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Potential of *Graptophyllum pictum* Leaf Decoction as an Immunomodulator: Modulation of Macrophage Phagocytosis and Lymphocyte Proliferation

Anidasari Irdanita, Selvia Natasya, Yumna Alifah, Aji Winanta*
Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta, Yogyakarta, Indonesia

*Corresponding author: ajiwinanta@umy.ac.id
Orcid ID: 0000-0002-2700-7873

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Abstract

***Background**: Red pudding leaves (Graptophyllum pictum) are commonly used by the Lebong community in Bengkulu as an immune system-enhancing drink containing flavonoids, glycosides, saponins, tannins, and triterpenoids. **Objective**: This study aimed to evaluate the potential of red pudding leaves as immunomodulatory agents in vitro and assess their total flavonoid content. **Methods**: The extraction method employed was a decoction, and the flavonoid content was measured using the TLC method by calculating the resulting Rf value and utilizing the LC-MS technique. The total flavonoid content was quantified using a colorimetric method, and immunomodulatory activity was assessed based on the phagocytosis capacity, phagocytosis index, and lymphocyte proliferation. **Results**: The results showed that red pudding leaf contained flavonoid compounds based on the LC-MS method in the form of trans-3-Indoleacrylic acid, schaftoside, adenine, corymboside, fraxetin and 4-coumaric acid. The total flavonoid content obtained at a concentration of 7.5% amounted to 74.937 mg QE/g; at a concentration of 15%, it amounted to 75.483 mg QE/g; and at a concentration of 30%, it amounted to 97.825 mg QE/g. All red pudding leaf infusion concentrations increased macrophage phagocytosis activity and lymphocyte cell proliferation. **Conclusion**: In conclusion, red pudding leaves show potential for development as an alternative beverage to enhance the immune system.*

***Keywords**: flavonoids, immunomodulators, lymphocytes, macrophages, red pudding leaves*

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INTRODUCTION

The immune system serves as the body's defense mechanism against external foreign substances, including parasites, bacteria, viruses, fungi, and other tumor cells (Kalsum, 2017). External support is necessary to enhance the defence capabilities of the immune system. Immunomodulators are biological compounds that influence or regulate the immune system by stimulating, modulating, or suppressing both innate and adaptive immune responses. Besides synthesized drugs, immunomodulators can come from natural ingredients, such as plants (Lestari, 2021).

Several studies related to the role of herbal plants as immunomodulators can explain the various effects that can be caused by herbal administration on the immune system. Herbal plants can affect T cells, mast cells, and exert anticancer and antimicrobial effects. Several active ingredients in herbal plants, such as flavonoid polysaccharides, are thought to enhance the immune system. The role of herbal plants as immunomodulatory agents can be immunostimulatory or immunosuppressive (Lestari 2021).

Macrophages play an important role in the immune system. Macrophages produce cytokines that play a role in various wound healing processes and antigen presentation. Monocytes are produced by the spinal cord and migrate through blood vessels to turn into monocytes and differentiate into macrophages (Wolska et al., 2019). Macrophages are phagocytic cells that play a major role in the defense against pathogen or microorganism attacks through phagocytosis mechanisms, which play an important role in adaptive and innate immune responses (Abbas et al., 2012). Phagocytosis is the ability of macrophages to phagocytise latex particles. Macrophage phagocytosis is used as the standard for a person's health or immunity. Macrophage activity in the phagocytosis of latex particles can be measured using two parameters: phagocytosis index (PI) and phagocytosis capacity (PC) (Hartini et al., 2013).

Proliferation is the process of mitotic cell division, a biological function of the body. Lymphocytes are part of the adaptive immune response that can recognize pathogens for the first time and increase the specific immune response when exposed to repeated exposure. Lymphocytes play a role in a specific immune response (T cells) for the body's defense against viruses, bacteria, and parasites. The lymphocyte proliferation response is used as a reference for describing lymphocyte function and the immune status of the human body (Meilandani & Makiyah, 2015).

The use of herbal plants as traditional medicine is often utilized by Indonesians. Traditional medicinal plants are a combination of natural ingredients derived from generations that have been used as treatments based on experience. Red Pudding Leaf is an ornamental plant commonly utilized by the residents of Bengkulu Province, especially in the Lebong district, as a traditional medicine (Permenkes RI, 2016). The factors used in this study were based on previous studies. The results showed that red pudding leaves were positive for flavonoids, alkaloids, steroids, tannins, and saponins after phytochemical screening using UV-Vis spectrophotometry. The ethanol extract of red pudding leaves in this study showed antibacterial activity. This was caused by the high content of secondary metabolites (Fauzi et al., 2016). The difficulty of health facilities in this area has led people in Lebong Regency to use boiled red pudding leaves as a first alternative for treating bleeding or bruising. Testing the effectiveness of red pudding leaves on wound healing in mice showed that administration of red pudding leaf extract at concentrations of 10% and 15% had a good wound healing effect on mice (Tukiran et al., 2014). Testing the effectiveness of red pudding leaves on wound healing in rats showed that red pudding leaf extract at concentrations of 10% and 15% had a good wound healing effect on rats (Andiyani et al., 2018).

Previous research related to phytochemical tests have shown that this plant contains non-toxic alkaloids, steroids, flavonoids, glycosides, calcium oxalate, saponins, tannins, formic acid, and fat. (Tukiran et al., 2014). Previous research on testing the total flavonoid content of ethanol extracts showed that it had high flavonoid levels of 402.88 mg / 100 g QE. There is a correlation between the flavonoid content in red pudding leaf extract and its capacity to diminish free radicals. As flavonoid content increases, so does its effectiveness in reducing free radicals (Rustini & Arianti, 2017). Furthermore, in the antioxidant testing of red pudding leaves, the results of red pudding leaf extract in ethanol have an IC50 value; therefore, it can be stated that red pudding leaf extract has the strongest antioxidant effect. In the anti-inflammatory test, it was found that 10% red pudding leaf extract produced the highest number of fibroblast cells (167.25 %) (Sartika & Indradi, 2021). To advance this research, additional studies will be conducted to explore the potential of red pudding leaf decoction as an herbal remedy to enhance the immune system.

The research conducted will discuss whether the *G. pictum* extract has flavonoid compounds based on the

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TLC method, Next, the study will determine the total flavonoid content of the *G. pictum* extract and investigate whether this extract exhibits immunomodulatory activity based on macrophage cell function and lymphocyte cell proliferation.

MATERIALS AND METHODS

Materials

Red pudding (*Graptophyllum pictum*) leaves were collected from Lebong, Bengkulu, China. Subsequently, plant determination tests were conducted at the Faculty of Biology, Ahmad Dahlan University, Yogyakarta. The selected red pudding leaves were separated and aerated without direct sunlight for several days until the leaves had dried completely. The dried leaves were then ground with a blender until they became a powder.

Extraction

An infusion of red pudding leaves at a concentration of 7.5% was made by putting 7.5 grams of dried red pudding leaf powder into a pot and then adding distilled water until all the dried red pudding leaf powder became wet. After standing for 10 min, 100 ml of water was added to the container. The mixture was heated for 15 min starting at a temperature of 90 °C while stirring. Subsequently, the infusion results were obtained. The process was carried out with the same thing at concentrations of 15% (15 g of red pudding leaves) and 30% (30 g of red pudding leaves) (Hamdan, 2017).

Analysis of compound with TLC method

The sample was dissolved in 70% ethanol, and the sample was dotted in the stationary phase (silica gel GF254 plate) to identify the compound. The TLC plate was sprayed with the FeCl3 reagent. The spots were detected using UV 254 nm, UV 366 nm, and visible light. To obtain the color reaction of flavonoid compounds, the silica plate was sprayed with ammonia and then left for 15 min to observe the color of the spots that appeared. The RF value was then calculated based on the TLC results. The Rf value was used to identify the content of chemical compounds in the TLC method by calculating the distance of spot displacement(Munawaroh et al., 2018).

Compound content test LC-MS method

Secondary metabolites in the red pudding leaf infusion were analyzed by liquid chromatography-mass spectrometry (LC-MS) using a Thermo Scientific Vanquish UHPLC Binary Pump coupled with a Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer. Chromatographic separation was achieved on an Accucore™ Phenyl-Hexyl analytical column (100 mm × 2.1 mm, 2.6 µm) with a mobile phase gradient of MS-grade water containing 0.1% formic acid (A) and MS-grade methanol with 0.1% formic acid (B) at a flow rate of 0.3 mL/min. Mobile phase B was initially set to 5% and gradually increased to 90% over 16 min, maintained for 4 min, and returned to the initial conditions, completing a 25-minute run. The column was held at 40 °C with a 3 µL injection volume. Data were acquired in the full MS/dd-MS^2 mode for untargeted screening using both positive and negative ionization. Nitrogen was utilized as the sheath, auxiliary, and sweep gases, with settings of 32, 8, and 4 units, respectively. The spray voltage was 3.3 kV, the capillary temperature was 320 °C, and the auxiliary heater was maintained at 30 °C. Scans ranged from 66.7–1000 m/z with a resolution of 70,000 for full MS and 17,500 for dd-MS^2. Instrument settings and tuning were managed with XCalibur 4.4 software, with weekly calibration for mass accuracy, ion transfer, and sensitivity using Thermo Scientific Pierce ESI calibration solution (Windarsih et al., 2022).

Total flavonoid level measurement

The maximum absorbance wavelength (λ_max) was determined using quercetin solution prepared at a concentration of 30 µg/mL. A 0.5 mL aliquot of 35 µg/mL quercetin was mixed with 0.1 mL of 10% $AlCl_3$ and 0.1 mL of 1 M sodium acetate in a 5 mL volumetric flask, and distilled water was added to a final volume of 5 mL. After brief incubation at room temperature, the absorbance was measured at λmax. A 500 µg/mL stock solution of quercetin was prepared by dissolving 5 mg of quercetin in 10 mL 70% ethanol, from which dilutions of 15, 20, 25, 30, and 40 µg/mL were prepared. To each dilution, 0.1 mL of 1 M sodium acetate, 1.5 mL of methanol, and 0.1 mL of $AlCl_3$ were added, followed by distilled water to a final volume of 5 mL. Incubation was conducted at room temperature for 30 min, and absorbance readings were recorded at 421.5 nm using a UV-Vis spectrophotometer. For sample analysis, 5 mg of freeze-dried red pudding leaf extract was dissolved in 5 mL of distilled water to yield a 10,000 µg/mL solution. A 0.5 mL aliquot was mixed with 1.5 mL of methanol, 0.1 mL of 10% $AlCl_3$, and 0.1 mL of 1 M sodium citrate, with distilled water added to reach 5 mL. The mixture was incubated at room temperature for 30 min, and the absorbance was measured at 421.5 nm (Ipandi et al., 2016). Each sample was analyzed in triplicate, and the average absorbance was used to calculate the flavonoid concentration based on a calibration curve, expressed as quercetin equivalents (mg QE/g extract) (Ahmad et al., 2017).

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Immunomodulatory activity test

Isolation and incubation of macrophage cells

Macrophages were isolated from male Balb/c mice (2–3 months old) following euthanasia by chloroform inhalation. The mice were positioned supine and their abdominal areas were disinfected with 70% ethanol. A small incision is made to expose the peritoneum. A total of 10 mL of RPMI 1640 medium was injected into the peritoneal cavity, and after a 5-minute wait with gentle shaking, macrophages were released into the medium. Peritoneal fluid was collected and centrifuged at 2000 rpm for 10 min, and the supernatant was discarded. The cell pellet was resuspended in 3 mL RPMI medium with 10% FBS, yielding a suspension of 2.5 × 10^6 cells/mL. Cells were seeded onto a 24-well plate, each well containing a coverslip and 200 µL of cell suspension (5 × 10^5 cells). Following a 30-minute settling period, the cells were incubated in a 5% CO_2 incubator at 37°C, washed three times with 250 µL of complete medium, and incubated for an additional 2 h. After washing twice with RPMI, 1 mL complete RPMI medium was added to each well, followed by a 24-hour incubation (Munawaroh et al., 2018).

Macrophage phagocytosis assay

24 hours after the cells were cultured, the medium was removed using a pipette so that only macrophages remained on the coverslip. The medium was removed using a drop pipette and the cells were washed twice with RPMI-1640. Latex (200 µL/well) was added to a sample concentration series (62.5, 125, 250, and 500 µg/mL extract) and LPS as a positive control, and three replicates were performed and incubated for 2 h in a 5% CO_2 incubator at 37 °C. The cells were then washed 3 times with PBS. The samples were then dried at room temperature for 30 s and fixed using methanol. The coverslip was then allowed to dry, and the methanol was removed. The cover slips were stained using 10 % (v/v) Giemsa for 20 min, washed with distilled water, and then the culture wells were removed and dried at room temperature. Using a 100 × magnification light microscope, observations were made on 100 macrophage cells were observed, and the number of macrophages that could phagocytose latex was counted using a microscope. SFA values were calculated using the amount of latex per 100 macrophages and phagocytosis capacity for the macrophage phagocytosis activity parameter (Munawaroh et al., 2018).

$$\text{Phagocytosis Index (IP)} = \frac{number\ of\ phagocytized\ latex}{\text{number of activated macrophages}(100)}$$

$$\text{Phagocytosis Capacity (KF)} = \frac{number\ of\ phagocytizing\ macrophages}{number\ of\ macrophages\ counted(100)} \text{x } 100\%$$

Isolation of lymphocyte organs

Lymphocyte cells were isolated from the spleens of mice because the spleen is a primary secondary lymphoid organ containing T and B cells and serves as a key site for the immune response to antigens (Abbas et al., 2017). The lymphoid organs were rinsed three times with PBS, after which 10 mL of RPMI medium was added to the spleen tissue. The resulting cell suspension was transferred to a centrifuge tube, adjusted to a volume of 15 mL, and centrifuged at 2000 rpm for 10 min. To lyse the erythrocytes within the pellet, 1 mL of ammonium chloride was added and thoroughly mixed, followed by a 5-minute centrifugation at 2000 rpm. The supernatant was discarded and the lymphocytes were resuspended in 1 mL of complete RPMI medium. Cells were counted using a hemocytometer and further diluted with complete RPMI to achieve a final cell density of 1.5 × 10^6 cells/mL (Hertiani, 2010).

Lymphocyte cells (1.5 x 106/mL) of 100µL were distributed into 96-well microplate wells and incubated for 48 h in an incubator with 5% CO_2 flow at 37 °C.One hundred µL of the sample extract was added to a concentration series of 62.5, 125, 250, and 500 µg/ml. LPS was used as a positive control. Next, 10 µL of 5 mg/mL MTT solution was added to each well. The cells were incubated for 4 h at 37°C. The reaction was halted by adding 50 µL of stop reagent in 0.001 N HCl. Incubation was continued for 24 h at room temperature, and the results were measured using an ELISA reader at a wavelength of 550 nm (Hertiani, 2010). The proliferation stimulation index (IS) was calculated using a microplate reader and the absorbance was measured at 550 nm (Sumardi et al., 2013).

$$\text{Stimulation Index (IS)} = \frac{\text{Absorbance (Sample} - \text{Control Medium)}}{\text{Absorbance (Normal Control} - \text{Medium Control)}}$$

Data analysis

The results of the data obtained were then processed by statistical analysis using SPSS to assess whether there were significant differences between the treatment groups. This analysis aimed to determine if there were significant differences between the independent variables.

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RESULTS AND DISCUSSION

Decoction

The decoction method was chosen because it has been empirically used in the community. The obtained decoction was then *freeze-dried* to obtain the water extract. The purpose was to remove water by sublimation at 0 °C. This method avoids the loss of compounds and damage to compounds due to the heating process (Reubun et al., 2020). From this process, the yields of the infusion extracts at 7.5% concentration were 0.466%, 15% was 0.44% and 0.263%. From this process, the yield of infuse extract at 7.5% concentration was 0.466%, 15% was 0.44% and 0.263%.

Analysis of compound content by TLC method

Based on the results of the TLC method using the Rf values. The Rf value was used to identify the content of chemical compounds with spots. The results showed yellow spots with an Rf value of 0.98 on the quercetin standard. The Rf of freeze-dried red pudding leaves at 7.5%, 15% concentration is 0.62, and 30% concentrations was 0.60, 0.62, and 0.91, respectively. Rf value obtained for the sample spot at a concentration of 30% was close to that of the standard spot of quercetin. The compounds in the red pudding leaves are thought to contain quercetin compounds, as determined by TLC. Red pudding leaf infuse extract is thought to contain flavonoids characterized by the appearance of a yellow spot color under UV light at 254 nm. At a UV light wavelength of 366 nm, no visible spots appeared due to a less clear light spectrophotometric lamp. In the quercetin standard solution, brownish-yellow spots were observed under visible light as well as under UV light at 254 nm and 366 nm. The presence of flavonoid compounds was confirmed by the visual greenish color observed with UV light at 254 nm.

LC-MS analysis of red pudding leaf decoction

Compound identification using LCMS yielded 171 compounds contained in the red pudding leaves. The compounds identified were assumed to be flavonoid compounds, with seven compounds having potential as immunomodulatory agents.

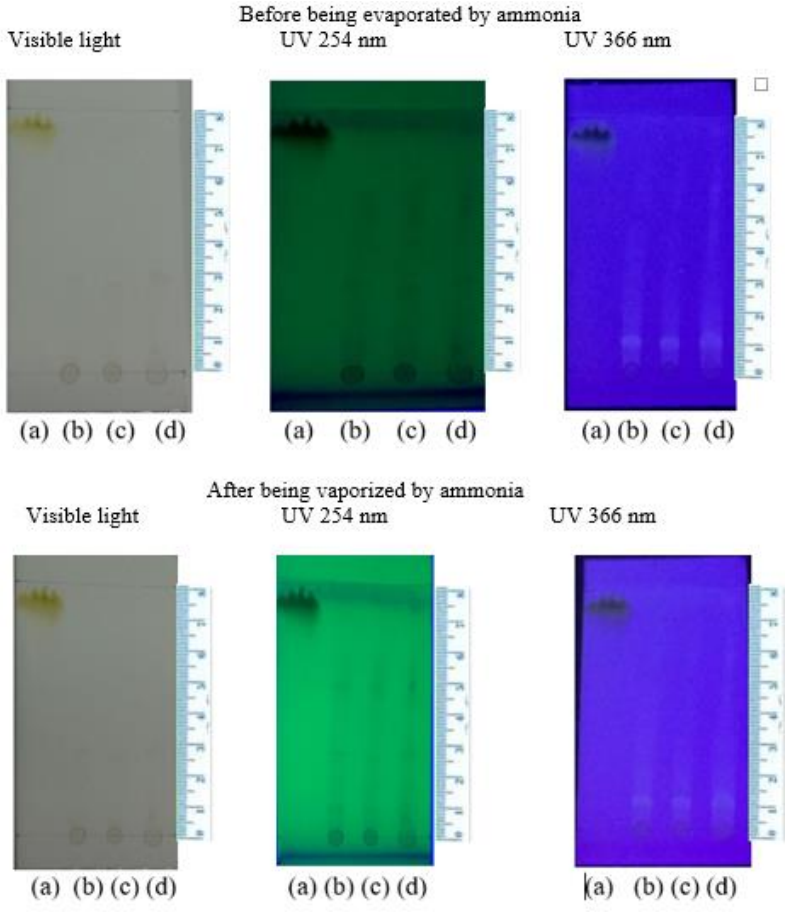

Figure 1. TLC profiles of quercetin (marked with arrow) standard (a), 7.5% red pudding leaf extract (b), 15% red pudding leaf extract (c), 30% red pudding leaf extract (d)

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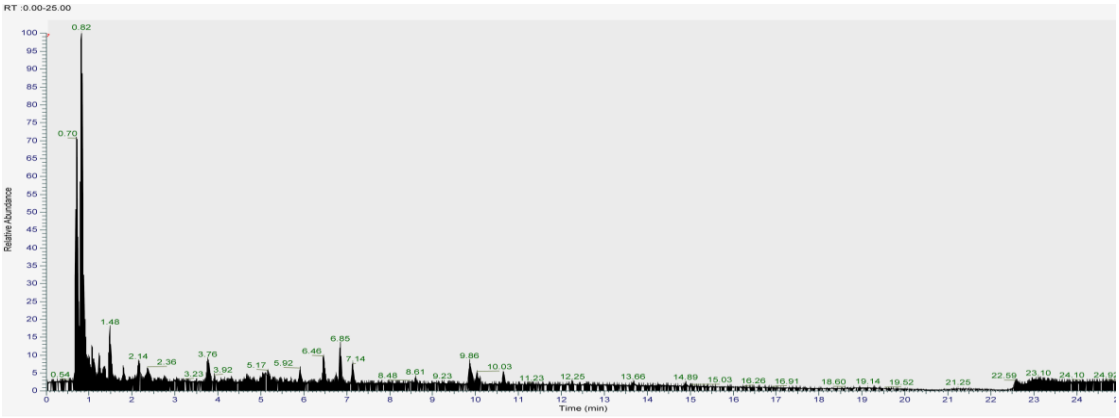
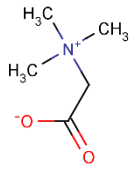
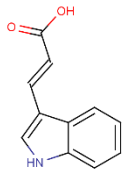
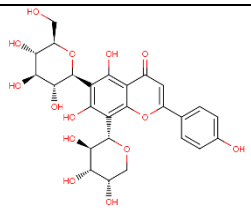
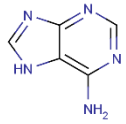
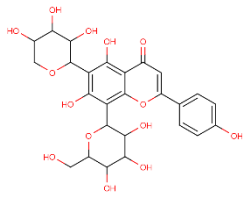
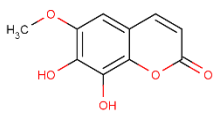
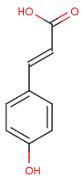


Figure 2. Chromatogram of red pudding leaves using LC-MS method

Table 1. Identification and detection results of flavonoid compounds using LC-MS

Name of compound	Chemical formula	Retention time (Rt)	Composition (%)	Structure
Betaine	$C_5 H_{11} N O_2$	0.828	58	
trans-3-Indoleacrylic acid	$C_{11} H_9 N O_2$	2.363	1,8	
Schaftoside	$C_{26} H_{28} O_{14}$	5.177	1,3	
Adenine	$C_5 H_5 N_5$	0.901	1,02	
Corymboside	$C_{26} H_{28} O_{14}$	5.304	0,59	
Fraxetin	$C_{10} H_8 O_5$	1.328	0,48	
4-Coumaric acid	$C_9 H_8 O_3$	4.29	0,22	

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As shown in Table 1, the compound contained in the infusion extract of red pudding leaves was betaine, with the highest content of 58%, which had a retention time of 0.828 with peak mass. *Betaine* and *trimethylglycine* are stable and non-toxic natural substances found in plants, animals, and microorganisms (Arumugam et al., 2021) and possess osmoprotective properties that are crucial for the immune, cardiovascular, nervous system, and kidneys (Ghasemi & Nari, 2020).

Wlodarska et al. (2018) indicates that *trans-3-Indoleacrylic acid* could enhance the function of the intestinal epithelial barrier and diminish inflammatory responses. Certain species of Peptostreptococcus produce indoleacetic acid metabolites that positively affect intestinal epithelial barrier function and reduce inflammation mediated by immune cells (Wlodarska et al., 2017).

Schaftoside is a flavonoid classified as a low-molecular-weight phenolic compound and a secondary metabolite. It is a flavonoid found in various Chinese herbal medicines including Eleusine indica, Rhizoma arisaematis, Lysimachia christinae Hance, Glycyrrhiza uralensis, and Dendrobium nobile (Zhou et al., 2019). A previous study by Yang Yi et al. (2018), involving proteomic analysis and cytokine assays, demonstrated that schaftoside also modulates the immune response and inflammation in host cells. Schaftoside exhibits safety and favorable pharmacokinetic properties, making it a promising candidate for the prevention and treatment of COVID-19 (Yi et al., 2022).

Adenine is a purine nucleoside produced by dephosphorylation of adenine nucleotides. *Adenine* markedly reduced lipopolysaccharide-induced release of inflammatory cytokines, including TNF-α, IL-1β, and IL-6, in THP-1 cells. The anti-inflammatory action of adenine may be linked to an increase in intracellular AMP, catalyzed by adenine phosphoribosyltransferase, which in turn activates AMPK (Wu et al., 2019).

Corymboside is a flavonoid compound that forms four hydrogen bonds and hydrophobic interactions with caspase 3 proteins. Previous research by Cristina *et al.* (2021) was related to the activity of corymboside in Phaleria macrocarpa (Scheff.) extract as a potential anti-cancer agent showed that corymboside had the highest TP53 expression enhancer and anticarcinogenic activity (Pa = 0.941 and 0.872, respectively) (Christina et al., 2021).

Fraxetin is a coumarin derivative extracted from the traditional medicinal plant Fraxinus rhynchophylla and is a key component in various herbal and dietary supplements. Previous studies investigating the impact of fraxetin on neuroinflammation following microglia-induced ischemic stroke have demonstrated that fraxetin effectively suppressed the expression of pro-inflammatory cytokines, including inducible nitric oxide synthase, tumor necrosis factor-α, interleukin-1 beta, and interleukin-6 in LPS-activated microglia. (Deng et al. 2022).

Cumaric acid (CA) is a secondary metabolite of phenol. Zhao et al. (2016) demonstrated that coumaric acid can inhibit the NF-kB and MAPK signaling pathways by blocking LPS-induced inflammatory cytokines. As a result, p-coumaric acid shows promise as an immunosuppressive agent for the treatment of autoimmune inflammatory diseases including rheumatoid arthritis (Kilani-Jaziri et al., 2017).

Total flavonoid measurement

The total flavonoid assay yielded a linear regression equation, $y = 0.0107x + 0.1188$, with an R^2 value of 0.9865. An R2 value close to 1 indicated a relationship between the concentration of the quercetin standard and the absorption value. The average total flavonoid content of red pudding leaf infusion extract at each concentration can be obtained through a linear regression equation, such as at a concentration of 7.5% of 74.937 mg QE/g, at a concentration of 15% of 75.483 mg QE/g, and at a concentration of 30% of 97.835 QE/g. The mean value was 82.75 mg QE/g, with a standard deviation of ± 13.06. Flavonoids are secondary metabolites widely present in several herbal plants. Flavonoids have immunostimulatory and immunosuppressive properties. Flavonoid compounds can boost the body's immune system and fight infection attacks from bacteria, viruses, fungi, or other types of microbes.

Immunomodulatory assay

Phagocytosis refers to the ability of macrophages to phagocytose latex particles. Macrophage phagocytosis is used as the standard for immunity. Macrophage activity in the phagocytosis of latex particles can be measured using two parameters: phagocytosis index (PI) and phagocytosis capacity (PC) (Hartini et al., 2013). Phagocytosis data were obtained by calculating the amount of latex phagocytosed before and after treatment with red pudding leaf infusion extract. From these data, the phagocytosis index and phagocytosis capacity were obtained, which shows that red pudding leaf extract has the ability to increase phagocytosis activity by increasing the value of phagocytosis capacity and phagocytosis index from several concentration series when compared to control cells.

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Immunomodulatory activity test results showed that the sample significantly increased the phagocytic activity of macrophages with LPS cell control. The 500 µg/mL concentration showed the highest activity, with a phagocytosis index of 1 1.701 ± 0.76, and a phagocytosis capacity of 94.833% ± 2.26.

Figure 3 (a) shows control cells without treatment, while Figure (b) shows cells treated with red pudding leaf infusion extract. Macrophages treated with red pudding leaf infusion extract phagocytose less latex than macrophages treated with red pudding leaf infusion extract.

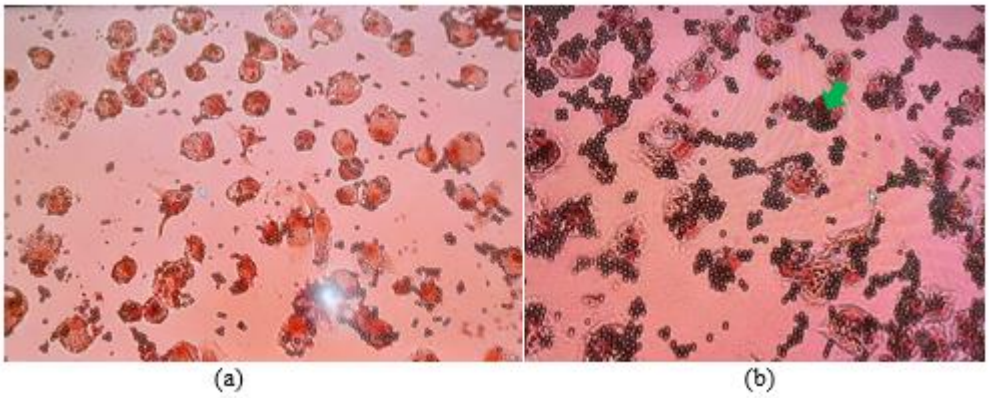

Figure 3. Comparison of the phagocytic activity of macrophages in control cells (a) and those treated with red pudding leaf infusion extract (b) at 100 × magnification. *The blue arrow reveals macrophage cells, and green arrow describes latexs wich is phagocyte by macrophage

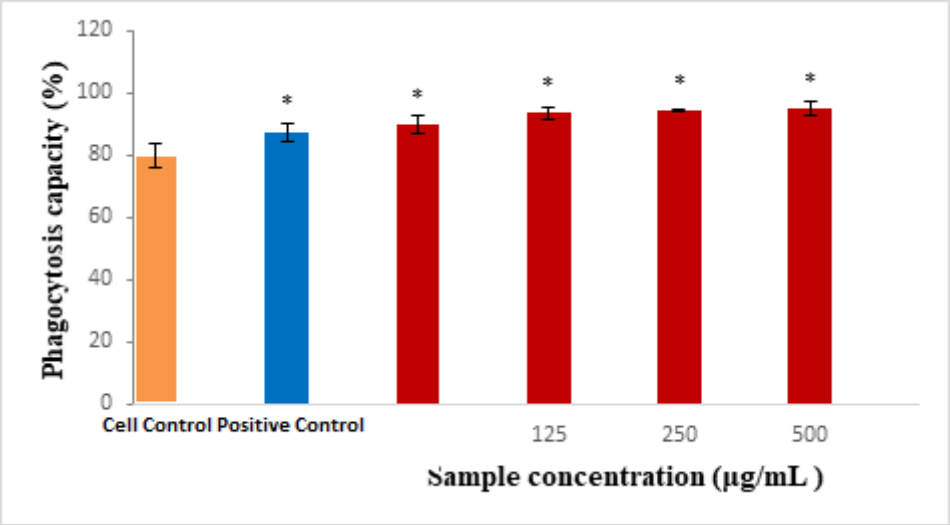

Figure 4 (a). Phagocytosis capacity profile. Phagocytosis capacity (%) at 100x magnification (mean±SD, n=3, α=0.05) *indicates a significant difference (P < 0.05) between the treatment and control groups. Cell control was macrophage cells with no treatment and treatment with LPS as a positive control

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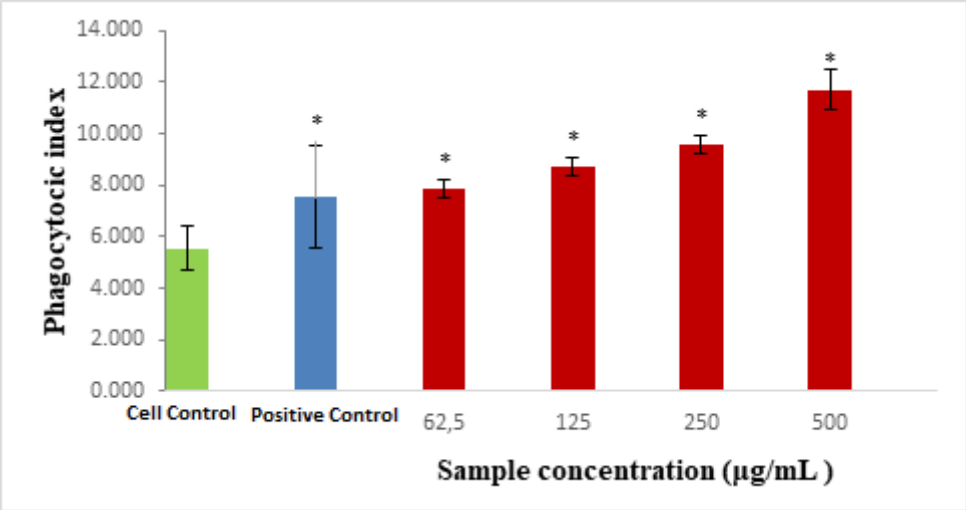

Figure 4 (b). Macrophage phagocytosis index profile at 100x magnification (mean±SD, n=3, α=0.05). *indicates a significant difference (P < 0.05) between the treatment and control groups. Cell control was macrophage cells with no treatment and treatment with LPS as a positive con control

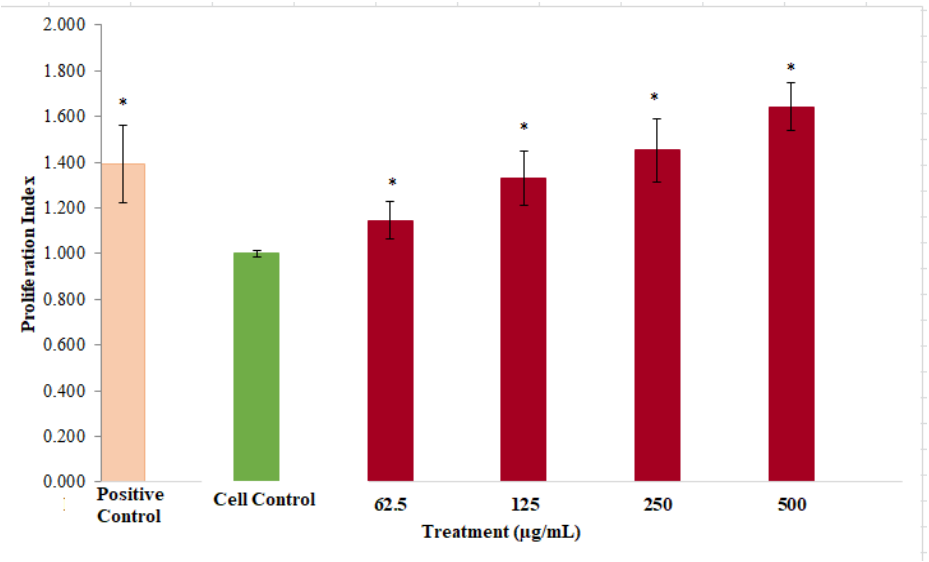

Figure 5. Lymphocyte proliferation activity at 100x magnification (mean±SD, n=3, α=0.05). * indicates a significant difference (P<0.05) between the treatment and control groups. Cell control was macrophage cells with no treatment and treatment with LPS as a positive con control

The results of this study (Figures 4 a and b) show that red pudding leaf extract can increase the phagocytic activity of macrophages characterized by an increase in the phagocytic capacity and phagocytosis index of several concentration series when compared to control cells. Compounds with IF>1 values are grouped as immunostimulant compounds, which means that these substances can increase or stimulate the body's immune system, whereas compounds with IF<1 values are grouped as immunosuppressant compounds, which means that these substances can sensitize the body's immune system (Kresno, 2007). Flavonoids have properties as immunostimulants and immunosuppressants. Flavonoid compounds can boost the body's immune system and fight infections by bacteria, viruses, fungi, or other microbial species (Erjon, 2022). Flavonoids act as immunomodulators by increasing the activity of IL-2 and lymphocyte proliferation. Additionally, flavonoids can activate NK cells, leading to stimulation of IFN-γ production. IFN-γ is the primary macrophage-activating cytokine among Macrophage Activating Cytokines (MAC), which plays a crucial role in the destruction of bacteria as part of cellular non-specific immunity (Abbas et al., 2012). The data analysis results indicated that each concentration exhibited a significant difference.

Proliferation is the process of mitotic cell division which is a biological function of the body. Lymphocytes are part of the adjuvant immune response that can recognize pathogens for the first time and increase the

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specific immune response if exposed repeatedly (Meilandani & Makiyah, 2015).

The lymphocyte proliferation assay was performed using the colorimetric method. The microtetrazolium (MTT) assay was used to determine the amount of potential possessed by natural ingredients using a microplate reader that will read the absorbance of formazan, which is generated from the reduction process by the enzyme succinate dehydrogenase found in the mitochondria of living cells (Amir & Murcitro, 2017).

The results depicting lymphocyte proliferation activity at different concentrations indicated that the administration of red pudding leaf infusion extract yielded a proliferation index <2. Generally, a stimulation index for lymphocyte proliferation between 2 and 3 is considered weakly positive, whereas an index value >3 is regarded as positive, especially if more than one concentration is obtained (Winanta et al., 2023). The results of this study show that red pudding leaf extract can increase lymphocyte proliferation activity marked by an increase in the value of the lymphocyte proliferation stimulation index from several concentration series when compared to the control cell. This suggests that a higher concentration leads to greater presence of flavonoid compounds. Flavonoids enhance lymphocyte proliferation by increasing IL-2 levels. IL-2 plays a crucial role in the proliferation of T lymphocytes, and antigen-stimulated T lymphocyte proliferation is regulated by the interplay between IL-2 and differentiation of B lymphocytes and Natural Killer (NK) cells (Ulfah et al., 2017). According to Makiyah and Wardhani (2017), flavonoids can boost lymphocyte proliferation, as evidenced by an increase in the diameter of the white pulp and area of the germinal center. The data analysis results indicated significant differences among the concentrations.

CONCLUSION

Red pudding leaf infusion extract (*G. pictum*) based on phytochemical screening of TLC and LC-MS methods contains flavonoid secondary metabolite compounds in the form of trans-3-Indoleacrylic acid, schaftoside, adenine, corymboside, fraxetin, and 4-coumaric acid. The highest average value of total flavonoids in *G. pictum* infusion extract was obtained at a concentration of 30%, with a value of 97.825 mg QE/g compared to the other two concentrations at 7.5% and 15%. The red pudding leaf infusion extract showed immunostimulant activity through macrophage phagocytosis and lymphocyte proliferation methods with an increase in phagocytic capacity value and the highest macrophage phagocytosis index at a concentration of 500 µg/ml (IF = 11.701 ± 0.761; % KF = 94.833 ± 2.268) compared to the cell control and positive control, but did not affect lymphocyte cell proliferation in vitro.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.I.; Methodology, A.I.; Software, S.N.; Validation, A.I.; Formal Analysis, S.N.; Investigation, A.I.; Resources, S.N.; Data Curration; Y.A.; Writing - Original Draft, A.I.; Writing - Review & Editing, A.I.; Visualization, Y.A.; Supervision, A.I.; Project Administration, A.W.; Funding Acquisition, A.W.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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