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Immunostimulant Activity of *Marchantia paleacea* Bertol. Herb Liverwort Ethanol Extract in *BALB/c* Mice

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ABSTRACT

Immunostimulants are compounds that can stimulate an immune response by increasing the activity of non-specific and specific components of the immune system (humoral and cellular) against certain infections and diseases. The liverwort plant species *Marchantia paleacea* Bertol. has long been used as a source of nutrition and empirical medicine. However, scientifically there is still not much research data on immunomodulators in these plants. This study aims to determine the activity of immunomodulators in the ethanol extract of the herb *Marchantia paleacea* Bertol. in male mice of *BALB/c* strain. Bioactive compounds from this plant were extracted by maceration method using 96% ethanol. Extract characterization and phytochemical screening were determined according to WHO guidelines and standard procedures from previous studies. The immunomodulatory activity of the extract was tested by carbon clearance method and lymphoid organ index (non-specific responses), primary and secondary antibody titer tests (humoral specific responses), IL-2 cytokine levels and IFN-ɣ from serum secondary antibodies and delayed-type hypersensitivity reaction/DTH (cellular specific response). The results of qualitative phytochemical screening contained flavonoid compounds, saponins, phenolics, tannins and steroids/triterpenoids. The results of the non-specific immune response immunomodulator test showed that the dose of 52 mg/kg bw had the largest phagocytic index of 1.52 which included strong immunostimulation (K > 1.5) and the organ spleen index of 0.55 ± 0.11 which increased significantly compared to the control ($p<0.05$). The data on the acquisition of specific immune responses in the primary and secondary antibody titer test in the three test extracts resulted in increased titer levels compared to the control and at a dose of 52 mg/kg bw could significantly increase the levels of IL-2 cytokines in the control group ($p<0,05$). Meanwhile, in DTH test, doses of 13 and 26 mg/kg bw could significantly increase the thickness of the soles of mice compared to controls ($p<0.05$).

Keywords: Immunostimulant, *Marchantia paleacea* Bertol., Liverwort, innate immune response, IL-2 Cytokine

INTRODUCTION

The immune system contributes significantly in helping to protect the level of human health. Some disorders of the immune system in various diseases such as: infectious diseases, cancer, arthritis, allergies and asthma. Several diseases caused by impaired modulation of the immune response have been of interest to researchers for many years. Compounds that can modulate the immune system are known as immunomodulators. Immunomodulators are classified as immune-stimulants or immunosuppressants. At present, many synthetic compounds and herbal products are used as immunomodulators in modulating non-specific and specific immune responses. The use of medicinal plants with immunostimulant effects in patients reported less toxicity and side effects, thus attracting researchers to focus on exploring medicinal plants and their derivative compounds from plants that can improve certain immune responses (Iwo, *et al*., 2014; Kumolosasi *et al*., 2018).

Therefore, medicinal plants and their various bioactive components have great potential as agents of immunomodulatory products. *Marchantia paleacea* Bertol. is one of the species of liverworts of the *Marchantia* genus which has long been used as an ethnomedicinal empirically in China, Europe, North America and Indonesia but research and utilization of its pharmacological activity are still not well explored (Fadhilla, *et al*., 2012; Jantwal, *et al*., 2019).

Various pharmacological activities were observed from this liverwort extract of the genus *Marchantia*, namely: antimicrobial, antifungal, antioxidant, antipyretic, diuretic, cytotoxic and apoptotic activity, cardiotonic, muscle relaxant and antihepatitis. Several bioactive components that play an important role in these activities are bis-bibenzyls (marchantin), terpenoids, flavonoids, saponins and phenolic compounds. In Indonesia, *Marchantia paleacea* Bertol. grows a lot in the highlands with cool climates at an altitude of more than 2,000 meters above sea level. This type of moss grows quickly and dominates among other plants so it is categorized as a weed (Asakawa, 2017; Fadhilla *et al*., 2012).

This study aims to explore immuno-modulatory activities, especially the immuno-stimulant efficacy of the whole plant liverwort (herba) *Marchantia paleacea* Bertol. against non-specific (innate) and specific (adaptive) immune responses in test animals of *BALB/c* male mice. However, the immunostimulant activity of this type of plant has not been reported in any scientific journal.

MATERIAL AND METHODS

The sample used in this study was the ethanol extract of the liverwort herb (EELH) *Marchantia paleacea* Bertol. in the form of a thick extract with a fixed weight. This plant material was obtained from the Kampung Padajaya, Sindangjaya Village, Cipanas District, Cianjur Regency, West Java, Indonesia. This plant was determined at the Research Center for Plant Conservation and Botanical Gardens – Cibodas Botanical Gardens, Indonesian Institute of Sciences (LIPI), Indonesia with No. B-0433/IPH.5/AP.0/II/2018. The test animals used were male mice of the *BALB/c* strain with a weight of 25-35 g aged 8 weeks and obtained from the Animal Breeding Laboratory of PT. Biofarma, Cisarua-Lembang, Bandung. The certificate of ethical approval in this study was obtained from the Ethics Commission for the Use of Test Animals, Bandung Institute of Technology with No. 04/KEPHP-ITB/10-2017. The test animal feed ingredients are standard laboratory feeds which are uniformly given to all samples of the test group. EELH *Marchantia paleacea* Bertol. test doses of 13, 26 and 52 mg/kg bw were used, levamisole (Askamex®, PT. Konimex) was used as a positive control immunostimulant at a dose of 2.5 mg/kg bw and methylprednisolone (PT. Gracia Pharmindo) at a dose of 40 mg/kg bb, sheep red blood cells 2%/SRBC 2% (as an antigen to induce antibody formation), ink containing colloidal carbon (Pelican China Ink B-17®), phosphate buffer saline (PBS), Mouse IL-2 ELISA kit (BioLegend®) and Mouse IFN-γ kit (BioLegend®).

Preparation Sample

Fresh herbs *Marchantia paleacea* Bertol. harvested, sorted, washed thoroughly (until there are no other types of plant contaminants, impurities and soil still attached). Then the samples were dried, sorted dry and ground into simplicia powder. The active compounds in simplicia were extracted by maceration of 1000 g of simplicia powder with 10 L of 96% ethanol as solvent, stirring periodically and allowed to stand for 1 day and repeated 2 times. The liquid extract was then filtered, evaporated using a rotary evaporator and a water bath to obtain a thick extract with a fixed weight (Fadhilla, *et al*., 2012).

Characteristic examination, phytochemical screening of ethanol extract of *Marchantia paleacea* Bertol.

The obtained extracts were characterized including: determination of water content, total ash content, water soluble extract content, ethanol soluble extract content and determination of specific gravity 5% (or 0.5 g/10 mL) and phytochemical screening in accordance with the guidelines of the research standards that have been carried out to guidelines from research standards that has been done by previous researchers (RI, 2000; Siregar *et al*., 2021; WHO, 2011).

Testing for Non-specific Immune Responses

Immunomodulatory testing of this non-specific immune response was carried out by carbon clearance test (phagocytic index) and determination of lymphoid organ indices. Test animals were randomly assigned to 6 groups, namely: EELH group with doses of 13, 26 and 52 mg/kg bw, positive control group immunostimulant (levamisole) 2.5 mg/kg bw, positive control group immunosuppressant (methylprednisolone) 40 mg/kg kg and the normal control group were given 0.5% Na-CMC carrier and the whole test group was given orally. Each test group consisted of 5 mice.

Carbon Clearance Test

Test animals that have been grouped randomly were given the test preparation for 7 days. On the 8th day, 20 µL of animal blood serum was taken through the tail vein at minute 0 (t_0), then the animals were injected with colloidal carbon (Pelican China Ink B-17®) in 1% w/v gelatin (in physiological solution of 0.9% NaCl) with dose 0.1 mL/10 g bw intravenously (Arunabha and Satish, 2014). The same amount of blood sample was taken back through the tail vein at 5 minutes (t_5) and 15 minutes (t_{15}) and put into a tube containing 1% acetic acid solution. Furthermore, the percent transmittance was determined at a wavelength of 675 nm, the rate of carbon elimination (K or kel) was calculated from the blood samples of the test animals and the phagocytic index was determined by the formula:

$$K = \frac{\text{Log OD5} - \text{Log OD 15}}{\text{T2} - \text{T1}}$$

Explanation: Log OD5 = log absorbance of blood at minute 5; Log OD15 = log absorbance of blood at 15 minutes; T1= initial pick-up point (t_5); T2 = final pick-up point (t_{15})

$$\text{Phagocytic index} = \frac{\frac{K}{3} \text{ x Mice weight (g)}}{\text{Liver weight (g)} + \text{Spleen weight (g)}}$$

(Yu, *et al*., 2018; Zhang, *et al*., 2018)

Determination of Organ Index

After administering the test substance on the 8th day, the test animal was sacrificed by inserting it into the CO_2 chamber, performing thoracic surgery and taking the liver, spleen and thymus gland from each test animal and weighing their organs. The organ index for the three organs is calculated and expressed against the body weight of each test animal with the following formula:

$$\text{Indeks organ (\%)} = \frac{\text{Organ weight (g)}}{\text{Body weight (g)}} \text{ x } 100$$

Testing for Specific Immune Responses

Efficacy testing for specific immune responses was determined through primary and secondary antibody titer tests, which are humoral immune responses using the hemagglutination method and measurement of IL-2 and IFN-ɣ cytokine levels from serum secondary antibody titers and determination of delayed type hypersensitivity (DTH) as a cellular immune response. The antigens used in the specific immune response assay were sheep red blood cells (SRBC) 2% in physiological NaCl 0.9%.

Determination of primary and secondary antibody titers using the Hemagglutination Method (Technique)

The mice tested were grouped randomly into 5 test groups, namely: a control group, levamisole and the three EELH test doses. The test extract was administered for 16 days, on the 4th day of administration of the test extract, an antibody formation induction process was also carried out by administering 2% SRBC suspension administered intraperitoneally at a dose of 0.1 mL/10 g bw. On the 11th day, after administering the test extract, the primary antibody titer was measured from all test groups using the hemagglutination technique. Blood serum of each test group as much as 20 µL was added with the same amount of PBS and 2% SRBC on a MicroWell 96-well plate on a "V" basis which was then diluted into series ½, ¼, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 and 1/2048. After being incubated for 24 hours, the mixed samples were examined for primary antibody titer levels using the hemagglutination technique. The antibody titer value was determined based on the last dilution

where the antibody was still detectable through visually formed hemagglutination. On the 11th day, apart from the primary antibody examination, reinduction (second injection) was also carried out with 2% SRBC (0.1 mL/10 g bw) to induce the formation of secondary antibody (IgG). Secondary antibodies were determined on the 16th day during the administration of the test extract (5th day after the second injection) with the determination of secondary antibody titer as carried out in the determination of the primary antibody (Faradilla and Iwo, 2014; Vikasari, *et al*., 2021).

Determination of IL-2 and IFN-ɣ Cytokine Levels

The efficacy of the test extract on the cellular immune response in this study was determined by determining the levels of IL-2 and IFN-ɣ cytokines. On the 5th day after the second injection with 2% SRBC for the determination of the secondary antibody titer, the blood serum was also determined for the levels of IL-2 and IFN-ɣ using the ELISA Reader (Kumar, *et al*., 2012; Yu, *et al*., 2018).

Delayed-Type Hypersensitivity (DTH) Test

Efficacy against cellular immune responses was also determined by the DTH assay. The test extract was given for a total of 9 days. Each test animal was immunized with 0.1 mL/10 g bw of 2% SRBC intraperitoneally (day 1). On day 7, the thickness of the left foot of the mice was measured (T_0), then the mice were injected again with 0.05 mL of 2% SRBC intradermally in the sole of their left foot. Measurement of left foot thickness was repeated at 24 (T_{24h}) and 48 (T_{48h}) hours after the injection. The thickness of the left foot before and after the 2% SRBC injection was calculated as the percentage change in the thickness of the left foot in each test group. The size of the DTH test response was obtained by comparing the thickness of the left leg of the test group with the control group (Singh, *et al*., 2012).

RESULT AND DISCUSSION

Fresh herb samples of *Marchantia paleacea* Bertol liverworts (Figure 1) were determined to ensure the correctness of the type of plant to be studied. The plant parts used in this study were herbs or the whole plant including thallus and rhizoids. Characteristic examination was carried out microscopically on this type of liverwort simplicia with the results observed (Figure 2) with 40x magnification using a light microscope. The results of the examination showed that the simplicia contained a ventral cavity of the talus, a smooth-walled and pegged rhizoid structure, calcium oxalate crystals that formed a rosette, oil cells, pore water, fiber cells as described by Lu and Huang (2017) from the results of his microscopic research on *Marchantia paleacea* Bertol. in Taiwan.

Figure 1. *Marchantia paleacea* Bertol. liverwort plant picture.

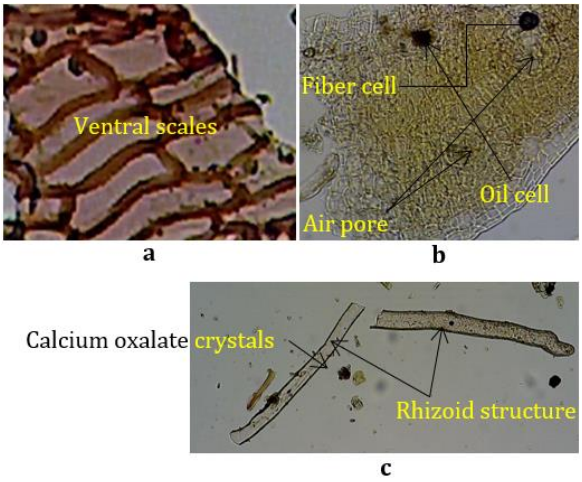

Figure 2. Microscopic characteristics of the simplicial of the plant *Marchantia paleacea* Bertol. which shows: a. ventral thalloid; b. thalloid dorsal; c. rhizoid structure and calcium oxalate crystals in rosette form.

The yield of viscous extract with a fixed weight obtained was 2.64% (w/w). The ethanol solvent used is a conventional organic solvent and has been considered a universal solvent in extracting various polar to less polar compounds (Karmakar, *et al*., 2020; Oomen, *et al*., 2020). The ethanol extract of liverworts obtained was in the form of thick gum with blackish green color and a characteristic aromatic odor.

The results of the examination of the quality characteristics of the simplicia and ethanol extract (Table I). The ethanol soluble extract content in the EELH extract has a greater content of 34.88% (w/w) than the water-soluble extract content of 7.49% (w/w). Extraction of ethanol and water-soluble extracts can also be useful for determining the purity of medicinal products. The total ash content in the examination of the quality characteristics of simplicia and extracts provides an overview of the inorganic composition and other impurities present in simplicia and extracts (Krishnamurthy, *et al*., 2016).

Qualitative phytochemical screening test results on simplicia and plant extracts of *Marchantia paleacea* Bertol. which contains flavonoid compounds, saponins, phenolics, tannins and steroids/triterpenoids. This phytochemical screening was carried out by standard methods as mentioned by previous studies from Depkes RI (2000), Siregar, *et al*. (2021) dan WHO (2011). The presence of a positive class of flavonoid, tannin and phenolic compounds causes its ability as a natural antioxidant that has free radical scavenging activity. Based on the content of other compounds such as bis-bibenzyl (Marchantin compound) and other bioactive compounds, this plant has great potential as herbal medicine to treat various types of diseases (Siregar, *et al*., 2021).

The data on the rate of elimination of carbon particles in all test groups (Figure 3). Carbon clearance test is one of the non-specific immune response tests used to examine the effect of EELH in modulating reticuloendothelial mediated phagocytosis (RES) (Iwo, *et al*., 2014). When an ink containing colloidal carbon (Pelican China Ink B-17®) is injected with dose 0.1 mL/10 g bw intravenously, macrophages and other phagocytic cells phagocytize the carbon particles of the ink. Carbon particles injected into *BALB/c* strain mice were mainly phagocytosed by Kupffer cells (90%) and followed by macrophages outside liver cells (10%).

Carbon particles in this colloid as markers/antigens that will be phagocytosed by phagocytic cells, such as: macrophage cells, neutrophil cells, monocyte cells and other phagocytic cells. The carbon used is Pelican China Ink B-17® which is used as an antigen which can also be replaced by carbon from other product preparations that have small particle sizes such as: Pelikan China Ink B-17® so that there is no blockage in the blood vessels (Aldi *et al*., 2016). This phagocytic activity was determined by observing the clearance rate of carbon particles that were given as foreign substances in the blood by determining the transmittance of blood samples (in 1% acetic acid) at certain time intervals. The transmittance was determined at a wavelength of 675 nm using a UV-Vis spectrophotometer. After the transmittance value is determined, then a graph curve of 100 - % Transmittance is made against the time interval that has been plotted as shown in Figure 3. The more sloping each line, the smaller the number of carbon particles in the blood. In turn, the carbon clearance rate can also be used to determine the phagocytic index. The phagocytic index was determined by comparing through the ratio method (Iwo, *et al*., 2014). In the graph curve (Figure 3) test extract doses of 13 and 52 mg/kg bw as well as levamisole have a greater slope than the control group and methylprednisolone.

The phagocytic index in this study can be observed (Table II). The phagocytic index in the range of 1.0–1.5 has a moderate immunostimulating effect and a phagocytic index of more than 1.5 has a strong immunostimulating effect. The phagocytic index at a dose of 52 mg/kg bw EELH had a phagocytic index of more than 1.5, namely 1.52 or had strong immunostimulation compared to controls. Meanwhile, EELH doses of 13 and 26 mg/kg bw had moderate immunostimulating effects (Iwo, *et al*., 2014).

EELH efficacy on lymphoid organ index is shown in Figure 4. The spleen organ index in EELH test animals at a dose of 52 mg/kg bw increased significantly compared to control by 0.55 ± 0.11 ($p<0.05$). While the thymus organ index for EELH doses of 13 and 52 mg/kg bw also increased but not significantly compared to controls. Lymphoid organ index is a non-specific immune response test method. The thymus gland is also an organ of the immune system where T lymphocytes develop, differentiate and mature (Yu, *et al*., 2018). The spleen is a secondary lymphoid organ that not only contains B and T lymphocytes, but also contains dendritic cells and macrophages that act as APCs (Antigen Presenting Cells). The increase in immune cells correlates with an increase in spleen weight as (Figure 4). The increase in the index of the spleen organ indicates an effect as an immunostimulant activity (Puspitaningrum, *et al*., 2017). Especially at a dose of 52 mg/kg bw there was a significant increase in spleen organ index compared to controls ($p<0.05$).

Table I. Parameter of simplicia quality characteristics and standardization of EELH

Examination	*Marchantia paleaceae* Bertol. Herb	
	Simplicia	EELH Extract
Moisture Content (% v/w)	8.00	4.98
Total Ash Content (% w/w)	18.08	8.77
Determination of Specific Gravity of Extract 5% (g/mL)	-	0.8140
Water Soluble Content (% w/w)	0.85	7.49
Ethanol Soluble Essence Content (% w/w)	0.26	34.88

Table II. Phagocytic index after administration of EELH

Group	Dose (mg/kg)	Elimination speed (k_{el})	Phagocytic Index	Classificaiton of Immunomodulatory Effects
Control	-	0.059±0.014	1	-
EELH	13	0.089±0.002	1.50	Immunostimulation
	26	0.082±0.025	1.37	Immunostimulation
	52	0.090±0.004	1.52	Immunostimulation
Levamisole	2.5	0.089±0.038	1.49	Immunostimulation
Methylprednisolone	40	0.042±0.031	0.71	Immunosuppressive

*Data are expressed as mean ± SD; EELH= ethanol extract of the liverwort herb of *Marchantia paleacea* Bertol.

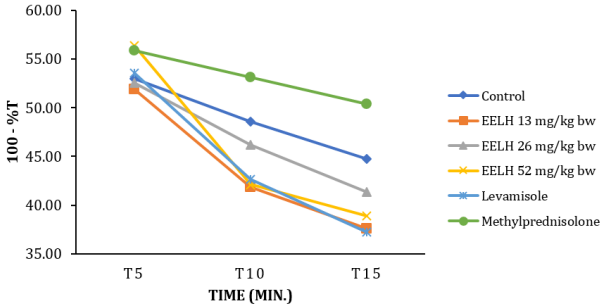

Figure 3. The rate of elimination of carbon particles after administration of the test extract. T5, T10 and T15 on the x-axis in the graph show the time of taking and examining the absorbance of carbon particles in the blood samples of mices at 5, 10 and 15 minutes in each group. Graph T5, T10 and T15 display data from the same mices. The more sloping each line, the smaller the number of carbon particles in the blood.

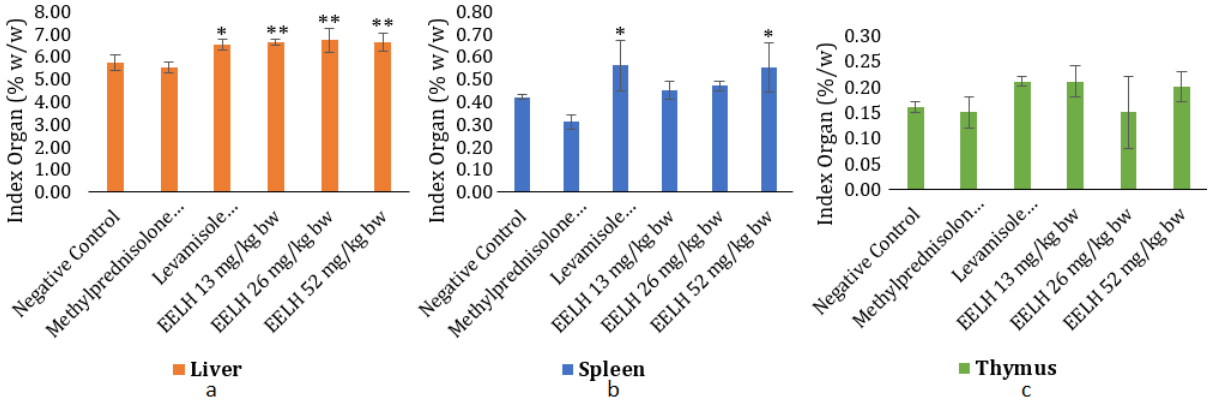

Figure 4. Lymphoid organ index (% w/w) after administration of test extract. a). Organ index data from liver, b). Organ index data from spleen and c). Organ index data from thymus. The bar charts in a), b) and c) display data from each test group. Data are expressed as mean ± SD; significant differences organ index are indicated: * = p<0.05 dan ** = p<0.01 compared to control (ANOVA with LSD Post Hoc test)

Table III. Antibody Titers Determined by Hemagglutination Inhibition (HI) for 2% SRBC in *BALB/c* Mice After Administration of EELH

Group	Dose (mg/kg bw)	Antibody Titers Determined by Hemagglutination Method (Technique) for 2% SRBC Antigen	
		Primary	Secondary
Control	-	1:128	1:256
EELH	13	1:256	1:1024
	26	1:256	1:512
	52	1:256	1:1024
Levamisole	2,5	1:256	1:512

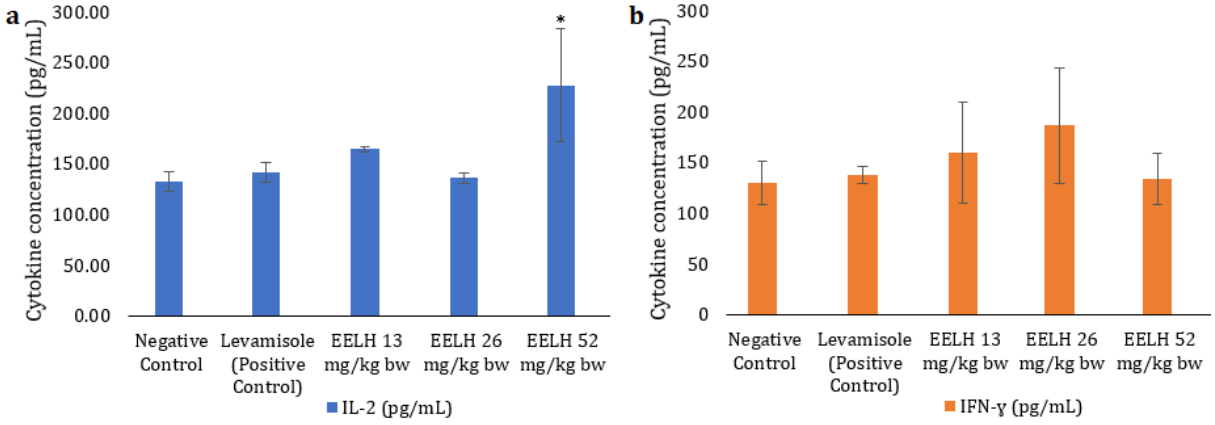

Figure 5. Cytokine concentration levels (pg/mL) of IL-2 and IFN-ɣ. The bar charts in a) and b) display data from each test group. Data are expressed as mean ± SD. Significant differences organ index are indicated: * = p<0.05 dan ** = p<0.01 compared to control (ANOVA with LSD Post Hoc test).

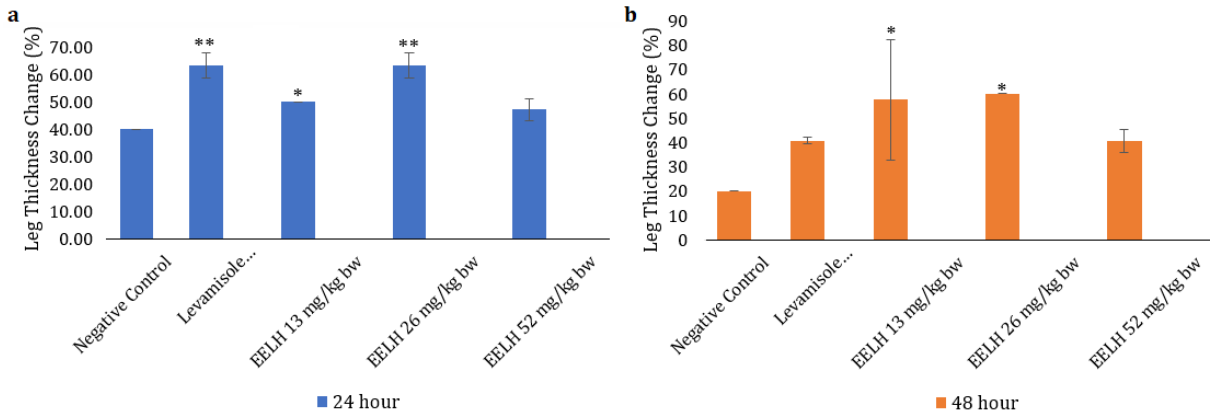

Figure 6. EELH activity on delayed-type hypersensitivity (DTH) reactions. The bar charts in a) and b) display data from each test group. Data are expressed as mean ± SD. Significant differences organ index are indicated: * = p<0.05 dan ** = p<0.01 compared to control (ANOVA with LSD Post Hoc test).

The efficacy of EELH on primary and secondary antibody titers determined by hemagglutination method (technique) responses can be seen in Table 3. Antibody titer testing shows the activity of the humoral immune response which involves the process of interaction between B lymphocytes and 2% SRBC antigen which results in the formation of proliferation and differentiation of B lymphocytes into plasma cells that secrete primary and secondary antibodies. Antibodies act

as effector humoral responses by opsonizing antigens so that antigen neutralization occurs and facilitates the process of eliminating antigens that are more easily phagocytosed by macrophage cells (Faradilla and Iwo, 2014). The results of primary antibody titers in the EELH group at doses of 13 and 52 mg/kg bw were higher than the control group and the activity was the same as in the levamisole group. An increase in the primary antibody titer indicates the formation of stimulation of B lymphocytes (secretion of Ig M antibodies). Meanwhile, an increase in secondary antibody titer indicates a stimulation of B cell memory in the process of Ig G antibody formation.

The levels of IL-2 cytokines from serum secondary antibody titers at 52 mg/kg bw EELH significantly increased compared to the control ($p<0.05$) which can be seen in Figure 5. Although the other two test extracts increased compared to the control, it was not significant. The three EELH test groups also experienced an increase in IFN-ɣ levels against the control although not significantly different. This is in accordance with the theory of immunostimulants that can increase the production of IL-2 and IFN-ɣ cytokines in splenocytes which will help maturation and activation of immune cells. IL-2 and IFN-ɣ are also typical cytokines in activating helper T cells (Th1) and these two cytokines exhibit antiviral, immunomodulatory (immunoregulatory) and antitumor properties. IFN-ɣ can activate resting macrophages to become active macrophages (Macrophage Activating Cytokine) and cellular immune responses (Nurkhasanah and Novitasari, 2019; Puspitaningrum, *et al*., 2017; Shin, *et al*., 2016). This enhanced effect of IL-2 has special relevance for its prophylactic and therapeutic potential in enhancing the immune response to various infectious diseases (infectious microorganisms) and antitumor with few side effects (Kumar, *et al*., 2012).

Cellular specific immune response data determined through DTH test can be seen in Figure 6. The DTH response requires specific recognition of the antigen (SRBC 2%) given to T lymphocyte cells which then proliferate and release certain cytokines. The release of cytokines from activated T cells will increase vascular permeability, induce vasodilation, macrophage accumulation and increase activation and increase macrophage activity. So that the concentration of granzymes produced by macrophage cells can accelerate the process of antigen elimination. In the initial stage, Th1 cells are activated by the first antigen exposure and proliferate. On subsequent exposure, the 2% SRBC antigen induces an effector response in which Th1 cells secrete several cytokines that activate macrophages and other non-specific inflammatory mediators. The delayed response time reflects the time required for cytokines to induce macrophage activation (Faradilla and Iwo, 2014). The percentage of changes in the left foot of the EELH group at doses of 13 and 26 mg/kg bw increased significantly compared to the control ($p<0.05$) both at the 24 and 48 h. This indicates that at both doses, EELH can stimulate a cellular immune response mediated by T lymphocytes.

CONCLUSION

Ethanol extract of the liverwort herb of *Marchantia paleacea* Bertol. (EELH) in this study has immunostimulant efficacy because EELH at the three test doses, especially the dose of 52 mg/kg bw for the whole test, both with a non-specific immune response test and specific immune response test can stimulate the entire test so that EELH has the potential to be developed as an immunostimulant product.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding this study.

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