ORIGINAL ARTICLE

The immunomodulatory activity of parijoto fruit (*Medinilla speciosa*) fraction against phagocytosis macrophages and lymphocyte proliferation

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ABSTRACT

The immune system's principal functions are to preserve health and defend against dangerous invaders (antigens). Parijoto fruit (*Medinilla speciosa*) is a plant that can potentially have immunomodulatory activity because it contains flavonoid and terpenoid compounds. The aim of this research is to ascertain the total flavonoid and phytochemical content of the parijoto fruit fraction, as well as its potential *in vitro* immunomodulatory activity. The extraction of powdered parijoto fruit was conducted using 70% ethanol, followed by the separation into n-hexane, ethyl acetate, and water fraction. The phytochemical content was analyzed with gas chromatography (GC)–mass spectrometry. The total flavonoid contents were determined by colorimetric analysis. In addition, the immunomodulatory activity assay was conducted *in vitro* to evaluate the phagocytic activity (phagocytic capacity [PC] and phagocytic index) of macrophages and the proliferation of lymphocytes (stimulation index [SI]). The GC results showed that parijoto fruit extract contains 9,12-Octadecadienoic acid-, and phthalic acid. The ethyl acetate fraction exhibited the greatest total flavonoid concentration at 7.4094 ± 0.49 mg QE/g sample. *In vitro*, immunomodulatory tests showed that all fractions could significantly increase macrophage phagocytic activity compared to control cells. The highest value of PC and phagocytic index was found in the n-hexane phase with a concentration of 750 g/mL of 82.75 ± 0.87 and a concentration of 500 g/mL of 6.62 ± 0.19, respectively. The ethyl acetate fraction exhibited the most significant SI for lymphocyte proliferation, recorded at a concentration of 750 g/mL with a value of 8.70 ± 1.01. The ethyl acetate fraction's SI >3 value in the lymphocyte proliferation test suggests that it exhibits lymphocyte proliferation activity. The parijoto fruit may enhance the phagocytic role of macrophages and promote lymphocyte proliferation, indicating its potential as an immunomodulatory therapy.

Key words: Immunomodulator, parijoto fruit fraction, phagocytosis macrophage, proliferation lymphocyte

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INTRODUCTION

Indonesia is rich in natural resources that contain bioactive components that are secondary metabolites in plants. These

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bioactive components are beneficial for health, such as alkaloids, flavonoids, terpenoids, and steroid compounds.[1,2] Flavonoids are natural ingredients with the constituent structure of phenolics, and phytonutrients (chemical compounds found in plants). They are present in almost all fruits, vegetables, and medicinal plants. This compound is used and applied in various fields of health, cosmetics, supplements, and medicine because of its capacity to enhance the immune system. Terpenoids and steroids also have immunomodulatory activity. Some plant from Borneo Island, Indonesia, has been proven to contain terpenoid and steroid compound and have immunomodulatory activity by boosting the phagocytic ability of macrophages, as evidenced by the ingestion of latex bead particles by the macrophage cells.[3,4]

Based on research, one of Indonesia's native plants with high anthocyanin content, namely flavonoid compounds that have the potential as immunomodulators, is the parijoto fruit.[5] Parijoto plant (*Medinilla speciosa*) is a species of the *Melastomataceae* family. There are secondary metabolites of parijoto such as saponins, glycosides, flavonoids, terpenoids, and tannins.[6-8] Parijoto has very strong antioxidant activity and the potential as an immunostimulatory.[5] Antioxidants may neutralize free radicals by providing electron pairs to atoms with unpaired electrons, rendering them nonreactive. Antioxidants can support the body's defenses toward pathogenic microbes such viruses, bacteria, parasites, protozoa, and helminths.[9]

According to research, the flavonoid, steroid, and terpenoid metabolites act as antioxidants in tumor growth and improve the immune system.[10] The results of *in vitro* tests of flavonoids and flavonols show an immune response, which can increase the immunity in the body and modulate lymphokines generated by T cells, thereby activating phagocytic cells to perform a phagocytic response.[5,11] It prompted the researchers to conduct this study on the extract fraction of parijoto fruit *in vitro* to figure out the immunomodulatory effect of this plant further after analyzing the immunomodulatory activities of the extract in previous studies and phytochemical compound by gas chromatography–mass spectrometry (GC-MS).

MATERIALS AND METHODS

Materials

The parijoto fruit was taken from Kudus Regency, Central Java, in April 2020. The sample was recognized in Laboratory of Biology, Faculty of Pharmacy UAD, under No. 195/Lab. Bio/B/VII/2020. Ethanol 70% (General Labora/technical grade), ethyl acetate, n-hexane, water, proanalytical methanol solvent, anhydrous quercetin (Sigma®), male Swiss Webster mice aged 2–3 months, RPMI-1640 (Sigma®) media, phosphate-buffered saline (PBS), latex beads (Sigma®), Giemsa (Merck®), methanol (Merck®), silica gel GF254 (Merck®), fetal bovine serum, quercetin (Sigma®), MTT reagent, tris-NH4Cl buffer (Sigma), hepatitis B vaccine antigen (Euvax B®), stopper reagent (SDS 0.01 N hydrochloric acid), sodium acetate (Merck®), and DMSO (Merck®).

Extraction and fractionation

The parijoto fruit was harvested and dried in an oven at 40°C for 24 h. Subsequently, the Parijoto fruit was ground using a blender, and then the extraction process using the maceration method referred to in the research of Vifta and Advistasari[8] with several modifications. Parijoto fruit simplicia was maceration method with a ratio of 1:10 between simplicia powder and 70% ethanol solvent. To get a thick extract, the macerate will be evaporated using a rotatory evaporator at 60°C.

The fractionation process in this study refers to the research of Munawaroh *et al.*,[12] The fractionation process by dissolving every 10 g of thick extract of parijoto fruit in 50 ml of methanol–water in a ratio of 9:1. The methanol–water phase was then liquid–liquid partitioned 6–7 times using n-hexane solvent. The n-hexane phase was subsequently isolated from the methanol–water mixture and evaporated. The suspension was then partitioned into liquid–liquid using ethyl acetate solvent and evaporated. The aqueous fraction was evaporated utilizing a freeze–dryer.

Determination of total flavonoid level

The total flavonoid content refers to research by Munawaroh *et al.*,[12] that began by analyzing the maximum wavelength of the standard quercetin compound with a ultraviolet-visible (UV-Vis) spectrophotometer. Measure 50 mg of quercetin and dilute it in distilled water to get a 500 ppm-stock solution. The stock solution dilutes to make a series of levels of quercetin at 2 ppm, 3 ppm, 4 ppm, 5 ppm, and 6 ppm. In the next step, 0.5 mL of each stock fraction solution is transferred to 1.5 mL. Methanol was added to aluminum chloride ($AlCl_3$), and sodium acetate solution was added to 100 L (0.1 mL) and added with distilled water, incubated for 30 min, and the absorbance was quantified at 430 nm for three replications with a UV-Vis spectrophotometer. The total flavonoid levels are calculated as milligrams of quercetin equivalent per gram of sample.

Gas chromatography–mass spectrometry analysis

The parijoto fruit extract was examined utilizing the GC-MS technology at LPPT UGM. The examination of sample was conducted using qualitative approaches with GC-MS, employing the Thermo Scientific Trace 1310 and ISQ Single Quadrupole, utilizing an HP-5MS UI column of 30 m × 0.25 mm × 0.25 µm. Ultra-High Purity Helium (He) serves as a transport gas with a flow rate of 1.0 mL/min. In addition, 0.5 g of parijoto fruit extract was diluted in 1.5 mL of ethanol. One milliliter of the sample vial was injected into the gas chromatography column using a syringe.

Macrophage phagocytic activity assay

Macrophages were isolated from the peritoneal cavity of 2–3 month old Balb/c mice. A total of 10 ml of cold RPMI-1640 used to isolate Macrophages. The number of cells was determined and subsequently suspended in RPMI medium to achieve a concentration of 2.5 × 10^6 cells/mL. In a 24-well plate, the cell suspension inoculates on coverslips, with 400 µL of cell suspension applied to each well and incubated for 30 min. Incubate each well with 600 µL of RPMI media for 24 h at 37°C. The process for determining macrophage phagocytic activity began with the injection of 500 ml of the fraction into the well with a successive concentration of 62.5, 125, 250, and 500 g/mL. Then put cells of control on each well. Each tube made three replications. The well was incubated using a CO_2 incubator. The temperature used in the incubation process was 37°C with an incubation duration of 4 h. The suspension was washed using RPMI-1640 media. Each well plate was washed by adding 200 L of latex suspension in RPMI media at a concentration of 2.5 × 107/mL, then incubated for 60 min at 5% CO_2. The incubation temperature was 37°C. Subsequently, the cells were rinsed with PBS and then air-dried at ambient temperature. Methanol was used to fix the cells for 30 s. After the coverslips had dry, they were stained using Giemsa 10%. After that, the quantity of latex that was phagocytosed by active macrophages as well as the number of macrophages that were able to phagocytose latex material were determined. The calculation by observing about 100 macrophages using a microscope with a magnification of ×1000. Afterward, evaluate macrophage phagocytic activity, determined by the Phagocytosis Index (PI) and phagocytic capacity (PC).[13]

Lymphocyte proliferation assay

Lymphocytes were isolated from the spleen organs of Swiss Webster strain mice carried out aseptically. The RPMI media was pumped into the spleen so that lymphocytes came out. The suspension of cells was put in a 10 mL centrifuge tube and concentrated for 5 min at 2,500 rpm 4°C. The pellet was suspended in 4 mL of Tris-NH4Cl solution. Cells were mixed for 15 min. The next step adds 4 mL of RPMI media to neutralize Tris-Buffered Ammonium Chloride, centrifuged for 4 min at 2500 rpm 4°C. Then, the pellets were washed twice using RPMI medium. After that, the pellets containing lymphocyte cells were suspended with an RPMI medium. Cells were collected at 1.5 × 106 cells/mL and cultured in a CO_2 incubator at 37°C.[14]

A total of 100 µL of lymphocyte cells (2.5 × 106 cells or 250,000 cells) were distributed into wells of a 96-well plate in the form of concentration series (125, 250, 500, 750 µg/mL) in the parijoto fruit fraction and control cells. Then, 10 µL hepatitis B vaccination was administered per well and incubated for 24 h. A volume of 100 mL of the test sample was added per well for each concentration series for three replications, so 300 µL was obtained for each concentration series and rounded to 500 µL. Then, it was incubated for 48 h and followed by adding 10 µL of MTT solution 0.5 mg/mL to each well. Then, it was incubated for 4 h at 37°C. The reaction with MTT stopped by adding a solution of 10% SDS in 0.01 N hydrochloric acids (HCl) as much as 50 µL in each well. The measurement absorbance was analyzed at 550 nm. Absorbance data are converted into stimulation index (SI) data about lymphocyte cell proliferation.[14]

RESULTS

Determination of total flavonoid level

The data indicate that the ethyl acetate fraction has the largest flavonoid, 7.409 ± 0.49 mg QE/g sample [Table 1]. Next is the n-hexane fraction with the largest total flavonoid content after the ethyl acetate fraction, while the water fraction with the lowest flavonoid content. The high levels of total flavonoids in the ethyl acetate fraction explained that the characteristics of the flavonoid compounds in the parijoto fruit extract were presumed to contain more compounds that had the same polarity as ethyl acetate. The presence of a methoxy group in the chemical structure of ethyl acetate is apparently responsible. Ethyl acetate is

Table 1: Test results of parijoto fruit fraction total flavonoid level

Sample	mg QE/g sample
N-hexane fraction	6.778±0.44
Ethyl acetate fraction	7.409±0.49
Water fraction	1.628±0.11

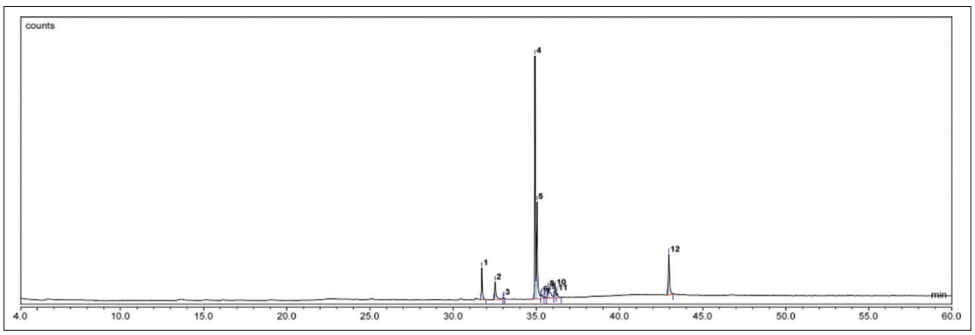

Figure 1: Gas chromatography–mass spectrometry chromatogram of parijoto fruit extract

a semipolar compound with the formula $CH_3CH_2OC(O)CH_3$ has a methoxy group (CH3O-), estimated that it can attract compounds with a wide polarity range, ranging from nonpolar or polar compounds to produce a variety of compounds belonging to this class of flavonoids.

Gas chromatography–mass spectrometry analysis

The determination of compound parijoto fruit extract based on GC-MS is shown in Figure 1.

The parijoto ethanol extract yielded 12 distinct main components [Figure 1 and Table 2]. The primary constituent in the extract is 9,12-Octadecadienoic acid (Z, Z)-, discovered at a retention time of 34.92 min. Nevertheless, based on the GC-MS identification, one component is very interesting. The compound is Ethyl iso-allocholate, classified under the terpene group. The chemical was seen with a retention time of 35.32. The ethyl iso-allocholate molecule exhibited an m/z of 436.

Macrophage phagocytic activity assay

The observation of macrophage phagocytic activity was carried out using phagocytic index (PI) and PC parameters. The immunomodulatory activity observed from the results of the PI and PC values of the parijoto fruit was higher than in the control cells.

Phagocytic capacity of macrophage cells

The final results of the analysis revealed that all samples of parijoto fruit fraction significantly increased the capacity of macrophages [Figure 2]. An increase in the concentration of the fraction of parijoto fruit correlates with an enhancement in the PC observed.

Macrophage cell phagocytosis index

Theoretically, a phagocytosis index (PI) value of <1 suggests that the test substance exhibits immunosuppressant activity, indicating its potential to suppress the immune system. Conversely, a PI value <1 signifies that the test substance demonstrates immunostimulant activity, implying its ability to stimulate or enhance the body's endurance.[15] The phagocytosis index data showed that it presumed that all of fraction could be classified as immunostimulants because they had an PI >1 [Figure 3].

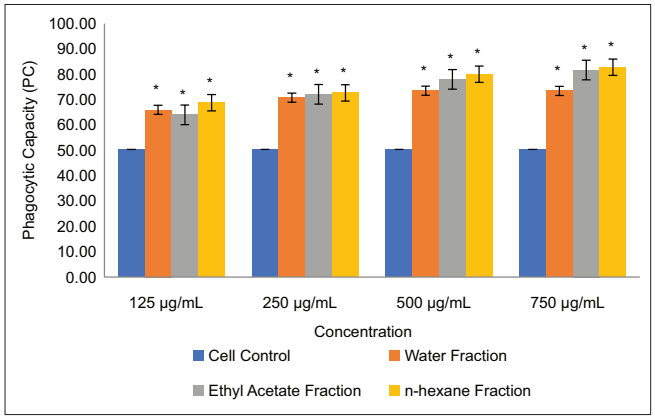

Figure 2: The phagocytosis capacity of macrophage against latex beads. *Demonstrates a statistically significant difference relative to the control ($P < 0.05$)

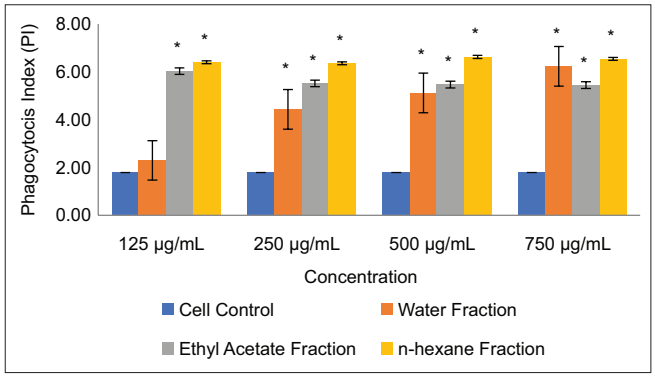

Figure 3: Macrophage phagocytosis index from parijoto fruit fraction against latex beads. *Demonstrates a statistically significant difference relative to the control ($P < 0.05$)

Table 2: Phytochemical compound in ethanolic extract of parijoto fruit using gas chromatography–mass spectrometry

Number	Retention time/min	Name of the compound	Chemical formula	Molecule weight	Retention area (%)
1	31.73	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	6.33
2	32.51	n-hexadecanoic acid	$C_{16}H_{32}O_2$	256	5.74
3	33.04	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.30
4	34.92	9,12-octadecadienoic acid (Z, Z)-, methyl ester	$C_{19}H_{34}O_2$	294	41.74
5	35.03	9,12,15-octadecatrienoic acid, methyl ester, (Z, Z, Z)	$C_{19}H_{32}O_2$	292	22.53
6	35.32	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	1.49
7	35.51	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.44
8	35.70	13-heptadecyn-1-ol	$C_{17}H_{32}O$	252	2.42
9	35.81	9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z, Z, Z)	$C_{21}H_{36}O_4$	352	3.71
10	36.12	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	308	2.21
11	36.24	9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z, Z, Z)	$C_{21}H_{36}O_4$	352	1.66
12	42.96	Phthalate acid	$C_{24}H_{38}O_4$	390	11.44

Lymphocyte cell proliferation index

The ethyl acetate fraction has highest SI compare with cell control and other fraction. The higher the resulting SI value indicates the higher the lymphocyte proliferative activity that occurs. The Stimulation Index (IS) for lymphocyte proliferation is considered positive when the index value stimulation (IS) value exceeds 3, indicating an increase in lymphocyte cell proliferation and it is said to be weakly positive if the SI value is between 2 and 3. Meanwhile, if the IS is smaller or <2 then it is said to be negative in providing an increasing effect on lymphocyte cell proliferation.[16]

DISCUSSION

The activity of macrophage cells evidences the innate immune reaction. In contrast, the adaptive immunological response is demonstrated by the lymphocyte proliferation and the administration of parijoto fruit fractions. The ethyl acetate and n-hexane fractions can enhance both the phagocytosis index and PC [Figures 2 and 3]. The liquid–liquid partition purification separates the crude extract into three portions according to varying polarity. The ethyl acetate fraction identified in this research shows the highest flavonoid concentration (7.409 ± 0.49 QE/g sample). The high levels of total flavonoids in the ethyl acetate fraction explained that the characteristics of the flavonoid compounds in the parijoto fruit extract were presumed to contain more compounds that had the same polarity as ethyl acetate. Based on various studies, flavonoid compounds can be well responded to by the body's immune system, thereby stimulating an increase in the secretion of cytokines produced by immunocompetent cells, including interleukin-1 (IL-1) and IL-6 that increase the phagocytic activity of macrophages. Flavonoid compounds also increase IL-2 which will activate lymphocyte proliferation. CD4+ cells will be influenced by lymphocyte proliferation, which will lead to the activation of Th1 cells. Activated Th1 cells will have an impact on specific macrophage activating factor (SMAF). SMAF comprises many chemicals, including the lymphokine IFN-γ, which is synthesized by T cells. The phagocytic activity of macrophages is enhanced by the activation of interferon-γ (IFN-γ). IFN-γ serves as the primary cytokine for macrophage activation, enhancing macrophage function and promoting increased phagocytic activity.[17,18]

The result of GC-MS showed the parijoto contain 9,12-Octadecadienoic acid (Z, Z)-, a methyl ester, phthalate acid, and ethyl iso-allocholate [Table 2]. The compound may be responsible for immunomodulatory effect. The ethyl iso-allocholate are steroidal derivate which have cytotoxic activity in cancer cell and reduce tumor growth. Some terpenoid compound have known immunomodulation effect by induction of phagocyte macrophage, increase activity of NK cell, and enhance proliferation of B and T cells.[19,20] The other effect of ethyl iso-allocholate are report by Malathi *et al.*,[21] methanolic extract from medicinal rice karungkavuni are steroidal which have the potent inhibitor of dihydropteroate synthase and antimicrobial activity.

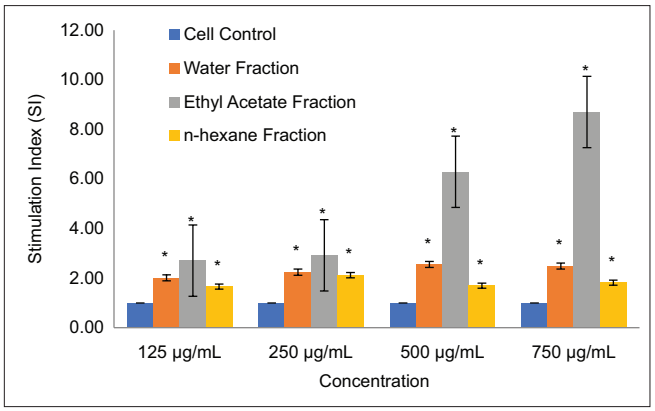

Figure 4: Lymphocyte proliferation stimulation Index from parijoto fruit fraction. *Demonstrates a statistically significant difference relative to the control ($P < 0.05$)

The ethyl acetate fraction has highest SI compare other sample [Figure 4]. There is a possibility that the ethyl acetate fraction attracts more active compounds of parijoto fruit extract that have characteristics similar to semipolar flavonoid aglycones such as catechins, anthocyanins, leucoanthocyanins, aurons, chalcones, and flavanonols which soluble in polar and semi-polar solvents. The bioactive compound aglycone flavonoid is presumed to increase the activity of lymphocyte proliferation, possibly caused by a bioactive compound in the form of a phenolic compound (C_6H_5OH), which is a compound that has one or more hydroxyl groups (-OH) which is attached to a phenyl ring ($-C_6H_5$) derived from a benzene molecule (C_6H_6) or aromatic hydrocarbons in the chemical structure of flavonoid aglycones.

These phenolic compounds act as antigens and can know by B-cell and T-cell receptors. The content of these compounds is presumed to interact with T-cell receptor-TCR through hydrogen bonding, while B cells can bind to surface receptors (immunoglobulins M. The binding of the antigen with the T-cell surface receptor and IL-1 from the antigen-presenting cell activates G-proteins and leads to the production of phospholipase C. This enzyme catalyzes the hydrolysis of phosphatidyl inositol bisphosphate (PIP2) to produce the reactive products diacylglycerol and inositol triphosphate. IP3 subsequently induces the release of Ca^{2+} into the cytoplasm, resulting in an enhance in Ca^{2+} concentration. The elevation of Ca^{2+} is implicated in the expression of protein kinase C and 5-lipoxygenase enzymes. Protein kinase C enhances IL-2 production, subsequently activating the proliferation of B and T cells.[22] This is presumed to be the cause of the ethyl acetate fraction being capable of increasing the activity of lymphocyte cell proliferation as an adaptive or specific immune response after administration of the hepatitis B vaccine to lymphocyte cells. A compound is said to be an immunostimulatory if it

can increase the immune responses formed previously due to exposure to an antigen.

Flavonoid compounds generate a positive response from the immune system, leading to an enhance in the secretion of cytokines by immunocompetent cells, such as IL-1 and IL-6, and enhancing the activity of macrophages. In addition, flavonoid compounds can also act on lymphokines (IFN-γ) generated by T cells hence enhancing the responsiveness of phagocytosis process and can stimulate lymphocyte proliferation, increase the number of T cells, and increase the release of IL-2, IL-1, IL-12, IL-6, and TNF which increase antigen phagocytosis by macrophages.[23]

CONCLUSION

The total flavonoid levels exhibit a correlation with the immunomodulatory activity of the n-hexane, ethyl acetate and the aqueous fraction derived from parijoto fruit extract. This investigation demonstrated that the parijoto fruit fraction may augment the phagocytic activity of macrophages and stimulate lymphocyte proliferation *in vitro*. The ethyl acetate fraction with is considered the most promising component of the parijoto fruit extract for further investigation as an immunomodulator. Further research is necessary to determine the optimal dose that can stimulate an immunological response, and undertaking additional toxicity studies for practical applications addressing this issue is essential.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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