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Immunostimulation effect of extract ethanol white oyster mushroom (*Pleurotus ostreatus* Jacq Fr. Kumm) on mice

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ABSTRACT

Acknowledging the immunostimulation effect of ethanol extract white oyster mushroom to animal experiments which were desensitized with red blood cell of lamb. This study is an experimental study, 14 healthy male swiss Webster mices were used. The subjects are randomly divided into two groups. The first group is a control group and the second group is a treatment group. The immunostimulation effect is assessed by specific immune response to desensitized of lamb's RBC. Humoral immune response was assessed through a hemagglutination test. Cellular immune response was assessed with a delayed type hypersensitivity test by measuring the thickness of edema of the mice's foot Specific immune response is assessed through total of blood lymphocyte using flowcytometry method. We found an increase of total blood lymphocyte level on the primary sensitization of placebo and Ethanol extract of white oyster mushroom (EEWOM). After secondary sensitization, the total blood lymphocyte level on placebo and EEWOM showed a significant

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difference. EEWOM increases the total antibody titre significantly compare to placebo. The level of serum gamma-globulin of treatment mice is significantly higher than placebo. On the 24th and 48th hour after the sensitization of lamb's RBC, the increase of thickness of the mice's feet which were given placebo and EEWOM have significant difference. EEWOM has an immunostimulating effect towards specific immune response.

Keywords**:** immunostimulating, lymphocyte, serum gamma-globulin, thickness of the mice's feet white oyster mushroom, *Pleurotus ostreatus*

1. INTRODUCTION

The environment around humans contains various types of pathogens that can cause infection in humans. Infections in people normally occur short and rarely leave permanent damage, because the human body has the immune system that can provide response and protect the body against the pathogens.[1] Synthetic drugs or traditional medicine can be used to maintenance, improve, and recovery of health or treatment of diseases. The utilization of medicinal plants as traditional medicine tends to increase, because traditional medicine is considered relatively safe compared to synthetic drugs. The use of traditional medicines as immunomodulators is based on the consideration that, according to traditional medicine systems, medicines derived from nature can influence the activity of the body's immune system.[2,3] The benefits of fungi for human health has been proven. Mushrooms are nutrient-rich plants contains of riboflavin, nicotinic acid, panthotenate, and biotin. Active compounds which contained in mushroom able to decrease blood pressure, prevent atherosclerosis, and increase the immune system (immune system) against diseases.[4] The main structure of the fungus, β-glucan, is known to have an immunomodulatory effect and significantly inhibit tumor activity. White oyster mushroom (*Pleurotus ostreatus* L.) contains pleural compounds, such as (1-3)-β-D-glucans derived from polysaccharides. β-glucans can stimulate nonspecific and specific immune responses by directly binding and activating macrophages and other white blood cells (neutrophils and NK cells). Activated macrophages secrete various cytokines and increase the number of leukocyte cells, including T lymphocytes and B lymphocytes.[5] Previous studies of the effects of white oyster immunostimulatest against nonspecific immune responses (innate) with macrophage phagocytosis index and the number of total leukocyte have been investigated, but no studies have been conducted on the immunostimulant effects of white oyster mushrooms on specific immune responses.[6] Based on this, the purpose of the research is to determine the effect of ethanol extract of white oyster mushroom on specific immune response. The active substance contained in the white oyster mushroom is β-glucan have a good solubility in ethanol.

2. METHODS

A. Extract of white oyster mushroom

White oyster mushroom obtained from Cidadap Village, Kabupaten Bandung Barat. Mushroom 40 days aged harvested, weighed and determined. Determination were done at Herbarium Bandungense School of Life Sciences and Technology ITB (Institut Teknologi

Bandung). To make extract, white oyster mushroom sliced thinly, then dried in an oven at 500-600 °C for 4 days. The dried material then grounded using a ground mill to obtain dried simplicia and ready to be extracted. The extract dissolved in 90% ethanol. Dry simplicia then put into extractor with ratio of 1: 6 (4 kg dried simplicia used 24 liters of ethanol 90%) for 18 hours continuously. Liquid extract then evaporated in a Rotary evaporator to obtain condensed extract, then inserted into cabinet oven drier for 18 hours at 500 °C to obtain dried extract. Yield for this process is 0.0682 gr extract per 1 gram dried simplisia. In the previous study the optimal doses used were 200 mg/kg BW for its efficacy on innate immune response.[7]

B. Lamb's Red Blood Cell Suspension

Used as an antigen and reagent hemagglutination in the examination of antibody titers. Taken from the fresh blood of 5 year old Lamb (*Ovis aries* Garut local line) in Animal Breeding of PT. Biofarma, Lembang. Blood is collected with anticoagulants (Na citrate), then centrifuged for 10 minutes at 3000 rpm. Then RBC was separated and washed with PBS (pH 7.2), three times. RBCs were prepared in 10% and 2% preparations with the addition of NaCl solution. The 10% suspension is used for desensitization and 2% is used for hemagglutination tests on antibody titers (anti-lamb's RBC).

C. Phosphate Buffer pH 7,2

Phosphate buffer pH 7.2 was obtained by dissolving 8 gr of sodium chloride, 0.2 gr of potassium chloride, 1.15 disodium phosphate, and 2 gr of potassium dihydrogen phosphate into distilled water up to 1 litre.

D. CMC (Carboxy Methyl Cellulose) 1%

CMC was obtained from Penelitian Antar Universitas-Bandung Institute of Technology (PAU-ITB). CMC is used as a solvent of the ethanolic extract of white oyster mushroom and as a placebo.

E. Mice

Swiss Webster mice aged 7-9 weeks, weight 25-40 gr obtained from PAU ITB, adapted to the new environment. Research objects were fed during the adaptation period. 14 mice were grouped into two groups by random block permutation. The control group of 7 mice were given 2 ml daily of 1% CMC solution, and the treatment group: 7 mice were given the extract at a dosage of 200 mg / kg BW /day dissolved in 1% CMC by oral daily.

F. Calculation of lymphocytes total population

Tail vein blood sample collection 0.5 ml on day 7, transferred into the EDTA tube. Samples were examined by flowcytometry method using *Hematology Counter Sysmex XE-2200* (Sysmex, Kobe, Japan).

G. Measurement of Total Antibodies Titer

Mice's blood serum were used and obtained by separated blood and serum by centrifuged for 10 minutes at room temperature. The antibody titer was determined by

hemagglutination method, used V bottom 96 well microplate. Dispensed 25 µl of phosphate buffer pH 7.2 on each well, 25 µl of a test serum on the first line. The Multichannel pipette was used to carry out two-fold serial dilutions across the plate until column 12 and disposed 25 µl from the last column. 25 µl of Lamb's RBC were dispensed on each well. The highest dilution which gives agglutination considered as antibody titer. The hemagglutination reaction is positive if the microwell is homogeneous. The hemagglutination reaction is negative if it appears precipitation in the bottom well of the microplate. The antibody titer is expressed as the opposite of the highest dilution rate which indicates hemagglutination.

H. Measurement level of γ serum globulin

0.5 ml of heart blood mice collected on the 15th day into a vacuette tube with a separator gel. Centrifuged at 2500 rpm for 10 minutes. Serum samples were examined using Sebia MG 300 electrophoresis protein at RSHS Clinical Pathology Laboratory. Procedures in accordance with manufacture instructions (Sebia Inc., Norcross, GA, USA).

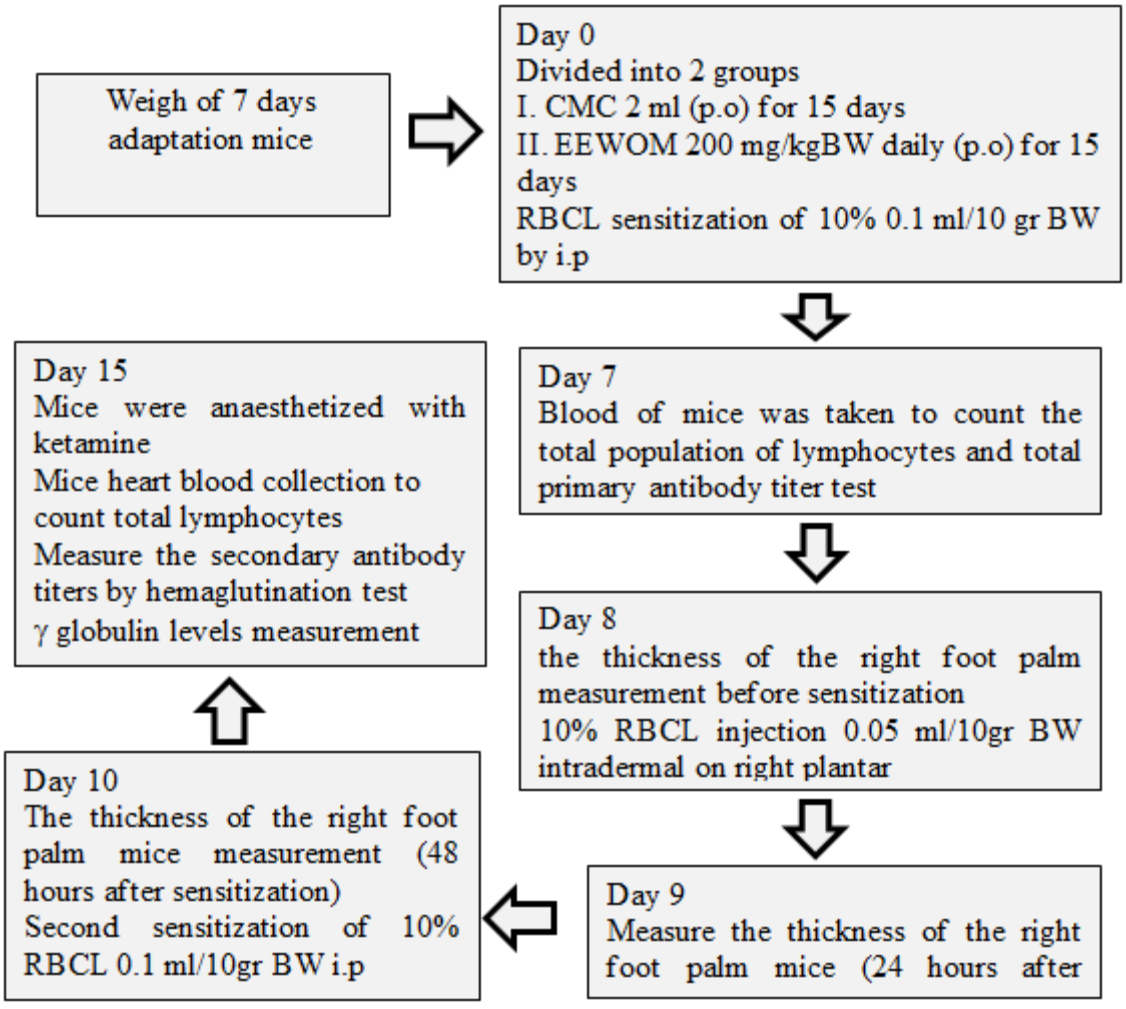

Figure 1. Treatment procedure flow.

I. Delayed-type hypersensitivity skin testing

Delayed-type Hypersensitivity responses represent the response of cellular immune to specific antigen from lamb's RBC that have been exposed previously. On the 7th day, second sensitized by injecting the same antigen of 0.05 ml of 10% lamb's RBC intradermal. Measurement of foot palm thickness using a calipers at the previous point of injection, 24 hours and 48 hours after injection. The difference in the foot palm thickness before and after the injection indicates of the Delayed-type Hypersensitivity responses.

J. Statistical Analysis

Implemented bivariable analysis data, mean value ± SD from each data. Analyzed using Independent t-test and Mann Whitney. Data obtained were analyzed using SPSS (Statistical Package for The Social Sciences) program for windows version 13.0

3. RESULT

A. Weight Measurement

First acclimatization of mice were done for 7 days. Then mice weighed and assigned randomly into two groups, control and treatment. The results of weight measurement of objects studied is described on Table 1.

Table 1. The Results of Weight Measurement of All Objects Research

Weight variable	Average		P Value*)
	Control	Treatment	
Prior to adaptation (gram)	27,64 ± 1,74	30,12 ± 2,83	0,072
Subsequent to adaptation (gram)	30,62 ± 0,90	32,05 ± 2,39	0,166

*) Independent T Test

Table 1 shows the statistical test results using Independent t-test. There is insignificant body weight difference of mice before and after adaptation in two groups (control and treatment). Therefore we can say that those two groups are homogeneous.

B. Comparison of total Lymphocyte

Mice that have been weighed and grouped were sensitized by injecting 10% lamb's RBC intraperitoneally, then taken serum on day 7 to measured total lymphocyte count..

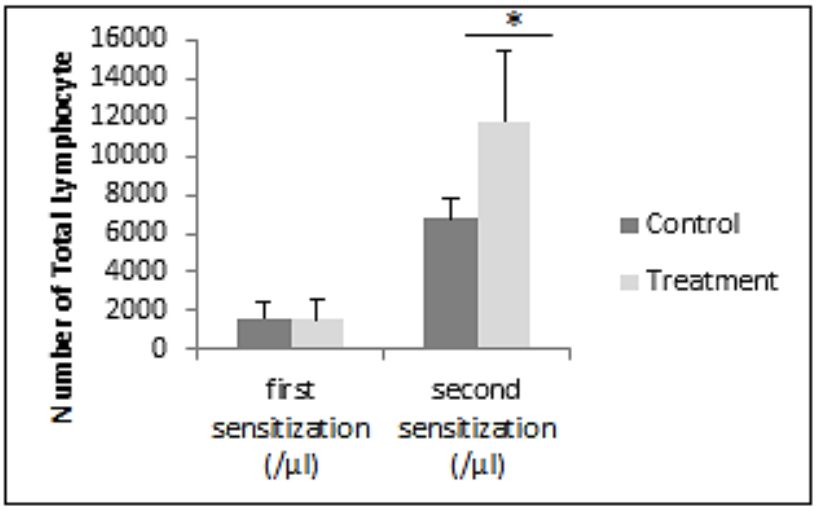

Figure 2. Comparison the number of total lymphocyte in treatment group compared to control group. Data is presented as mean ± SD of the treatment group which is compared to control group using Independent T-test. The p value ≤0,05 shows significant difference compared to control group (*significant compared to control group).

The results showed that there is a significant difference of total lymphocyte after secondary sensitization between treatment group and control group of white oyster mushroom (p value = 0.001, p≤0,05). However, there is insignificant difference of lymphocyte level after primary sensitization between those two groups (treatment and control) (p = 0.870, p> 0.05).

C. Comparison of Total Antibody Titer

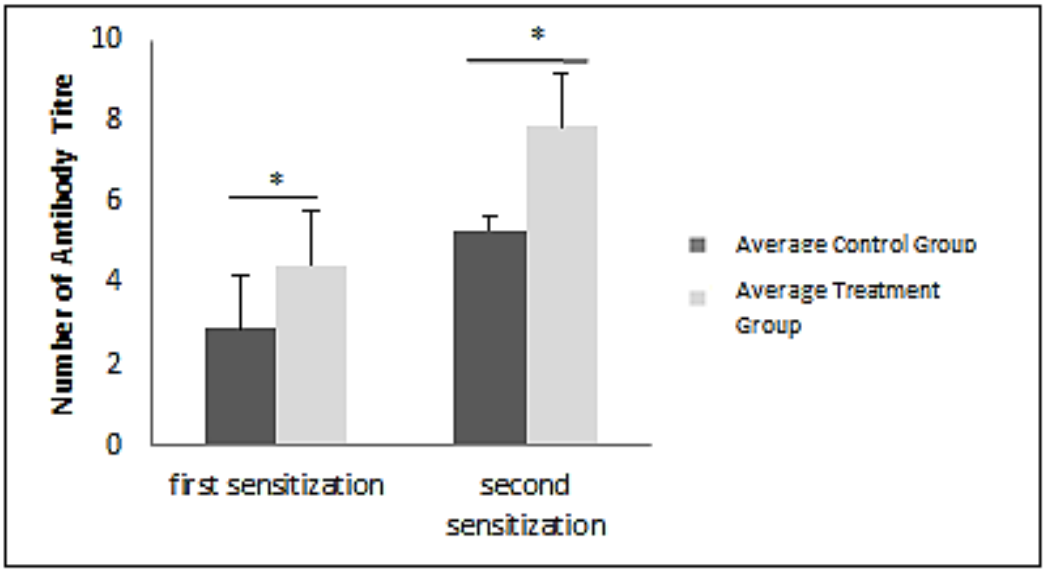

Figure 3. Comparison of antibody titer between treatment group and control group. Data is presented as ± SD of treatment group compared to control group using Independent T

Test. The p value ≤0,05 shows significant difference between two group (*significant compared to control group)

Mice that have been weighed and grouped were sensitized by injecting 10% lamb's RBC intraperitoneally on the day 0 and day 8, then serum taken on day 7 and day 15 to measured total titer antibody. The comparison of antibody titer after first sensitization and second sensitization is described on Figure 3. Based on a statistical result, there is a significant differences in antibody levels after primary and secondary sensitization between two groups namely treatment group and control group (p value = 0.050, p≤0.05 for primary sensitization and p = 0.004, p≤0, 05 for secondary sensitization)

D. Measurement of γ Globulin level

γ Globulin test is carried out to measure specific antibody against the foreign antigen. Total γ globulin fraction is an indication of the humoral immune response measured quantitatively. Measurement of γ Globulin was carried out on day 15 after sensitization. The result shows the whole object studied has mean about 0,34 g/dL and γ Globulin level of control and treatment groups about 0,28 ± 0,07 g/dL and 0,41±0,06 g/dL, respectively. In addition, there is a significant difference of globulin level between two groups namely treatment and control groups using *Independent T test* (p value = 0,006, p≤0,05).

E. Comparison of the Footprint Thickness

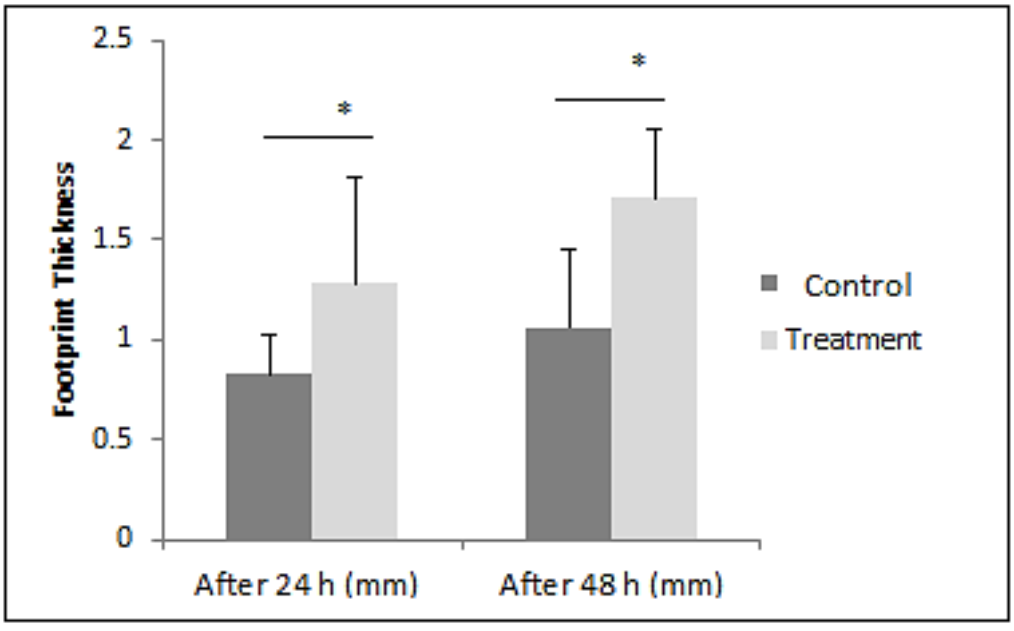

Figure 4. The comparison of footprint thickness between treatment and control groups. Data is presented as ± SD of treatment group compared to control group using Independent t-test dan Mann Whitney Test. The p value ≤0,05 shows significant difference between two group (*significant compared to control group)

Delayed-type Hypersensitivity test in this study illustrated cellular immune responses to specific antigens from previous exposure. Mice's foot thickness measurements using caliper are done 24 hours and 48 hours after injection. Based on a statistical tests (see Fig 4), there is a significant differences in the footprint thickness after 24 hours and after 48 hours between two groups namely treatment group and control group, respectively (p value = 0.010, $p \leq 0.05$ after 24 hours & p = 0.018, $p \leq 0, 05$ after 48 hours

4. DISCUSSION & CONCLUSIONS

Macrophage is one of the most important in immune response, both functionally in phagocytosis and antigen presenting cells. lamb's RBC will be destroyed after it passes through the physical barrier and enter to the bloodstream and confront the phagocytic cells.[8]

Lamb's RBC will be converted into small immunogenic peptide and presented for T and B cells. This process will trigger Thelper cell to proliferate and differentiate into Th1 and Th2 cells. These two cells are kind of third body defense, namely, adaptive immunity.[6, 9, 10]

The increasing of lymphocyte in this study indicate the raising of proliferation and differentiation of lymphocyte after sensitization due to the present of β-glucan compound in the extract of white oyster mushroom.[5] The sensitization of lamb's RBC will subsequently trigger the process of proliferation and differentiation of B cells to become plasma cells and memory cells. Humoral immunity is often regarded as the formation of antibody by plasma cells. In the first exposure to lamb's RBC, specific IgM antibodies can be detected in the body circulation after five to seven days. IgM production then followed by IgG production.[11]

In this study, the antibody titer after the primary sensitization in the treatment group is higher than in the control group. Morevover, the antibody titer examination after the secondary sensitization in the treatment group is also higher than in the control group. This phenomenon is caused by the second lamb's RBC exposure triggers a secondary immune response. The antibody is produced faster and greater in quality because of the existence of memory cells which it does not need a process of differentiation.[12]

The production of antibodies will be enhanced by β-glucan which is compounds of white oyster mushroom ethanolic extracts. β-glucan, increased phagocytosis by granulocytes and macrophages, can stimulate cytokine production by Th cells. Th2 cells, which do on humoral immunity, will stimulate B lymphocyte activity into plasma cells and B cells memory and it cause the increasing of antibody production.[6] Intraplantar lamb's RBC will stimulates the inflammatory response which ultimately cause the raising of macrophages in the system.[13]

Lamb's RBC is cleared from the tissues through the lymph vessels to the nearest lymph nodes. The dendritic cells and macrophages which existed in the lymph nodes will phagocytosis the lamb's RBC and will be functioned as APC. Moreover, Th cells will differentiate into Th1 and Th2 cells. Th1 cells act on the process of cellular immunity, then secretes IL-2. IL-2 to stimulate Th1 cells to proliferate and differentiate into T_{DTH} memory cells. As a result, the activity of T lymphocytes can be seen through the reaction of Delayed type Hypersensitivity-DTH. The occurence of DTH can be regarded as a CD4 T lymphocyte response of stimulation of antigen in the skin epithelium, which is characterized by local inflammatory reactions such as edema and erythema in tissues that peak 2-3 days after antigen sensitization Lamb's RBC on the skin.[14, 15]

In this study, the Delayed type Hypersensitivity reaction was allegedly enhanced by β-glucan compounds in white oyster mushroom ethanolic extracts. This β-glucans are able to stimulate macrophage activity, Th cells and to induce T cell differentiation. Th cell subpopulations ie Th1 and Th cells will stimulate the process of proliferation and differentiation of T lymphocyte cell, among other, T_{DTH} cells. It subesquently will induce the release of IFN-sit cytokines which it has a role in the macrophage activity, processing and presentation of antigens through MHC class I and II activation and ultimately stimulate lymphocyte proliferation. Finally, β-glukan is able to stimulate cellular immune response.[16, 17]

In conclusion, the ethanol extract of white oyster mushrooms will increase the population of lymphocytes, specific antibody titers, γ-globulin levels in the blood of mice due to sensitized LAMB'S RBC and increasing specific cellular immune response which is shown by the thickness of the footprint. Further research is needed on the safety of white oyster herbs by means of acute, subacute and chronic toxicity tests.

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