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The Potency of Guava *Psidium guajava* (L.) Leaves as a Functional Immunostimulatory Ingredient

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Abstract

The potential of natural substances to improve the immune system has long been the subject of investigation. The purpose of this research was to study Guava (*Psidium guajava* L.) leaf extract as a functional ingredient for immunostimulant. The study used water and ethanol as solvents to obtain optimum active compounds of the extracts. The result showed that the higher the content of phenol total was found in the extract, the higher the stimulation index value was obtained for both solvents. However, the stimulation index value was not only influenced by antioxidant activity. The reason was that the type of active compound in Guava leaf extract responsible for immunostimulatory activity was probably not only polyphenolic antioxidant.

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Keywords: Antioxidant; functional ingredient; immunostimulant; phenol total; *Psidium guajava* (L.) extract

Nomenclature	
GAE	Gallic Acid Equivalents, is an expression of total phenol content (mg · g^{-1})
SI	Stimulation Index, is average optical density of treatment (with stimulation) / average optical density without stimulation (medium only), [%]
RSA	Radical Scavenging Activity, [mg BHA equivalent · g^{-1}]
rpm	revolutions per minute, 1 hertz is equal to 60 rpm

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1. Introduction

Guava (*Psidium guajava* L.) has been used traditionally in the treatment of various diseases. In Indonesia, Guava leaf is commonly used to treat diarrhea, gastroenteritis and other digestive complaints, while the Guava fruit has been used to increase platelets in patients with dengue fever. Many studies have been done to scientifically prove efficacy in the treatment of guava leaf. Among them were the benefits of guava leaf as a remedy antiarthritison animal testing using hydro alcoholic extract[1].

Another study proved that the flavonoids content in extract of guava leaves acts as an antibacterial activity, while the antidiarrheal properties of guava leaf extract caused by quercetin content. Quercetin is one of the most abundant flavonoids found in guava leaf. It is able to relax intestinal smooth muscle and inhibit bowel contractions[2]. Extract of Guava leaves showed anti proliferative activity in vitro tests using leukemia cells. Its activity was 4.37 times more than the activity of vincristine[2]. Moreover, water extract of guava leaves was described to be effective against a number of microbial strains and anti-rotavirus activity[3]. Genotoxic studied of the *P.guajava* leaf has been done by Ofodile et al.[4]. Genotoxicity and mutagenicity testing were an important part of the hazard assessment of chemicals for regulatory purposes[5]. The water extract of Guava was effective in inactivating the mutagenicity of direct acting mutagens[3].

The ability of guava leaf extract on the treatment of various diseases has been proven scientifically, but the mechanism hasn't been fully explained. In general, biological properties of guava have been already associated with its polyphenolic compounds, such as protocatechuic, ferulic, ascorbic, gallic and caffeic acids and quercetin[6]. Polyphenols are secondary metabolites of plants. In the last decade, there has been much interest in the potential health benefits of dietary plant polyphenol as antioxidant[7]. The polyphenol compounds in the extract of guava fruits and leaves can act as an immunostimulant that may lead to an increase in the immune system. Increasing the body's immune system can keep the body from various infectious diseases. A well-functioning immune system is crucial for staying healthy. Therefore, the potential of natural substances to strengthen the immune system has long been the subject of investigation[8]. There were many synthetic and natural preparations claiming to be immunostimulants. They seemed to represent useful alternative to vaccination and chemotherapy in the control of disease. Immunostimulants from natural substances could enhance the specific immune respone[9].

The presence of active compounds in food plants or herbs that are beneficial to health can be used as a source of functional ingredients. Functional ingredient is a bioactive compounds present as natural constituents or as fortification in food having the potential to provide health benefits beyond the basic nutritional value of the product[10]. Some natural substance that can act as an immunostimulant was polysaccharides[11], peptides[12], oligo-nucleotide[13], and antioxidant[14].

The modulation of immune response by using medicinal plant products as a possible therapeutic measure has become a subject of active scientific investigation. This study aim was to determine the potential of guava leaf as an immunostimulant. In addition we would also determine a group of active compounds that play a role in immune enhancement

2. Material and methods

2.1. Materials

Dried mashed of guava (*P.guajava*) leaves, Folin-Ciocalteau's reagent, and gallic acids (Sigma-Aldrich, St. Louis, MO, USA) as standard, ethanol absolute, Na_2CO_3, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Ficol-Hypaque solution form Sigma, Roswell Park Memorial Institute medium (RPMI)-1640, concovalin A (Con A) (Sigma), lipopolysaccharides (LPS) (Sigma), penicillin-streptomycin, 3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazoliumbromide(MTT) (Sigma), tryphan blue, EDTA-$Na_2H_2 \cdot 2H_2O$, phosphate buffer saline (PBS), Tris-HCl buffer**,** 3-tetra-butyl-4-hydroxyanisole (BHA)**.**

2.2. Preparation of the extract

2.2.1 Water extracts

1 g dried mashed of Guava leaves was extracted with 50 mL of boiling water 100 °C for (5, 10, 15 and 20) min. The obtained Guava leaf extract was filtered using a vacuum pump with filter paper (Whatman No. 1) and evaporated using rotary evaporator to obtain the final extract volume of 10 mL.

2.2.2 Ethanol extracts

1 g dried mashed of Guava leaf was extracted with 100 mL of 96 % ethanol and shaked at room temperature with periodical mixing (240 rpm) for (1, 6, 12 and 24) h, and then obtained Guava leaf extract was filtered using a vacuum pump with filter paper (Whatman No. 1). The residual matter on the filter paper was added with another ethanol solvent and the extraction was repeated three times. All extracts were collected and mixed into one bigger glass, and were evaporated under reduce pressure using rotary evaporator at around 50 $^{\circ}$C to 55 $^{\circ}$C until the solvent had evaporated (9 mL). The evaporated extract was added with aqueous 96 % ethanol until 10 mL. The supernatant as the ethanol extract solution was used in this study

2.3. Determination of phenol total content

The phenolic total content was determined by the method of Singleton and Rossi[15], by using gallic acids as a standard.

2.3.1 Preparation of reagents.

Gallic acid standard solution (5 mg · mL^{-1}) : 0.25 g of gallic acid was added with 5 mL of 96 % ethanol and distilled water to a volume of 50 mL.

20 % Na_2CO_3 solution : 5 g Na_2CO_3 was added with 20 mL of distilled water, and heated to boiling. Let stand for 24 h, filtered and diluted with distilled water to 25 mL

2.3.2 Preparation of the calibration curve of gallic acid with Folin-Ciocalteu reagent

Stock solution was prepared by dissolving 5 mg of gallic acid in 1 mL of distilled water. (300, 400, 500, 600, and 700) mg · L^{-1} gallic acids standard solutions were prepared by diluting stock solution step by step with distilled water (6 mL, 8 mL, 10 mL, 12 mL, 1 mL of stock solution was diluted with distilled water to a volume of 100 mL).

Assay: 0.0395 mL of each standard was added with distilled water up the volume to 0.5 mL and dissolved with 2.5 mL of Folin-Ciocalteu's reagent (diluted in water 1 : 10) was placed in tubes and, after 8 min, 7.5 mL of sodium carbonate (20 %) were added. The tubes were kept away from the light and, after two h, the absorbance was read in a spectrophotometer (Hitachi, Japan) at 765 nm. The total phenolic content was expressed as mg/g gallic acid equivalents (GAE).

2.3.3 Determination of total phenolic content

Determination of total phenol content was done based on the reaction between phenol compounds with phosphomolybudate-phosphotungstate reagent (Folin-Ciocalteu solution) and will give a yellow color, and the addition of an alkali will produce a blue color.

A volume of 0.5 mL of the extract and 2.5 mL of Folin-Ciocalteu's reagent (diluted in water 1 : 10) was placed in tubes and, after eight min, 7.5 mL of sodium carbonate (20 %) was added. The tubes were kept away from the light and, after 2 h, the absorbance was read in a spectrophotometer (Hitachi, Japan) at 765 nm. The total phenolic content was expressed as mg · g^{-1} of GAE.

2.4. Measurement of DPPH radical scavenging activity (RSA)s

2.4.1 Preparation of reagents

0.2 mM DPPH solution: Dissolved 19.7 mg of DPPH in 250 mL of absolute ethanol. Freshly prepare every time before use.

100 mMTris-HCl buffer: Dissolved 12.1 g of Tris in 800 mL of distilled water and adjusted the pH to 7.4 with HCl, then fill up a volume to 1 000 mL

BHA standards: 5 mM stock solution was prepared by dissolving 90 mg of BHA in 100 mL absolute ethanol. Standard solutions (50 μM to 500 μM) were prepared by diluting stock solution with ethanol.

2.4.2 Assay

The scavenging activity of Guava leaf extracts on the DPPH radical were determined by a spectrophotometer assay based on procedure described by Yamaghuci[16].

The Guava leaf extract solution or BHA (0.2 mL) was mixed with 0.8 mL of 100 mMTris-HCl buffer. Add 0.2 mM DPPH ethanolic solution (1 mL), the mixture was vortex for 1 min and then left to stand at room temperature for 20 min in the dark, and its absorbance was red at 517 nm. The ability to scavenge the DPPH radical was calculated as BHA equivalent from the standard correlation obtained from BHA standards. Alternatively, the activity is revealed as percentage according to the following formula given by Yamaguchi. The content of antioxidant was expressed as mg BHA equivalent/g.

$$\text{Formula:} \quad \%\ \text{RSA} = \frac{A - B}{B} \times 100\% \tag{1}$$

% RSA which was calculated as a percentage of DPPH discoloration, A is absorbance of control and B is absorbance of sample.

2.5. Immunostimulatory test

Immunostimulatory activity was tested in vitro using lymphocyte proliferation test by assay[17].

2.5.1 Lymphocyte isolation

Human lymphocyte was isolated from peripheral blood by centrifugation based on Ficol-Hypaque differential density. First centrifugation at 514 g for 10 min was aimed to separate cellular components. Red blood cells which were heavier would be at the bottom while blood lymphocytes would be concentrated at the top of the solutions. Buffy coat layer which mainly composed of human lymphocyte would be located in between those layers was taken carefully and dissolved into 3 mL of RPMI basic media. The next step was separating the lymphocyte suspension in basic media by following the suspension slowly on top of the Ficol-Hypaque solution to form two layers. Centrifugation was done at 1 430 g for 30 min to get granulocyte and red blood cells in the bottom while lymphocyte, monocyte and platelets on the top. The top layer was washed 2 times with basic media and centrifuged at 228 g for 30 min to get the lymphocyte on the precipitate. Lymphocyte cells were counted by trypan blue method and dissolved into RPMI media to get 10^6 cells · mL^{-1}.

2.5.2 Lymphocyte proliferation response analysis with MTT assay

80 μL lymphocyte suspension cells (10^6 cells · mL^{-1}) grown on RPMI were dispensed into micro wells. Into each well, 20 μL pomegranate extract with various concentrations were added. Final concentration for each extract

was 0.1 µg . mL^{-1}, 0.25 µg . mL^{-1}, and 0.5 µg . mL^{-1}. As control, RPMI 1 640 media without extract was used. Incubation was done for 72 h at CO_2 incubator. Following incubation, the cells were treated with MTT and were incubated further for 4 h. Lymphocyte proliferation activity was expressed as % Stimulation Index (% SI). Measurement was done using ELISA reader.

Formula:
$$SI = \frac{T}{C} \times 100\ \% \quad (2)$$

Where SI is stimulation index, T is optical density of treatment (with stimulation) and C is optical density of control (without stimulation, medium only), [%]

3. Results and discussion

Extraction is influenced by the type of solvent, temperature and time. Solvents often used for extraction of phenolic compounds are methanol, ethanol, acetone, water, ethyl acetate, propanol and combinations of these solvents. However, there is no suitable solvent used for isolation of the whole phenolic components. In this research, the extraction process had been done by using water and 96 % ethanol. The result of different extraction times and solvents used on optimization of extraction can be seen in Figure 1 (a) and (b). In water solvent, the highest phenol total content occurred in the extraction time 20 min. While extraction used ethanol solvent, the highest phenol total content was achieved at extraction time 6 h. In both conditions, the phenol total content of water and ethanol extract were not significantly different, 101.93 and 101.20, respectively. However, parameters measured in this study could not explain why the polyphenolic compounds in the water and ethanol extracts were similar.

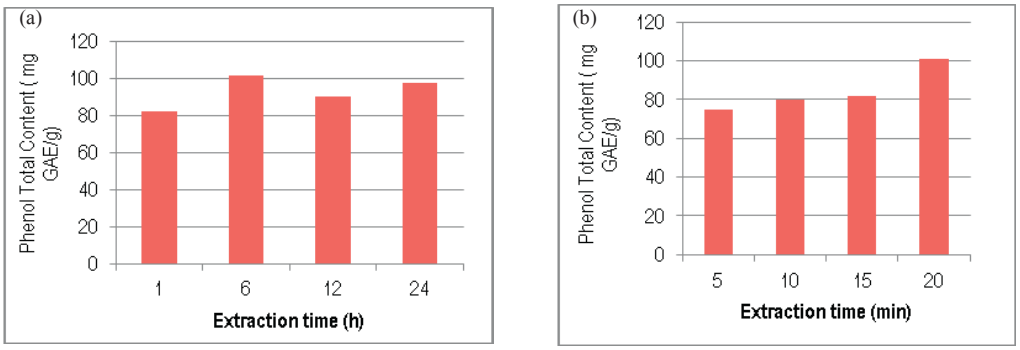

Fig.1. (a) average of phenol total content in ethanol extract at different time extractions;
(b) average of phenol total content in water extract at different time extractions.

The time required to extract the active compounds depend on the amount of active compounds to be extracted and the solvent used. The more active compounds content, the extraction time required would be longer. The optimum time required to extract polyphenolic compounds from guava leaves with ethanol was 6 h. In the subsequent extraction time, the amounts of polyphenolic compounds were relatively stable or decrease. Decreased levels of phenol content in the extraction of 12 h and 24 h (Fig. 1a), may be caused by the destruction of polyphenolic compounds due to prolonged contact with solvents.

Deny et al.[6] found that the phenolic total content of ethanol extract from Guava pomace was (3.40 ± 0.09) mg GAE · g^{-1}. In this study, the extraction time used was 30 min by ethanol solvent. While other researchers used 75 % acetone as a solvent for 24 h extraction time at room temperature produced phenolic content of Guava extract was 44.05 mg GAE · g^{-1} [18]. Such difference between the values of phenol total obtained showed that the values of phenol total produced depend on the type of solvent, extraction time and part of the plant used. In this study we used Guava

leaf, while other studies used waste Guava[6] and Guava fruit[18]. Guava fruit had high levels of polyphenolic compounds such as myricetin and apigenin, ellagic acid, and anthocyanins[16]. While Guava leaf contained polyphenolic compounds such as isoflavonoids, gallic acid, catechin, epicathechin, rutin, naringenin, kaempferol[19].

The measurement result of antioxidant content in ethanol and water extracts showed difference. Antioxidant content of ethanol extract was higher than water extract (Table 1). Water is a common solvent used in the extraction process, but ethanol has a greater polarity than water, so it can dissolve more polar compounds contained in the sample than water solvent. Kim et al.[20] mentioned that the extraction efficiency of the bio-active ingredients was correlated with the solvent polarity.

Table 1. Comparison of stimulation index, antioxidant and phenol total contents in water and ethanol extracts

Parameters	Water extract	Ethanol extract
Phenol total content (mg GAE · g^{-1})	101.20	101.93
Antioxidant content (mg BHA eqv · g^{-1})	78.6	201.71
Stimulation Index (%)	1 567	1 539

The level of antioxidant in the leaf extract was higher than that in fruit extract (1.426 mg · g^{-1} and 0.722 mg · g^{-1} in white and pink pulp[18]. While the level of antioxidant in the steam bark extract was 1.12 mg · g^{-1} [21]. Barbalhoet et al.[19] have reported the presence of higher amounts of phenolic compounds with antioxidant activity in the leaf of white (*P.guajava* var. pyrifera L.) and red guava (*P.guajava* var. pomifera L.) when compared with other vegetable species.

There were no linier correlation between radical scavenging activity and phenolic content (Table 1). This result was in contrast to Asha et al.[22] which had found that phenolic compounds mainly responsible factor for the high antioxidant activity in Guava, a strong positive correlation was found between antioxidant activity and phenolic compounds. Asha et al.[22] used Guava fruit, while in this study we used Guava leaf. Joseph and Priya[2] mentioned that there were differences in the active compounds in the Guava leaf and fruit.

Many researchers have been demonstrating the presence of a wide variety of bioactive compounds in the leaf of *P.guajava* that are capable of showing beneficial effects on human health. Extract of Guava leaves has analgesic, anti-inflammatory, antimicrobial, hepato protective and antioxidant activities. These effects are probably due to the presence of polyphenolic compounds[19].

The potential extract of Guava leaves as an immunostimulatory agent had been measured in this study. The immunostimulatory activity was expressed as % Stimulation Index (% SI). The result of immunostimulatory activity of the water and ethanol extract showed on Table 1. There were no differences in percent of stimulation index of the water and ethanol extract. These values had correlation to the phenol content in these extracts, but not with the antioxidant content. Immunostimulatory activity of the extract was influenced by the type of active compounds. The active compounds in guava leaf extract that contributes to the immunostimulatory activity were probably not only polyphenolic antioxidant.

Guava leaf had high contents of polyphenolic compounds and antioxidant, and high activity of immunostimulant (Table 1). Compared with LPS as a comparison sample, the percent of stimulation index of water and ethanol extracts were 12.7 times and 12.5 times, respectively. While for Con A as a comparison sample, the percent of stimulation index of water and ethanol extracts were 5.4 times and 5.3 times, respectively. These indicated that guava leaf was excellent source of active compounds for immunostimulatory functional ingredient additives.

4. Conclusion

Guava leaf has great potential to be developed as functional ingredients. Firstly, they are widely available, with a guaranteed supply. Secondly, guava leaf naturally occurring compounds, and their extraction is relatively cost effective. Lastly, they contained high level of antioxidant, phenolic compound and biological activities as immunostimulatory agents.

Based on the measurements results of phenol total content, antioxidant and immunostimulant activity, the active compounds of the guava leaf expected to have immunostimulatory activity were probably not only polyphenolic

antioxidant compounds. Further research is needed to determine the active compounds that act as immunostimulatory agents from extract of guava leaves.

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