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| RESEARCH ARTICLE

Ethyl Acetate Fraction of Momordica Charantia L. Fruits Induce the Phagocytosis Activity and Capacity of Rat Peritoneal Macrophages

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| ABSTRACT

Phagocytosis is one of the body's immune mechanisms in the elimination of antigens, including bacteria. Its mechanism is affected by many factors, such as the inducers. The present study aims to elaborate on the effect of ethyl acetate fraction of *Momordica charantia* L fruits on the activity and capacity of rat peritoneal macrophages on a non-A *Staphylococcus aureus,* including the white blood cell percentage. A series of *M. charantia* ethyl acetate extract concentrations of 25 mg/kg BW, 50 mg/kg BW, and 100 mg/kg BW were used to identify the phagocytosis activity and capacity, as well as the percentage of white blood cells (WBC). The result showed that the concentration of 50 mg/kg BW had the highest phagocytic activity and capacity compared to other concentrations ($P < 0.05$), while for the WBC percentage, there were no significant differences among the concentrations ($P > 0.05$). The conclusion of the present study is *M. charantia* ethyl acetate extract has the potential to be used as a natural immunostimulant.

| KEYWORDS

Ethyl acetate fraction, *Momordica charantia,* phagocytosis, white blood cells, immunostimulant.

| ARTICLE INFORMATION

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1. Introduction

For decades, many new infectious diseases (new emerging and re-emerging diseases/NERD) have appeared that have affected humans, animals, and the environment. The human population is very vulnerable to various respiratory, digestive, urogenital and other organ system diseases (Abbas et al. 2007). Immunity is defined as a defense against infectious diseases or toxins by the actions of specific antibodies or white blood cells (Beutler 2004). Macrophage also enables the production of various chemical intermediates such as nitric oxide (NO) and hydrogen peroxide (H_2O_2), as well as inflammatory mediators that can eliminate bacteria or cause infection in the host (Mirshafiey et al. 2004; Eze et al. 2014). Immunomodulatory agents are used to suppress or stimulate an organism's immune response to an antigen, and these compounds are mostly obtained from plants. Furthermore, immunomodulators have activities that stimulate specific and non-specific immunity (Beutler 2004).

Medicinal plants can inhibit or stimulate immune responses, which could be useful in the treatment of various human diseases. In particular, medicinal plants capable of inhibiting cellular and humoral responses could have useful applications in the treatment

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of immunological disorders. One of the herbal medicines that have been known as an immunomodulator is *Echinaceae purpurea* herb. *Echinacea purpurea* has a worldwide reputation for its immunomodulatory and anti-inflammatory properties, capable of modulating various immune system pathways. There are different classes of secondary metabolites of the plant showing immunostimulatory activity, such as alkamides, caffeic acid derivatives, polysaccharides, and glycoproteins (Barnes et al. 2005). *E. purpurea* has been widely used as an immunomodulator for the products used.

Another herbal medicine that is expected to have immunomodulatory activity is *M. charantia* L., also known in Indonesia as *Pare*. Dwijayanti et al. (2019) showed the anti-inflammatory effects of *M. charantia* extract, especially in its ethyl acetate fraction. Up to date, only a few reports about the immunomodulatory activity of *M. charantia*, especially its effects on macrophage activity; thus, the present study aims to elaborate on *M. charantia* fruit as an immunomodulator.

2. Materials and Methods

2.1 Plant Material Collection and Determination

The fruits of *M. charantia* L. were collected from a commercial plantation in Bogor, West Java, Indonesia. Plant determination was carried out at the Biology Laboratory, Center for Biological Research, National Research and Innovation Agency (BRIN) Bogor-Indonesia. As a positive control, a commercial extract of *E. purpurea* obtained from commercial herb medicine producers under the trade name of Imboost® was used.

2.2 Experimental Animals

A total of 30 heads of Sprague Dawley rat (*Rattus norvegicus*) 7 weeks old male, 200-250 grams weight were used according to Federer's formulation. Animals were placed in a plastic box measuring 50 cm x 25 cm x 20 cm and covered with wire. Each treatment group consisted of 2 (two) boxes containing 3 (three) rats each and placed in an air-conditioned room with room temperature 22 ± 2 °C and optimal ventilation. The lighting is set in a 12-hour dark cycle (the light cycle starts at six in the morning until six in the evening). Rats were fed twice a day using a commercial rat feed, and drinking water was given ad libitum. The treatment was given 2 (two) hours before the rats were fed; this was related to gastric emptying and drug absorption. Wood shaving as the base for the plastic rat cage is replaced at least 2 (two) times a week or as needed. All experimental procedures were approved by the Animal Ethics Committee School of Veterinary Medicine and Biomedical Sciences, IPB University Indonesia (certificate No. 044/KEH/SKE/XI/2021).

2.3 Non- A Staphylococcus aureus

A Non-A *S. aureus* bacteria isolate was obtained from Bacteriology Laboratory, Department of Animal Diseases and Veterinary Public Health, School of Veterinary Medicine and Biomedical Sciences, IPB University Indonesia. The inoculum was incubated at 37 °C for 24 hours in a Nutrient Broth liquid medium and centrifuged at a speed of 5000 rpm for 1 hour. Furthermore, the pellet was then separated from the supernatant, and sterile phosphate buffer saline (PBS) was added. The turbidity was measured using a UV-Vis spectrophotometer (Shimadzu UV-Vis) at a wavelength of 580 nm. The adjustment procedure was conducted by adding PBS gradually to reach 25% transmittance (equivalent to 10^8 cells/0.5 mL).

2.4 Preparation for Extraction and Fractionation of M. charantia L. Fruit

The different solvents (ethanol, ethyl acetate, *n*-hexane) were studied for their effects on the extraction yield. A total of 25 kg wet weight of fresh fruits of *M. charantia* L were extracted through several steps involving weighing, washing, draining, separating them from the seeds, and cutting into a size of approximately 1 cm. The cubes were then put in a maceration vessel and soaked in 96% ethanol in a ratio of 1:2. The maceration was conducted for 24 hours, after which the residue was separated from the filtrate. Fractionation was done only to the ethanolic extract of *M. charantia* L fruit, while the other 2 extracts (ethyl acetate and n-hexane extracts) were kept for another purpose. The ethanolic extract was then dissolved in 100 mL of water: 96% ethanol (1:2) until completely dissolved. Then, 100 mL of ethyl acetate with different polarities was added to the extract solution in a separating funnel. The mixture was shaken to form 2 layers and separated the formed phases. The filtrate was concentrated, and the ethyl acetate fraction was obtained.

2.5 Ethanol Extract Quality Assay

The 96% ethanol extract of *M. charantia* L was assayed to determine its quality by several steps. A dry yield, ash content, acid un-soluble ash content, water-soluble substance, and ethanol soluble water substance are determined by a Gravimetri method. A water content is assayed using a Karl Fischer method. Ethanol solvent residual is determined using a GC-FID method. The heavy metal content of Cd and Pb is determined by the SSA method, while Hg is by the ASS method and As using ICP-OES method. The content of yeast and mold is determined by a colony count. The MPN Coliform number is assayed by a method that includes the multiple-tube fermentation (MTF) technique and the membrane filter (MF) technique.

2.6 In Vitro Immunomodulation Studies.

2.6.1 Lymphocyte Cell Isolation

The isolation of lymphocyte cells was obtained from the spleen organ of Balb/C strain mice which was conducted aseptically. The mice were sacrificed, and with the surgical method, the spleen was taken out and placed in a 50 mm diameter petri dish containing 10 mL of RPMI medium. The RPMI medium was pumped into the spleen to remove the lymphocytes, and the cell suspension was put in a 10 mL centrifuge tube and centrifuged at 1200 rpm 4ºC for 5 minutes. The pellet obtained was suspended in 5 mL of tris ammonium chloride buffer to lysis erythrocytes. The cells were mixed until homogeneous and allowed to stand at room temperature for 15 minutes or until the color changed to slightly yellowish. Then 10 mL of RPMI was added and centrifuged at 1200 rpm 4 °C for 5 minutes; the supernatant was then discarded. The pellets obtained were washed 2 times with RPMI medium, and the cells were counted with a hemocytometer. Furthermore, the lymphocyte cells were cultured in a 5% CO_2 incubator at 37 °C and tested for activity (Bafna and Mishra 2004; Mirshafiey et al. 2004).

2.6.2 In Vitro Lymphocyte Cell Proliferation Assay

A total of 100 µL of lymphocytes with a concentration of 1.5 x 10^6 cells/mL were distributed into a 96-well microplate. This microplate underwent a three-stage incubation: first, each well was added with 10 µL of ethanol/ethyl acetate/n-hexane extracts of *M. charantia,* and levamisole as a control in each concentration group was added (150, 300, and 450 µg/mL) and incubated in a 5% CO_2 incubator at 37 °C for 24 hours. Each well was added with 10 µL of Phytohaemagglutinin (PHA) and incubated again for four days in a CO_2 incubator. Each well was added with 10 µL of MTT 5 mg/mL, followed by four hours of incubation at 37 °C. The living cells would react with MTT, producing a purple color. After a stopper reagent (10% SDS in 50 µL HCl 0.01 N) was added to each well, the microplate was stored at room temperature for 12 hours in a dark condition. The absorbance values were assessed using an ELISA reader at a wavelength of 595 nm (Ang et al. 2014).

2.6.3 In Vivo Phagocytosis Activity and Capacity Assays and White Blood Count

For in vivo leukocyte mobilization, the ethyl acetate fraction from ethanol extract was used as a tested substance. A method described by Ribeiro *et al*. (1991) was used with few modifications to study the effect of the fraction on in vivo leukocyte migration induced by the inflammatory stimulus. The experimental animals were divided into 5 groups, and each group consisted of 6 rats. As a negative control group, rats were treated with a sterile aquadest only, while the positive group was treated with *E. purpurea* extract. Three groups were acted as treatment groups, which treated orally for 7 days with ethyl acetate fraction of *M. charantia* L fruit at the dose of 25, 50, and 100 mg/kg body weight (BW).

On day 8 (eight) after treatment, the rats were then infected with a non-A *S. aureus* intraperitoneally. The rats were then waited for 2 (two) hours for the phagocytosis process in the abdominal cavity to run optimally. The rats were then injected with 0.5 mL sterile distilled water intraperitoneally, and the abdominal cavity was massaged slowly for a while to mix the macrophages evenly. The rats were anesthetized using ketamine and xylazine; after the rats were anesthetized, they were euthanized by taking as much blood as possible intracardially. The collected blood is placed in a vial to be tested for white blood cell count (lymphocytes, monocytes, neutrophils, basophils, and eosinophils) using an automatic hematology analyzer "Mindray BC 5000 Vet") (Ribeiro et al. 1991; Mahamat et al. 2018).

For the macrophage cell activity and capacity assays, the euthanized rat abdomen was dissected using a sterile scalpel and tweezers, and the peritoneal fluid was taken using a micropipette as much as possible. The fluid was smeared on a clean and dry microscope slide, fixed with methanol for 5 minutes, and stained with Giemsa. The slide was then observed for the phagocytosis under the microscope with a magnification of a 10x-100x objective lens, and the activity and capacity of phagocytosis were calculated (Besung et al. 2016).

The formulation for calculating activity and capacity phagocytosis are:

1. Percentage of phagocytosis activity: Number of active macrophages that engulfed *S. aureus* bacteria in 100 macrophages X 100%
2. Phagocytosis capacity of macrophage cells: Number of engulfed bacteria *S. aureus* from 50 active macrophages.

2.7 Data Analysis

All collected data were statistically processed using ANOVA Version 26 (*One Way ANOVA*). A *p*-value of less than 0.05 ($p<0.05$) was considered as significant. Results are expressed as mean ± standard deviation as indicated. Graphical presentations were made using Microsoft Office Excel.

3. Results

3.1 Determination of M. charantia L.

The plant was identified as M. charantia L according to the identification certificate No. B-431/V/DI.05.07/2/2022 from the Biology Laboratory, Center for Biological Research, National Research and Innovation Agency (BRIN) Bogor-Indonesia (Figure 1). The variety of the M. charantia L plant was identified as Yunan F1varian, MC 2604**,** according to the Ministry of Agriculture Decree No.014/Kpts/SR.120/D.2.7/2/2015.

Figure 1. A dried sample of the identified *M. charantia* L plant

3.2 Extraction and Fractionation of M. charantia L. Fruit

The yield of *M. charantia* extract with different solvents (ethanol, ethyl acetate, *n*-hexane) resulted in a significant difference, as shown in Table. 1.

Table 1. The percentage yield of the various solvents of *M. charantia* L.

Solvent	Initial weight (Gram)	Final weight (Gram)	% Yield
Ethanol 96%	700	17.45	2.49
Ethylacetate	700	3.67	0.5
n-Hexane	700	0.19	0.027

The different yields of every solvent used indicated that the compound is more extracted using a semi-polar solvent (96% Ethanol) compared to the other solvents. The 96% ethanol extract was then used for the fractionation using an ethyl acetate solvent.

The evaporation result from the *M. charantia* L ethyl acetate fraction of *Momordica charantia* L is a condensed dark brown material similar to the condensed ethanol extract. The yield of the ethyl acetate fraction from the original ethanol extract is 6.97 %.

3.3 Quality of The M. charantia L Ethanol Extract

Quality requirements are all test parameters listed in the monograph and extract simplicia in question. A simplicial or extract cannot be said to be of quality according to the Indonesian Herbal Pharmacopoeia (FHI) if it does not meet the quality requirements. These quality requirements apply to simplicial and its extracts for health purposes and do not apply for other purposes. Table 2 shows the ethanol extract of *M. charantia* L quality assay that meets the quality requirements of "General Standard Parameters for Medicinal Plant Extract".

Table 2. Ethanol extract of *M. charantia* L quality assay

No	Test Parameters	Results
1.	Description	Thick extract, dark brown
2.	Total Aflatoxin	Not detected
3.	Arsen (As)	Not detected
4.	Cadmium (Cd)	Not detected
5.	Mercury (Hg)	0,02 ppm
6.	Lead (Pb)	Not detected
7.	Ash content	10,73 %
8.	Acid-insoluble ash content	6,46 %
9.	Water content	7,77 %
10.	Water-soluble compound levels	83,48 %
11.	Ethanol-soluble compound levels	34,44 %
12.	Ethanol solvent residual	0,18 %
13.	Dry yield	18,86 %
14.	Total Plate Count	5 CFU/gr
15.	Yeast Mold Content	< 10 CFU/gr
16.	Most Probable Number Coliform	< 3 APM/gr

3.4 Phytochemical Analysis
Phytochemical screening tests were performed to detect the presence of bioactive components in the mentioned plant extracts. Table 3 shows the phytochemical analysis for the extract of *M. charantia* L.

Table 3. Phytochemical analysis for the extract of *M. charantia* L. from the three solvents

Phytochemical compounds	Ethanolic Extract	Ethylacetate Extract	n-Hexane Extract
Alkaloids	+	+	-
Flavonoids	+	+	-
Saponin glycosides	+	-	-
Tannin	+	-	-
Quinone	+	-	-
Steroids	-	-	+
Triterpenoids	-	-	+

3.5 In Vitro Lymphocyte Cell Proliferation
Each extract was tested on lymphocyte cell proliferation. Figure 2 shows the absorbance values in lymphocyte cultures treated with the addition of ethanol extract, ethyl acetate, and n-hexane compared to normal control cells.

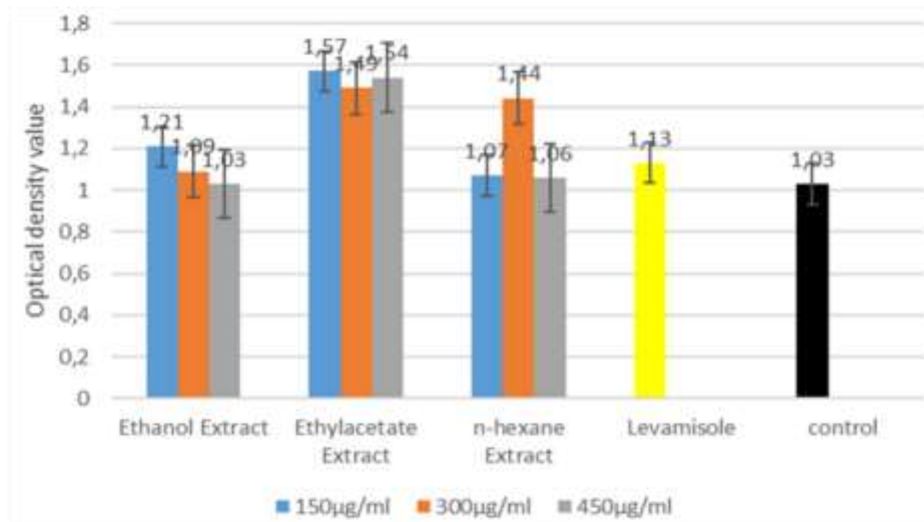

Figure 2. Profile of optical density value due to the addition of *M. charantia* extract on the lymphocyte cells proliferation. Results are expressed as mean ± SD.

Based on the in vitro result above, we decided that the ethanol extract of *M. charantia* L would develop to the fractionation using ethyl acetate solvent, and the resulting fraction would be used for the in vivo immunomodulatory assay.

3.6 In Vivo Phagocytosis Activity & Capacity

In the phagocytic activity (Figure 3), the highest activity was achieved at the dose of 50 mg/kg BW with an activity of 80.35%, followed by the dose of 100 mg/kg BW with an activity of 76.11%. This activity is a little bit lower compared to the control group of *E. purpurea,* with an activity of 83.16%. When compared within the treatment group, the dose of *M. charantia* extract at 25 mg/kg BW was the lowest, with an activity of 66.56%, but still high compared to the negative control group (59.90%). As shown in Figure 4, the negative control group and the treatment group had significantly different macrophage phagocytic activity ($p < 0.05$). This indicated that the *M. charantia* L fraction increased the phagocytosis activity of macrophages.

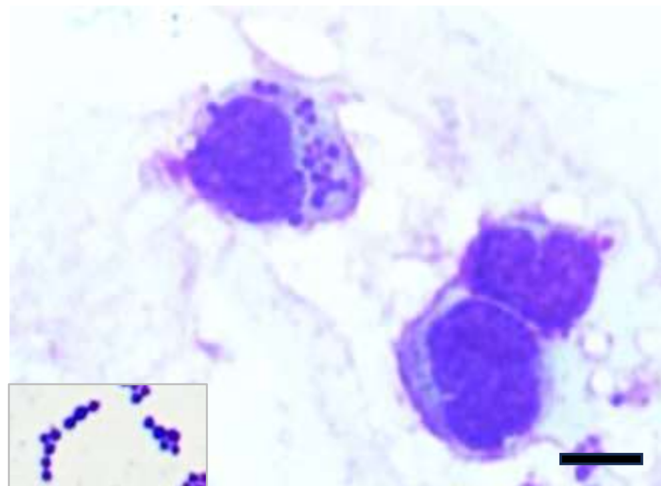

Figure 3. The phagocytic activity of macrophages. The active macrophage was engulfed *S. aureus* within the cytoplasm, while the other two macrophages are not active. Insert is the bacteria of *S. aureus* from the pure culture. Bar = 5 μm

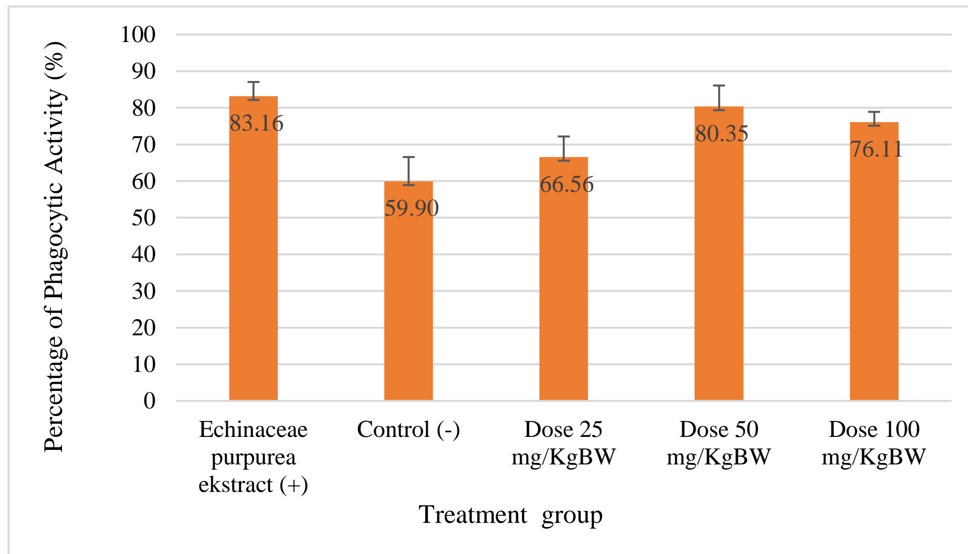

Figure 4. Percentage of the phagocytosis activity of the macrophage after administration of *M. charantia* fraction. Results are expressed as mean ± SD.

A similar pattern to that of phagocytosis activity was also observed in the phagocytic capacity of macrophage cells (Figure 5). Among the treated groups, the highest capacity was achieved at the dose of 50 mg/kg BW, followed by the dose of 100 mg/kg BW, and the lowest was at the dose of 25 mg/kg BW. The control positive of *E. purpurea* extract was the highest capacity compared to all treated groups.

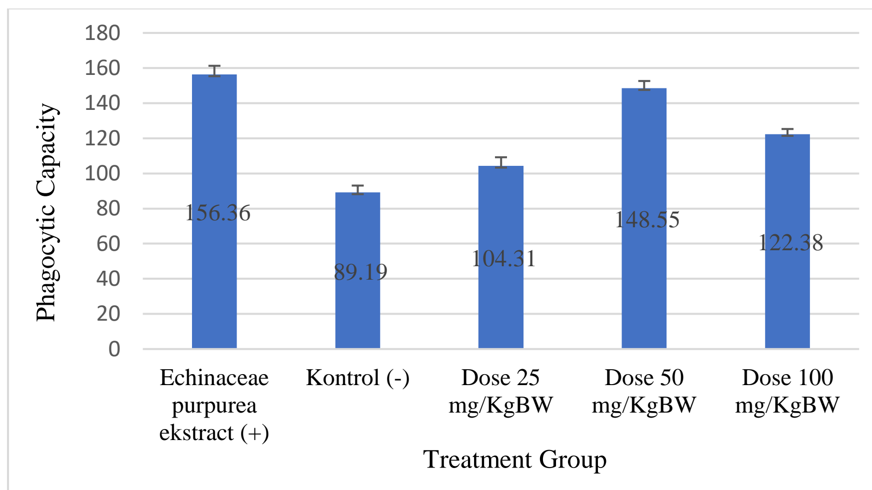

Figure 5. Phagocytosis capacity of peritoneal macrophage after exposure to the *M. charantia* ethyl acetate fraction. Results are expressed as mean ± SD.

The one-way ANOVA test results showed that the overall group on the macrophage phagocytosis activity and capacity had a statistically significant difference compared to the negative control group ($p < 0.05$). The administration of ethyl acetate fraction of *M. charantia* fruit at a dose of 50 mg/kg BW had a greater effect on the phagocytic activity and capacity of macrophages than the doses of 25 and 100 mg/kg BW.

3.7 White Blood Cell Count

On the white blood cell count among the treated groups, the highest average lymphocyte count was shown at the dose of 50 mg/kg BW (6.62%), while the lowest lymphocyte count was at the dose of 25 mg/kg BW (4.00%), 100 mg/kg BW (5.85%). Figure 6 shows the number of monocyte cells in the dose of 50 mg/kg BW yielded 1.27%, which did not differ from the normal control (1.22%). The lowest monocyte count was at a dose of 100mg/kg, followed by 25 mg/kg BW and the boost group (0.88%). For the neutrophils count, the highest number of neutrophils was achieved at a dose of 50 mg/kg BW (2.82%), followed by the control group (2.57%), at 100 mg/kg BW (2.02%), the boost (1.53%), and a dose of 25 mg/kg BW (1.38%). Furthermore, the highest number

of eosinophils happened in the *E. purpurea* extract group (0.43), followed by the control group (0.20%), the 50 mg/kg BW (0.22%), 25 mg/kg BW (0.17%), and a dose of 100 mg/kg BW (0.15%).

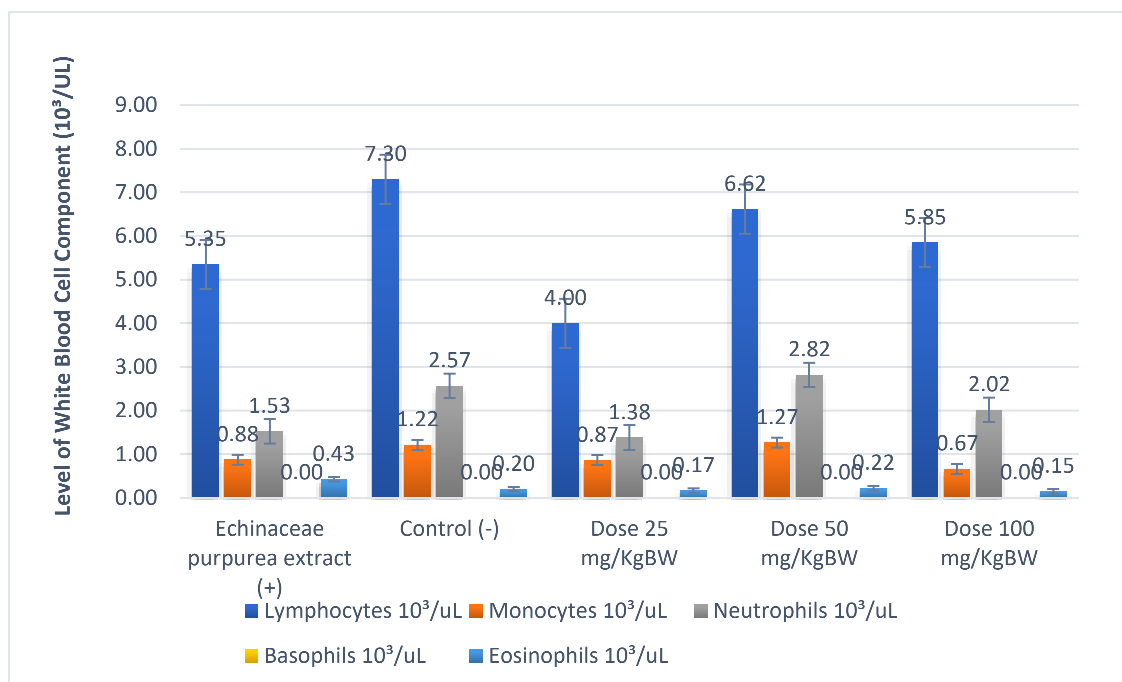

Figure 6. White blood cell counts after exposure to the *M. charantia* ethyl acetate fraction. Results are expressed as mean ± SD.

Among the treatment groups, the statistical analysis using a one-way analysis of variance showed that the effect of 50 mg/kg BW was significantly different ($P < 0.05$) for neutrophils, eosinophils, segment neutrophils, lymphocytes, and monocyte cells. Figure 6 showed a higher number of lymphocytes, monocytes, neutrophils, basophils, and eosinophils in the 50 mg/kg BW *M. charantia* extract compared to the 25 mg/kg BW, 100 mg/kg BW and *E purpurea* extract doses.

4. Discussion

The yields of *M. charantia* ethanol extract were higher than those of ethyl acetate and n-hexane. The variation in the yields was attributed to the difference in solvent polarities used and increased solubility of phytochemical compounds (Ribeiro et al. 1991; Ang et al. 2014). Furthermore, the differences in the structure of phytochemical compounds also determine their solubility in solvents of different polarities (Table 3). The phytochemical screening showed the ethanol extract of *M. charantia* contains chemical groups of alkaloids, flavonoids, saponin glycosides, tannins, and quinones. The ethyl acetate extract contains chemical groups of alkaloids and flavonoids, while the n-hexane extract contains chemical groups of steroids and triterpenoids. It is suspected that the immunomodulatory effect of the ethyl acetate extract of *M. charantia* is due to the content of alkaloids and flavonoids (Table 3). *M. charantia* L. is known to have both immunosuppressive and immunostimulant activities (Mahamat et al., 2020). Plant fruits were demonstrated to promote the phagocytic activity and activation of splenocytes (Tiwari et al. 2004; Ang et al. 2014). Several bioactive compounds of *M. charantia* fruit have been recorded in the literature. *M. charantia* contains triterpenoids (Grover and Yadav 2004; Liu et al. 2009), saponins (Xianyuan et al. 2001; Ma et al. 2014), polypeptides (Deng et al. 2014), flavonoids (Grover and Yadav 2004).

Immunomodulation, through stimulation or suppression, maintains a disease-free state. The agents of host defense mechanisms activators in the presence of impaired immune responsiveness provide supportive therapy to conventional chemotherapy (Beutler 2004; Mirshafiey et al. 2004). Furthermore, there has been a growing interest in identifying herbal immunomodulators ever since their possible use in modern medicine was suggested (Eze et al., 2014). In this study, the immunomodulatory effect of *M. charantia* was conducted in vitro by MTT reduction test at 96-well multiple. Furthermore, the power is determined based on the ability of cells to proliferate when *M. charantia* fruit extract is given, which acts as an immunostimulant. Figure 2 shows all groups of extracts capable of providing an immunomodulatory effect with lymphocyte cell proliferation. The ethyl acetate extract group of *M. charantia* had a higher immunomodulatory effect than that of ethanolic and n-hexane of *M. charantia*. This was evident from the higher OD value compared to the ethanolic and n-hexane extracts, control, and levamisole groups. Levamisole is a heterocyclic compound that is an effective anthelminthic agent and is also immunoregulatory. Its likely immuno-regulatory mode of action is by mimicry of the thymic hormone thymopoietin (Goldstein 1978).

In the observation between concentration groups, the highest OD results were found at a concentration of 150 μg/mL compared to that of 300 and 450 μg/mL; this is probably because concentrations of 300 μg/mL and 450 μg/mL are more immunosuppressant (Figure 2). *In vivo,* immunomodulatory activity level showed that the dose of ethyl acetate fraction (50 mg/kg BW) had a better immunomodulatory effect compared to the dose of 25 mg/kg BW and 100 mg/kg BW and was comparable to the *E. purpurea* extract which was shown by the increased phagocytic activity and capacity of macrophages (Figure 4 and 5). The process of phagocytosis by macrophages includes opsonization of the foreign particulate with antibodies and complement C3b, leading to the more rapid clearance of foreign particulate matter from the blood. The extract of *M. charantia* L., although produced dose-dependently, increased phagocytic activities, but no significant value was obtained with selected doses. However, higher doses could seem to have potentiated the phagocytic activity, and a higher dose of 100 mg/kg BW was more likely to seem immunosuppressant. The ability of its activation increases morphologically, metabolically, and functionally in eliminating infectious agents in the body. This is characterized by increased activity, phagocytic capacity, and interleukin production (Ribeiro et al. 1991; Ang et al. 2014). The activation of these macrophages has a high ability to ingest foreign objects through the process of phagocytosis. These cells destroy all foreign objects, such as germs, damaged cells, tumor cells, colloidal materials, and large molecules (Besung et al. 2016). Furthermore, the results in Figures 2, 4, 5 and supported by Figure 6, where the ethyl acetate fraction *M. charantia* group at a dose of 50 mg/kg BW showed a higher number of lymphocytes, monocytes, neutrophils, basophils, and eosinophils compared to that of 25 mg/kg BW, 100 mg/kg BW and *E. purpurea* extract. Immunologically, lymphocytes are competent cells and assist phagocytes in the body's defense against infection and other foreign invasions. These results showed that an increase in the granulocyte component has a strong correlation with phagocytic activity. These components are non-specific immune systems, and they consist of neutrophils, basophils, and eosinophils. The increased levels of neutrophils in the peripheral blood are strongly correlated with phagocytic activity. They are components of leukocytes, also known as polymorphonuclear (PMN), because of their various shapes. Furthermore, it is the most common leukocyte in peripheral blood and the first line of defense against infectious pathogens, such as bacteria, fungi, and protozoa. Neutrophils are the first leukocytes to migrate from the blood to the infected area to kill and phagocytose pathogens (Chanana et al. 2007; Besung et al. 2016).

This study has proved that the fraction of *M. charantia L* has an immunomodulatory effect based on the results of Mahamat et al. (2020), where diethyl ether and methanol extracts from *M. charantia* leaves had an effect on macrophages and neutrophils in a mouse model infected with *Salmonella typhi*. This was indicated by a significant increase in titers, antibodies, and mobilization of leukocytes, especially lymphocytes, at concentrations of 500 and 1000 mg/kg. Furthermore, Deng et al. (2014) found that intragastric administration of MCP 150 or 300 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ significantly increased carbolic particle clearance index, serum hemolysin production, spleen index, thymus index, and NK cell cytotoxicity to normal control levels in mice induced with cyclophosphamide (Cy). In addition, *M. charantia* is an effective immunomodulator due to its secondary metabolites, such as alkaloids, flavonoids, tannins, saponins, and triterpenoids. The mechanisms of the alkaloids and flavonoids are similar to those in other plants. Generally, flavonoid compounds can repair the immune system, while alkaloids have an immunomodulatory ability (Parawansah et al., 2018). Previous studies showed that flavonoid compounds can stimulate lymphocyte proliferation and increase the T-cell count and interleukin-2 (IL-2) activity. Flavonoids also work against lymphokines produced by T cells that will stimulate phagocytes to respond to phagocytosis, one of which is macrophages (Wagner and Proksch 1985).

5. Conclusion

The ethyl acetate extracts and fraction of *M. charantia,* which contains alkaloids and flavonoids, have immunomodulatory activity by inducing phagocytic activity and capacity of macrophages and white blood cell differentiation in vitro and in vivo. Therefore, alkaloids and flavonoids in *M. charantia* are expected to be responsible for the immunomodulatory activity of *M. charantia*. However, this result needs to be confirmed by the isolation of the pure compounds from *M. charantia* to elucidate their role and mechanism.

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References

[1] Abbas AK, Lichtman AH and Pilai S. (2007) Antibodies and antigen. Philadelphia: WB Saunders.

[2] Ang HY, Subramani T, Yeap SK, Omar AR, Ho WY, Abdullah MP and Alitheen NB. (2014). Immunomodulatory effects of *Potentilla indica* and *Dendrophthoe pentandra* on mice splenocytes and thymocytes. Exp Ther Med. 7(6):1733–1737. doi: 10.3892/etm.2014.1657.

[3] Backer CA, van den Brink Jr RCB. (1968) Flora of Java (Spermatophytes Only) - Vol. III. Groningen: Wolters-Noordhoff N.V.

[4] Bafna AR and Mishra SH. (2004). Immunomodulatory activity of methanol extract of flower-heads of *Sphaeranthus indicus* Linn. Ars Pharm. 45(3):281–291.

[5] Barnes J, Anderson LA, Gibbons S and Phillipson JD. (2005). Echinacea species (*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt., *Echinacea purpurea* (L.) Moench): a review of their chemistry, pharmacology and clinical properties. J Pharm Pharmacol. 57(8):929–954. doi: 10.1211/0022357056127.

[6] Besung INK, Astawa NM, Suata K and Suwiti NK. (2016). Relationship between the macrophage activity with interleukin-6 levels and titers of antibodies against *Salmonella typhi*. J Kedokt Hewan - Indones J Vet Sci. 10(1):1–4. doi: 10.21157/j.ked.hewan.v10i1.3359.

[7] Beutler B. (2004) Inferences, questions, and possibilities in Toll-like receptor signaling. *Nature. 430*(6996):257–263. doi: 10.1038/nature02761.

[8] Chanana V, Ray P, Rishi DB and Rishi P. (2007). Reactive nitrogen intermediates and monokines induce caspase-3 mediated macrophage apoptosis by anaerobically stressed *Salmonella typhi*. Clin Exp Immunol. 150(2):368–374. doi: 10.1111/j.1365-2249.2007.03503.x.

[9] Deng YY, Yi Y, Zhang LF, Zhang RF, Zhang Y, Wei ZC, Tang XJ and Zhang MW. (2014) Immunomodulatory activity and partial characterisation of polysaccharides from *Momordica charantia*. Molecules. 19(9):13432–13447. doi: 10.3390/molecules190913432.

[10] Dwijayanti DR, Okuyama T, Okumura T, Ikeya Y and Nishizawa M. (2019). The anti-inflammatory effects of Indonesian and Japanese bitter melon (*Momordica charantia* L.) fruit extracts on interleukin-1β-treated hepatocytes. Funct Foods Heal Dis. 9(1):16–33. doi: 10.31989/ffhd.v9i1.560.

[11] Dwijayanti DR, Shimada T, Ishii T, Okuyama T, Ikeya Y, Mukai E and Nishizawa M. (2020). Bitter melon fruit extract has a hypoglycemic effect and reduces hepatic lipid accumulation in ob/ob mice. Phytother Res. 34(6):1338–1346. doi: 10.1002/ptr.6600.

[12] Eze C, Shoyinka V, Okoye J, Ezema W, Ogbonna I, Eze D, Okwor E and Ikejiofor O. (2014) Comparison of the serum proteins and immune responses of velogenic Newcastle disease virus infected chickens and ducks. Open J Vet Med. 4(6):122–128. doi: 10.4236/ojvm.2014.46014.

[13] Goldstein G. (1978) Mode of action of levamisole. J Rheumatol Suppl. 4:143–148.

[14] Grover JK and Yadav SP. (2004). Pharmacological actions and potential uses of *Momordica charantia*: a review. J Ethnopharmacol. 93(1):123–132. doi: 10.1016/j.jep.2004.03.035.

[15] Liu J-Q, Chen J-C, Wang C-F and Qiu M-H. (2009). New cucurbitane triterpenoids and steroidal glycoside from *Momordica charantia*. Molecules. 14(2):4804–4813. doi: 10.3390/molecules14124804.

[16] Ma L, Yu AH, Sun LL, Gao W, Zhang MM, Su YL, Liu H and Ji T. (2014). Two new bidesmoside triterpenoid saponins from the seeds of *Momordica charantia* L. Molecules. 19(2):2238–2246. doi: 10.3390/molecules19022238.

[17] Mahamat O, Christopher T, Gilbert A, Teke Gerald N and Albert K. (2018). Immunological in vivo and in vitro investigations of aqueous extract of stem bark of *Pterocarpus erinaceus* Poir (Fabaceae). Am J Med Sci. 356(1):56–63. doi: 10.1016/j.amjms.2018.02.006.

[18] Mahamat O, Flora H, Tume C and Kamanyi A. (2020). Immunomodulatory activity of *Momordica charantia* L. (Cucurbitaceae) leaf diethyl ether and methanol extracts on *Salmonella typhi*-infected mice and LPS-induced phagocytic activities of macrophages and neutrophils. Evid Based Complement Alternat Med. 2020:5248346. doi: 10.1155/2020/5248346.

[19] Mirshafiey A, Rehm BHA, Sahmani AA, Naji A and Razavi A. (2004). M-2000, as a new anti-inflammatory molecule in treatment of experimental nephrosis. Immunopharmacol. Immunotoxicol. 26(4):611–619. doi: 10.1081/IPH-200042362.

[20] Parawansah P, Nurtamin T, Mulyawati SA, Nuralifah N and Misnaeni WOA. (2018). Immunomodulatory effect of *Momordica charantia* L. fruit ethanol extract on phagocytic activity and capacity of mice peritoneal macrophages. Indones Biomed J. 10(2):144–147. doi: 10.18585/inabj.v10i2.390.

[21] Ribeiro RA, Flores CA, Cunha FQ and Ferreira SH. (1991). IL-8 causes in vivo neutrophil migration by a cell-dependent mechanism. Immunol. 73(4):472–477.

[22] Tiwari U, Rastogi B, Singh P, Saraf DK and Vyas SP. (2004). Immunomodulatory effects of aqueous extract of *Tridax procumbens* in experimental animals. J Ethnopharmacol. 92(1):113–119. doi: 10.1016/j.jep.2004.02.001.

[23] Wagner H and Proksch A. (1985). Immunostimulatory drugs of fungi and higher plants. In: Wagner H, Hiroshi H, Farnsworth NR, editors. Economic and medicinal plant research. London: Academic Press; p. 113–153.

[24] Xianyuan W, Hong J, Zhiqin X and Lanxing G. (2001). Effects of *Momordica charantia* L. saponins on immune system of senile mice. Nutr Sin. 23(3):263–266