 200mg/kgbb, 300mg/kgbb, 400mg/kgbb and 500mg/kgbb showed an increase with increasing dose given.

Interferon gamma produced by lymphocytes showed an increase with increasing dose given Ashitaba extract. The mean levels of

gammainterferon produced visible in the control (13.534 pg), dose 100mg/kgbb (15.222 pg), dose 200mg/kgbb (15.745 pg), dose 300mg/kgbb (16.749 pg), dose 400mg/kgbb (17.116 pg) and dose 500mg/kgbb (17.278 pg). Statistical analysis showed a significant difference ($p < 0.05$) between treatment dose, but the dose 400mg/kgbb with 500mg/kgbb dose showed no significant difference ($p > 0.05$). This indicated that the antigen is able to induce cellular immunity. The results are consistent with research Lambot, et al. [5] that the gammainterferon can be produced in large quantities in vitro with rabies vaccine. Interferon-gamma produced by lymphocytes T-helper/Th-1. Rabies vaccine antigen is processed and distributed to the cell surface via the MHC-1 molecules are presented to cytotoxic T cells (CD8+). In response Th-1 cells will divide and produce interferon gamma (IFN- γ) [1]. The number of cells that secrete cytokines, indicating the activity of TH-1 indirectly as an indicator of specific immune rabies mediated by CD8+ T cells. Presence of signal TCD8+typical rabies virus and cytokines produced by Th-1 in mice (*Mus musculus*) which disensitasi with rabies vaccine will inhibit the replication of rabies virus and destroy the rabies virus-infected cells. Interferon gamma is secreted by Th-1 function to activate macrophages and NK cell, which is able to inhibit the spread of the virus to surrounding cells, increasing the expression of MHC-I molecules and MHC-II, as well as inhibiting the growth of Th-2 cells [10]. Interferon gamma will increase T cells to differentiate. Here interferon gamma helps naïve CD4+subsets to differentiate into Th1-and Th-2 inhibits proliferation in experiments with mice. This effect may occur because the activation mediated by mononuclear phagocytic cells that release IL-12 and T-cells that express IL-12 receptors. IFN- γ is also required for the maturation of CD8+ cytotoxic T cells [8]. IFN- γ function to stimulate B cells to IgG1 and IgE swiching to. IFN- γ will affect the IgG subtypes bind to IFN- γ on selfphagositosis. Thus IFN- γ to induce antibody responses that ultimately affect the elimination of microbes by phagocytes [8]. IFN- γ activates neutrophils to perform respiratory Brust although less powerful effect than TNF and LT [2]. IFN- γ spur sitolitik activity of NK cells play an important role in tumor imunologi [14]. Ashitaba extract role in stimulating an increase in IFN- γ is the ability to promote the inductive phase of the immune response.

Interferon gamma also increased the expression of MHCI and $\beta 2$ microglobulin molecules on the surface of various cell types. IFN- γ helps to improve the activity sitolitik T

lymphocytes sitolitik (T-CD8+) recognize antigen only when the destination cell antigens or presented with MHC molecule-1 (Samuel, 2001). Vaccination with rabies virus causes acid RNA double stranded core which is a by-product of viral replication. The interferon gamma induces and activates enzymes 2-5 oligoadenilate. Enzymes that have enabled ATP to linked oligamer mempolimerase will activate cell RNA and subsequent RNA of these cells to degrade RNA single stranded and inhibits cell wall [11].

IV. CONCLUSION

From results and discussion can be summed up as follows:

1. Extract Ashitaba (*Angelica keiskei*) can increase the number of cells that produce interleukin-2 are higher in Balb/C mice were vaccinated with rabies.
2. Extract Ashitaba (*Angelica keiskei*) can increase the number of cells that produce gammainterferon higher in Balb/C mice were vaccinated with rabies.

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