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Immunomodulatory Activity of Parijoto Fruit (*Medinilla speciosa*) on Macrophage Phagocytosis, Lymphocyte Proliferation, and IgG Production

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Abstract
Parijoto fruit (*Medinilla speciosa*) contains flavonoid compounds that are potentially immunomodulators because they increase macrophage phagocytosis activity and lymphocyte proliferation in vitro. This study aims to determine the phytochemical compound content of parijoto fruit and its potential as an immunomodulatory agent in vivo. Parijoto fruit was extracted using 70% ethanol solvent. The ethanol extract of parijoto fruit (EEPF) was qualitatively identified by phytochemical screening. The immunomodulatory activity test in vivo was conducted using BALB/c mice, which were separated into five groups: untreated, *Phyllanthus niruri* L. (PN) extract (positive control), and test groups (125mg/KgBW; 250mg/KgBW and 375mg/KgBW). The treatment duration was 18 days, with the induction of the hepatitis B vaccine on days 7 and 14. An immunomodulatory activity test was conducted on the 19th day, which included a macrophage phagocytosis activity test, lymphocyte proliferation, and measurement of IgG levels. The identification results showed that EEPF contains flavonoids, tannins, and saponins. Macrophage phagocytosis activity showed that EEPF increased macrophages' phagocytosis capacity (PC) and phagocytosis index (PI). EEPF increased lymphocyte proliferation activity and IgG levels, significantly different from control and not substantially different from positive control. Collectively, EEPF increases immunomodulatory activity *in vivo*.

Keywords: IgG, Immunomodulator, in vivo., lymphocyte, macrophage, Medinilla speciosa

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Aktivitas Imunomodulator Buah Parijoto (*Medinilla speciosa*) terhadap Fagositosis Makrofag, Proliferasi Limfosit dan Produksi IgG

Abstrak
Buah parijoto (*Medinilla speciosa*) mengandung senyawa flavonoid yang berpotensi sebagai imunomodulator karena mampu meningkatkan aktivitas fagositosis makrofag dan proliferasi limfosit secara *in vitro*. Tujuan dari penelitian ini adalah untuk mengetahui kandungan senyawa fitokimia buah parijoto dan potensinya sebagai agen imunomodulator secara *in vivo*. Buah parijoto diekstraksi menggunakan pelarut etanol 70%. Ekstrak etanol buah parijoto (EEPF) diidentifikasi secara kualitatif dengan skrining fitokimia. Uji aktivitas imunomodulator dilanjutkan dengan menggunakan mencit galur BALB/c yang dibagi menjadi lima kelompok: kontrol tanpa perlakuan, kontrol positif (ekstrak *Phyllanthus niruri* L.), dan kelompok uji (125mg/KgBB, 250mg/KgBB, dan 375mg/KgBB). Durasi perlakuan adalah 18 hari, dengan induksi vaksin hepatitis B pada hari ke-7 dan 14. Uji aktivitas imunomodulator dilakukan pada hari ke-19 yang meliputi uji aktivitas fagositosis makrofag, proliferasi limfosit, dan pengukuran kadar IgG. Hasil identifikasi menunjukkan bahwa EEPF mengandung flavonoid, tanin, dan saponin. Aktivitas fagositosis makrofag menunjukkan EEPF meningkatkan kapasitas fagositosis (PC) dan indeks fagositosis (*phagocytosis index*/PI) makrofag. Perlakuan dengan EEPF mampu meningkatkan aktivitas proliferasi limfosit dan kadar IgG, yang berbeda bermakna dengan kontrol normal dan tidak berbeda bermakna dengan kontrol positif. Berdasarkan temuan tersebut, EEPF dapat meningkatkan aktivitas imunomodulator *in vivo.*

Kata Kunci: IgG, imunomodulator, *in vivo*, limfosit, makrofag, *Medinilla speciosa*

1. Introduction

Indonesia is a country with the 7th most diverse flora in the world. This flora diversity is fascinating to study, especially in the health sector. In recent years, research on herbs has increased along with the increasing public interest in herbal medicine.[1] Parijoto plant (*Medinilla speciosa* (Reinw.ex Bl) is one type of herbal plant widely cultivated in the Mount Muria area, Kudus City, Central Java. Previous studies have reported that the phytochemical screening from ethanol extract of parijoto fruit contains flavonoids, alkaloids, tannins, and saponins.[2-3] Previous in vitro research showed that flavonoid compounds in parijoto fruit could increase immunomodulatory activity through increased response in lymphocyte cells and trigger cell division, resulting in proliferation.[4,5] In addition to being an immunomodulator, other studies have also mentioned that parijoto fruit has the potential to be antioxidant and antidiabetic. As an antidiabetic, the ethyl acetate fraction of parijoto fruit can reduce glucose levels optimally with a percentage of 50.21 ± 0.74%. The results of antioxidant activity testing with IC50 showed that the flavonoid content in parijoto fruit (ethyl acetate fraction) has an IC_{50} value of 4.14 ± 0.08 ppm, which means it can be a powerful antioxidant. Antioxidants in the immune system function to counteract free radicals.[3] An immunomodulator is a compound that enhances the functionality of the human immune system and is classified into immunostimulants that increase the body's infection resistance by increasing the primary immune response level, while immunosuppressants work the other way around, suppressing the work of the immune system.[6]

The mechanism of immunomodulatory activity of parijoto fruit ethanol extract is by increasing IL-12 activity and lymphocyte proliferation. Increased IL-2 production by T helper 1 (Th1) cells affects SMAF (specific macrophage activating factor), including cytokines like IFN-γ, which activate macrophages. Macrophage activation will increase phagocytosis activity quickly and efficiently and produce nitric oxide to kill bacteria.[7,8] Currently, no *in vivo* research is related to the immunomodulatory activity of parijoto fruit. Therefore, this study was conducted to acquire empirical evidence of the benefits of parijoto fruit as an immunomodulator *in vivo*. This research aims to elucidate the advantages of parijoto fruit within the community as an immunomodulator.

2. Method

2.1. Tools and Materials

The tools used were glassware, powder machine (Germany), porcelain dish, analytical balance (Sartorius®, Germany), oven (Memmert®, Germany), water bath, micropipette (Socorex®, Switzerland), vortex (Shimadzu®, Japan), stainless steel tweezers, stainless steel scissors, Class II Biosafety Cabinet (Esco®, North America), 24-well multiwell plate, petri dish, 96-well multiwell plate (Nunc®, UK), microplate reader (Bio-rad Benchmark®, Japan), coverslip (2001212mM) (SPL®, Korea), 5% CO_2 incubator (Heraeus®, Germany), injection syringe (Terumo®, Japan), inverted microscope (Olympus®, Germany, centrifuge (Sorvall®, America), hemocytometer (Neubauer®, Germany), IgG (total) mouse uncoated ELISA Kit (Thermo Fisher Scientific®, America).

The materials used were parijoto fruit, BALB/c male mice, 70% ethanol, fetal bovine serum (FBS) (Gibco®, South America), DMSO, MTT [3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium- bromide] (Merck®, Germany), phosphate-buffered serum (PBS), sodium dodecyl sulfate (SDS) stopper reagent (Merck®, Germany), hepatitis B vaccine (Biofarma®, Indonesia), tris-ammonium chloride Buffer, Phyllanthus niruri Linn. (PN) extract (Stimuno®), RPMI (Rosewell Park Memorial Institute) 1640 medium (Sigma-Aldrich®, Germany), Giemsa (Merck®, Germany), latex (3 mm) (Sigma- Aldrich®, Germany), 95% ethanol, Aquabidest, HCl, Mayer's reagent, Dragendorff reagent, Wager reagent, NaOH, $FeCl_3$, n-hexane, and Liebermann-Burchard reagent.

2.2. Methods

2.2.1. Extraction

Briefly, 550 mg of parijoto fruit powder was macerated using 5.5 L of 70% ethanol. The maceration process was conducted over five days at ambient temperature and shielded from direct sunshine. The macerate was evaporated with a rotary evaporator and water bath at 60°C to obtain ethanol extract of parijoto fruit (EEPF).[4]

2.2.2. Phytochemical Screening

Alkaloid Test

A 50 mg extract was dissolved in a few mL hydrochloric acid and filter. Later, it was added with 1 or 2 drops of Mayer, Wagner, or Dragendorff reagent. A positive reaction (+) was identified by the appearance of a white or yellowish precipitate on the Mayer reagent, a red-black color on the Wagner reagent, and an orange precipitate on the Dragendorff reagent.[9]

Flavonoid Test

The extract solution was poured into a test tube, and

up to two drops of 10% NaOH were added and agitated briskly. The test is positive if the solution shifts to yellow, red, or brown.[9]

Tannin Test

The extract was dissolved in distilled water and placed in a test tube. Then, 2-3 drops of a 1% $FeCl_3$ solution were added. The reaction was said to be positive if it produced a blackish-green or ink-blue color containing tannin.[9]

Steroid Test

The extract was added with ten drops of acetic acid, two drops of concentrated hydrogen peroxide, and 0.5 grams of the extract. After giving the solution a light shake, it was allowed to sit for a few minutes. A positive solution was indicated if the solution turned blue or green color.[9]

Saponin Test

One drop of HCl 2 N was added to the extract dissolved in hot distilled water. The test was positive if foam was formed and lasted more than 5 minutes.[9]

2.2.3. In Vivo Immunomodulatory Activity Test

Animal Conditioning and Treatment

his study used 30 BALB/c mice aged 2-3 months with ethical approval from Health Research Ethics Committee No. 116/EC-KEPK FKIK UMY/VIII/2022. BALB/c mice were adapted for one week before treatment. The temperature of the cages was kept between 28 and 32°C, while the relative humidity was maintained at 98%. Mice were given adequate feed and RO (reverse-osmosis) water *ad libitum*. The test animals were kept in cages with husk bedding, which was replaced every three days. BALB/c mice were allocated into five dosage groups, each comprising six mice. One group served as the normal control (baseline), whereas three groups received dosages of 125, 250, and 375 mg/KgBW (p.o), and one group was administered *Phyllanthus niruri* L. (PN) extract (Stimuno®) at a dose of 0.585 mg/KgBW (p.o). The treatment was administered for 18 days. All mice received the hepatitis B vaccine (intraperitonial) on days 7 and 14. The activity was ascertained on day 19.[10-11]

Macrophage Activity Assay

Macrophages were extracted from the peritoneal fluid of mice by the addition of 10 mL of cold RPMI 1640 into the peritoneal cavity while gently shaking for 5 minutes with the aim that macrophage could be released and suspended in the media. Approximately 1 mL of the entire medium was administered. The cells were enumerated and suspended in a medium to achieving a 2.5×10^6 cells/mL concentration. The cell suspension was inoculated into 24-well plates with glass coverslips and incubated at 37°C in 5% CO_2 for 30 minutes. Then, 1 mL of complete media was added to the wells and incubated for 2 hours. After pouring the medium, the cells were washed twice with RPMI 1640, after which 1 mL of complete media was introduced and incubated for 24 hours. Latex beads were suspended in phosphate-buffered saline (PBS). The macrophage culture underwent two washes with RPMI 1640. Two hundred microliters of latex bead suspension per well was applied and incubated in a 5% CO_2 incubator at 37°C for 60 minutes. Later, the cells were fixed with methanol for 30 seconds, followed by evaporation of the methanol. After that, it was allowed to dry, followed by a 30-minute application of 2% Giemsa stain, rinsed with distilled water, and then allowed to dry again. The phagocytic activity of macrophages was quantified as phagocytic capacity and phagocytic index. The quantity of latex phagocytosed by macrophages was quantified under a microscope.[11]

$$\text{Phagocytosis Activity} = \frac{\text{Number of activated macrophages}}{\text{Total macrophage count}} \times 100\%$$

$$\text{Phagocytosis index} = \frac{\text{Number of phagocytosed latex}}{\text{Number of activated macrophages}}$$

Lymphocyte Proliferation Assay

Lymphocytes were extracted from mice and transferred to a petri dish with RPMI 1640 medium. The suspension was centrifuged at 3200 rpm and 4°C for 4 minutes. The pellet was isolated from the supernatant and resuspended in tris-buffered ammonium chloride, then maintained at room temperature for 15 minutes.

The RPMI medium was used to create a suspension, followed by a 4-minute centrifugation, after which the supernatant was removed, after a second wash with RPMI, suspended in a complete medium. Cells were incubated in 5% CO_2 at 37°C. Following the suspension of the cells in full press, 100 µL per well of the suspension was added to a 96-well plate. The plate was incubated in a CO_2 incubator at 37°C for 72 hours. Following incubation, 10 mL of 5 mg/mL MTT solution in sterile PBS was added and incubated for 4 hours. Subsequently, 100 µL of stopper solution (10% SDS in 0.01 N HCl) was incorporated. Following the overnight incubation, the cells were measured for absorbance using a microplate reader at a wavelength of 550 nm.[11]

The results of the lymphocyte proliferation test were calculated by measuring the absorbance value, which

will be used to assess phagocytosis activity. The greater the optical density (OD) value generated, the more live cells were contained in the lymphocyte cell culture results.[12]

Measurement of Immunoglobulin G Level

A 0.5 mL blood specimen was extracted from the orbital vein of the eye. The sample was centrifuged at 5000 rpm for 10 minutes, after which the serum was recovered and stored at -20°C until required. Subsequent antibody titer was quantified with an ELISA IgG Kit and subsequently assessed with an ELISA reader at a wavelength of 450 nm. The optical density (OD) was used to calculate the antibody concentration in the serum. IgG antibody levels were calculated using a linear regression equation obtained from the IgG antibody standard curve.

2.2.4. Statistical Analysis

The mean ± standard error was used to characterize the results. The statistical analysis was conducted using a one-way ANOVA with post-hoc Tukey's HSD and a significance level of $p<0.05$.

3. Result

The findings produced by the phytochemical analysis of the ethanol extract of parijoto fruit were known to contain flavonoids, tannins, and saponins (Table 1).

Two assessment parameters are used in the macrophage phagocytosis activity test: phagocytosis capacity and phagocytosis index. Macrophage phagocytosis capacity (PC) is the number of macrophages that can phagocytose latex particles. Meanwhile, the macrophage phagocytosis index (PI) is the quantity of latex phagocytosed by active macrophages.[14]

The phagocytic activity of macrophages *in vivo* was known by an increase in the value of macrophage phagocytic capacity and macrophage phagocytosis index. Figure 1 showed that administering ethanol extract of parijoto fruit (EEPF) with various doses to test animals increased phagocytosis capacity and index compared to untreated and positive control. Phagocytosis capacity in the 125mg/KgBW, 250 mg/KgBW, and 375 mg/KgBW EEPF treatment groups was 58.67 ± 5.51%; 86.00 ± 1.00%; and 95.33 ± 0.58%, respectively. The administration of EEPF 250 mg/KgBW and 375 mg/KgBW showed better macrophage cell activation ability than the positive control (82.67 ± 7.51%). The administration of extract also increased the phagocytosis index of macrophages. The administration of EEPF 250 mg/KgBW (2.22 ± 0.08) and 375 mg/KgBW (2.73 ± 0.17) was able to increase the phagocytosis index better than the positive control (2.06 ± 0.10) (Figure 2).

Compared to the standard control, the number of optical density (OD) values increased in the EEPF treatment group. The untreated group had the lowest OD value of 0.1505 ± 0.0306, while the positive control had the highest OD value of 0.3886 ± 0.0815 (Figure 3). The administration of EEPF showed an increase in OD value at various doses, namely 0.1684 ± 0.0111 (EEPF 125 mg/KgBW), 0.2190 ± 0.0130 (EEPF mg/KgBW) and 0.3241 ± 0.0302 (EEPF 375 mg/KgBW). Parijoto fruit ethanol extract increased lymphocyte proliferation activity, although not as good as the positive control. In previous research in vitro, parijoto fruit ethanol extract was also shown to have lymphocyte proliferation activity, with the results of research giving EEPF concentrations of 125 ug/mL and 500 ug/mL giving positive results 67 increasing lymphocyte proliferation activity with stimulation index values of 3.63 ± 0.07 and 3.58 ± 0.16.[4]

The last test was the measurement of immunoglobulin G levels. Immunoglobulin G (IgG) is an antibody formed from antigen stimulation of B lymphocyte cells. Antigen stimulation was given in the form of hepatitis B vaccine vaccination through intraperitoneal administration. This stimulation causes B cells to undergo two development processes. First, B cells differentiate into plasma cells that form IgM (primary immune response). Then, when given a second antigen exposure, memory B cells will cause a faster reaction than

Table 1. Phytochemical Screening Results in Ethanol Extract of Parijoto Fruit

No	Metabolite Compounds	Testing Results	Changes that Occur
1	Alkaloid		
	- Mayer	-	White, white precipitate
	- Dragendorff	-	Orange red
	- Wagner	-	Chocolate
2	Flavonoid	+	Red brown
3	Steroid	-	Blue or green
4	Saponin	+	Dark blue or black
5	Tanin	+	Formed froth

Note: (+) metabolite compounds were present, and (-) metabolite compounds were undetected.

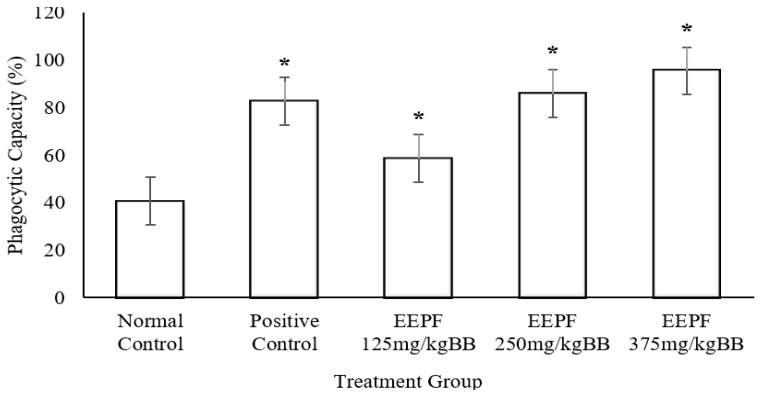

Figure 1. Phagostiosis capacity of macrophages after treatment with ethanol extract of parijoto fruit. The data was expressed as mean ± SD from 6 mice (*=p<0.05 compared to control group)

the first reaction and cause B cells to proliferate into plasma cells that will secrete specific Ig, namely IgG, giving rise to a secondary immune response.[10]

There was no significant difference in the number of antibodies produced by the treatment group compared to the group that served as the positive control. The lowest IgG level was the untreated with 149.791 ± 8.703. The highest level was the positive control, which was 202.967 ± 16.734. These results are not significantly different from EEPF 125 mg/KgBW, 250 mg/KgBW, and 375 mg/KgBW, which have levels respectively 186.761 ± 21.667, 197.118 ± 40.343 and 201.873 ± 21.001 (Figure 4), hence it can be concluded that the ability of parijoto fruit ethanol extract in increasing immunoglobulin G levels is equivalent to positive control.

4. Discussion

Parijoto fruit ethanol extract has been known to contain flavonoids, tannins, saponins, and alkaloids.13 Hence, the current study assessed the immunomodulatory activity based on macrophage phagocytosis, lymphocyte proliferation activity, and Immunoglobulin G levels. The macrophage phagocytosis activity test is one of the immunological parameters to assess immune function. Macrophage cells are phagocytic cells that play a role in counteracting pathogen attacks as a natural immune or adaptive response through phagocytosis, an elimination process from swallowing to destroying pathogens entering the body.[14]

The results of this study were directly comparable to previous research in vitro; the content of flavonoid compounds in methanol extracts of parijoto fruit has the potential to become immunomodulators of immunostimulant groups with the highest phagocytosis capacity value at a dose of 1000 µg/mL which is 61.50% and 74.00%. The highest phagocytosis index value occurs at a concentration of 500 µg/ml with a value of 2.53 and 3.16.[5] Research on other herbal plants as immunomodulators has stated that the flavonoid compounds present in red betel leaf extract (Piper scrotum) can increase the phagocytic activity of macrophages. Parijoto fruit also contains flavonoid compounds based on phytochemical screening. Flavonoid compounds cause an increase in IL-2 activity. Increased production of IL-2 by T helper one cells affects SMAF, for example, cytokines such as IFN-γ, which then activates macrophages. Macrophage activation will increase nitric oxide production, which kills bacteria.[8] Another study also mentioned that much evidence shows that flavonoids significantly increase phagocytosis in mouse macrophages both in vitro and in vivo. Liquiritigenin 7-O-glucuronide and genistein 7-O-glucuronide are types of flavonoids that greatly enhance phagocytosis of mouse macrophages, as well as increase the expression of functional receptors (CD88 and CD192)

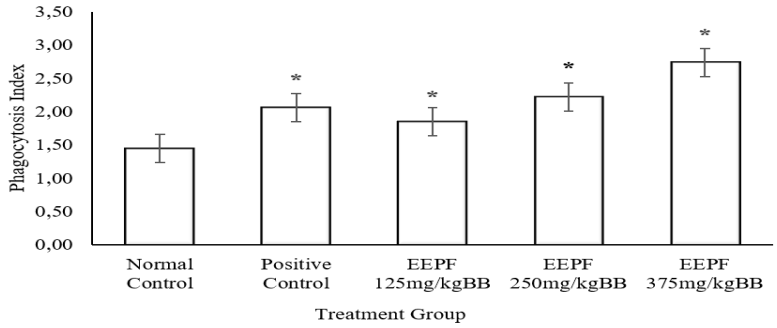

Figure 2. Macrophage phagocytosis index after treatment with ethanol extract of parijoto fruit. The data was expressed as mean ± SD from 6 mice (*=p<0.05 compared to control group)

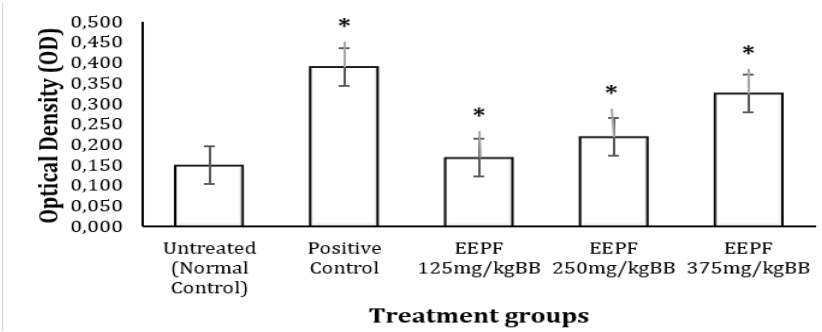

Figure 3. Lymphocyte proliferation index (PI) after treatment with ethanol extract of Parijoto fruit. The data was expressed as mean ± SD from 6 mice (* = p<0.05 compared to untreated group)

on macrophages in vitro.[15] Lymphocyte proliferation is the lymphocyte response to antigen stimuli, explicitly recognizing and responding to foreign antigens and acting as a mediator of humoral and cellular immunity. The antigen stimulus given is the administration of the hepatitis B vaccine. This vaccination stimulates an immune response; the first will trigger a primary immune response, and the second will trigger a secondary immune response.[11]

Another study on herbal plants stated that flavonoid glycoside compounds isolated from the *Alchornea floribunda* plant can modulate intracellular IFN-gamma and IL-2 expression in spleen T lymphocyte cultures. Results showed that stimulation with all compounds increased the proportion of $CD8^+/IFNg^+$ and $CD4^+/IFNg^+$ T lymphocytes up to twofold compared to the control.[16]

In other studies, natural materials containing flavonoid compounds are known to have the ability to increase immunoglobulin G. Ethanol extract of parijoto fruit is also known to contain flavonoid and phenolic compounds. Flavonoid compounds can increase the production of T helper one cell and produce IL-2, producing the cytokine IFN-γ, which causes differentiation and proliferation of B lymphocytes, which produce antibodies.[17,18] Another study mentioned that the types of flavonoid compounds that can modulate the proliferation of B lymphocyte cells produced by the immune system are villain, astilbin, and quercetin.[19] Phenolic compounds function as antigens and are recognized by B and T cell receptors. The interaction of the antigen with the T cell surface receptor and interleukin 1 (IL-1) from the antigen-presenting cell (APC) activates G-proteins, producing phospholipase C. This process involves the expression of protein kinase C and 5-lipoxygenase enzymes. Protein kinase C increases interleukin-2 (IL-2) production, which in turn activates the proliferation of B and T cells.[20]

Elevating antibody and immunoglobulin titers, rutin and catechin, two naturally occurring flavonoid compounds, demonstrated their ability to defend the humoral immune system. In a mouse model of oesophageal cancer, flavonoids can elevate the quantity of CD19+ cells in peripheral blood by stimulating an increase in IL-10. Additional investigations demonstrated the inhibition of serum IgG, the suppression of Th17 cells, and the elevation of Treg cells generated by baicalin. Quercetin is a flavonoid that improves immunotoxicity by stimulating the release of IgM and IgG in serum and elevating CD20 expression in the spleen. Furthermore, quercetin elevated the number of CD19 cells.[15]

5. Conclusion

The ethanol extract of parijoto fruit (EEPF) contains flavonoids, tannins, and saponins based on the

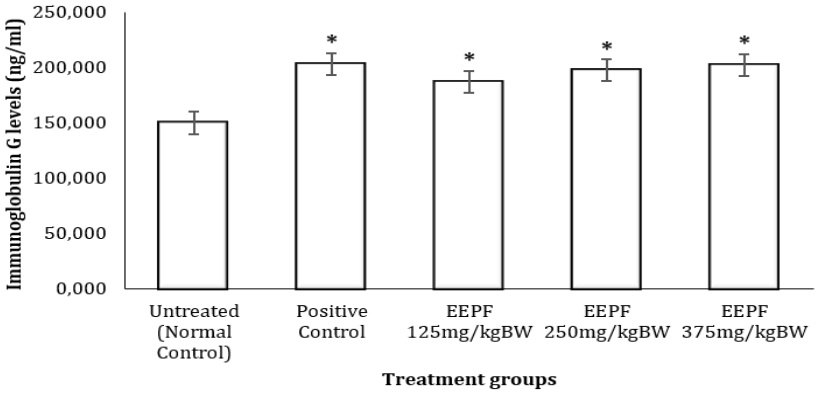

Figure 4. Immunoglobulin G (IgG) levels after treatment with ethanol extract of Parijoto fruit. The data was expressed as mean ± SD from 6 mice (* = p<0.05 compared to untreated group)

results of the phytochemical screening. In the immunomodulatory activity test in vivo, EEPF revealed the ability to generate non-specific immune responses. Phagocytic activity occurs in both the quantity of activated macrophages and the functional capacity of each macrophage. This study also showed an increase in the activity of specific immune responses by increasing the phagocytic activity of macrophages. The measurement of immunoglobulin G levels also increased, although not significantly.

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Conflict of Interest

The authors declare no conflicts of interest related to this work.

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