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Optimization of Extraction Solvent for Analysis of Total Phenolic and Antioxidant Activity of Guava Leaves (*Psidium guajava*) using Simplex Centroid Design

Optimasi Pelarut Ekstraksi untuk Analisis Total Fenolik dan Aktivitas Antioksidan Daun Jambu Biji (*Psidium guajava*) menggunakan *Simplex Centroid Design*

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ABSTRACT

Guava (*Psidium guajava*) is a popular tropical plant widely used as processed food and medicine for various diseases. This plant contains highly bioactive compounds like quercetin, flavonoids, and ferulic acid. The bioactive content can be influenced by the extraction method. However, the solvent used for extraction can also affect the bioactive content. Therefore, this study aims to determine the best solvent or combination of extraction solvents to extract guava leaves based on total phenolic content (TPC) and antioxidant activity using simplex centroid design (SCD). This research used guava leaf samples with various solvent combinations of water, ethanol, and acetone. The determination of phenolic content was determined using the TPC method, while the determination of antioxidants was conducted using the FRAP and ABTS methods. The research results showed that the highest TPC levels were produced by the solvent water : ethanol : acetone. Then the highest levels of FRAP and ABTS antioxidants were produced by the water : ethanol solvent. The solvent optimization solution obtained was using water : ethanol: acetone with a ratio of 0.462:0.436:0.102, respectively.

Keywords: antioxidant, phenolic, optimization, solvent, *Psidium guajava*

ABSTRAK

Jambu biji (*Psidium guajava*) merupakan tanaman tropis populer yang banyak dimanfaatkan sebagai makanan olahan maupun obat berbagai penyakit. Tanaman ini mengandung senyawa bioaktif yang tinggi seperti kuersetin, flavonoid, dan asam ferulat. Kandungan bioaktif tersebut dapat dipengaruhi oleh metode ekstraksi. Namun, pelarut yang digunakan untuk ekstraksi juga dapat mempengaruhi kandungan bioaktifnya. Oleh sebab itu, penelitian ini bertujuan menentukan pelarut atau kombinasi pelarut ekstraksi terbaik untuk mengekstrak daun jambu biji berdasarkan kadar total fenolik (TPC) dan aktivitas antioksidan menggunakan *simplex centroid design* (SCD). Penelitian ini menggunakan sampel daun jambu biji dengan berbagai kombinasi pelarut air, etanol, dan aseton. Penentuan kadar fenolik ditentukan menggunakan metode TPC, sementara penentuan antioksidan dilakukan menggunakan metode FRAP dan ABTS. Hasil penelitian menunjukkan bahwa kadar TPC tertinggi dihasilkan oleh pelarut air : etanol : aseton. Kemudian kadar antioksidan FRAP dan ABTS tertinggi dihasilkan oleh pelarut air : etanol. Solusi optimasi pelarut yang didapat yaitu penggunaan air : etanol : aseton dengan rasio berturut-turut 0,462:0.436:0.102.

Kata Kunci: antioksidan, fenolik, optimasi, pelarut, *Psidium guajava*

INTRODUCTION

Guava (*Psidium guajava*) is a popular tropical plant that is widely grown from the phylum Magnoliophyta, class Magnoliopsida, and family Myrtaceae (Dakappa et al., 2013). This plant has the characteristics of a small tree up to 10 m high with wide-spreading branches, oval leaves 5–15 cm long, and pinnate leaf veins. The flowers of this plant consist of four to six white petals and white stamens with yellow anthers. The color of the fruit skin and flesh varies between cultivars depending on the type and amount of pigment (Flores et al., 2015). Guava fruit is popularly consumed throughout the world because it tastes delicious and has various health benefits. Apart from that, guava fruit is also an important ingredient used for drinks, juices, and wine.

Guava has been widely used from various parts, such as leaves, roots, stems, bark, and fruit, to treat stomach aches, diarrhea, and other health ailments in many countries (Díaz-de-Cerio et al., 2017). Guava leaves, together with the pulp and seeds, can be used to treat various diseases, such as certain respiratory and digestive disorders, as well as to increase platelets in patients suffering from dengue fever. Guava leaves are also widely used as anti-spasmodic, cough sedative, anti-inflammatory, anti-diarrheal, anti-hypertensive, anti-obesity, and anti-diabetic. Animal Model studies have determined that the role of seed leaf isolates can also be as a powerful anti-tumor, anti-cancer, and cytotoxic agent (Kumar et al., 2021). Guava leaf extract also has pharmacological activity because it has bioactive compounds that can also treat dysentery, flatulence, gastric problems, and regulate blood glucose levels (Shaheena et al., 2019).

Guava is known to contain quite high levels of bioactive compounds. The unique and diverse bioactive polyphenolic compounds from guava leaves are essential oils that are rich in cineol, triterpenoids, tannins, eugenol, kaempferol, malic acid, gallic acid, chlorophyll, and mineral salts (Shaheena et al., 2019). Apart from that, this plant also contains quercetin, flavonoids, ferulic acid, and caffeic acid which determine bioactive and therapeutic properties. These phenolic compounds are known as secondary metabolites which show strong antioxidant and immunostimulant activity (Kumar et al., 2021).

The extraction results and antioxidant activity of guava leaves are influenced by the extraction method used and depend on the solvent used for extraction. This is because various antioxidant and phenolic compounds have different chemical characteristics and polarities, so they may or may not dissolve in certain solvents (Do et al., 2014). Extraction using solvents with three different polarities is more effective than using just one solvent because it can adjust to the level of polarity of the sample being evaluated (Wuryatmo et al., 2021). Thus, optimization of extraction using solvents on guava leaves has not been done much. Therefore, this research aims to optimize the guava leaf extraction process using a simplex centroid design to obtain the highest optimum extraction solvent and type of compound in guava leaves.

METHODS

1. Sample Preparation and Extraction

Guava leaves are ground with a blender until they become a fine powder, then sifted through a sieve. Guava leaf extract was obtained using the MAE (Microwave Assisted Extraction) technique. Four grams of guava leaf powder was put into seven Erlenmeyer flasks to which 20 mL of solvent had been added each. The composition of the solvent used can be seen in **Table 1**.

Table 1. Optimization of *Psidium guajava* extraction solvent using the simplex centroid design method

Run	Solvent (mL)			Variable response		
	Water (A)	Ethanol (B)	Acetone (C)	TPC (mg GAE/g)	FRAP (µmol TE/g)	ABTS (µmol TE/g)
1	450	0	0	32.23	212.07	441.66
2	225	225	0	59.78	386.40	1014.47
3	225	0	225	52.26	341.23	812.05
4	0	450	0	39.14	186.07	536.67
5	0	225	225	52.83	255.40	602.77
6	0	0	450	9.74	125.90	59.34
7	150	150	150	62.83	332.73	297.06

2. Total Phenolic Content Test (Nurcholis et al., 2022 with modifications)

The total phenolic content was determined using the Follin-Ciocalteu method. First, 20 µL of sample extract was mixed with 120 µL of 10% Follin-Ciocalteu in a microplate and incubated for 5 minutes. Then, 80 µL Na2CO3 was added to the sample and incubated for 30 minutes in the dark. Absorbance was measured using a microplate reader (Spectrostar Nano, BMG LABTECH) at a wavelength of 759 nm. The calibration curve used gallic acid with varying 20-300 ppm concentrations. The total phenolic content was then expressed in milligram gallic acid equivalents per gram of extract (mg GAE/g).

3. Antioxidant Activity Test (Nurcholis et al., 2022 with modifications)

Antioxidant activity was determined using the FRAP and ABTS methods. In the FRAP test, the solution was prepared by mixing acetate buffer (pH 3.6), 10 µM tripyridyl-s-triazine (in 40 mM HCl), and 20 mM $FeCl_3$ in a ratio of 10:1:1 (v/v/v) and stored in a dark bottle. After that, 20 µL of sample extract was mixed with 290 µL of FRAP in a microplate and incubated for 30 minutes. Absorbance was measured using a microplate reader (Spectrostar Nano, BMG LABTECH) at a wavelength of 583 nm. A calibration curve was made using Trolox concentrations of 100-600 µM. Antioxidant activity was further expressed as the micromoles of Trolox equivalent per gram of extract (µmol TE/g).

Next, the antioxidant activity test was determined using the ABTS method. The ABTS stock solution was made by adding 90 mg of ABTS with aquabidest to 25 mL into a volumetric flask, then stored in a dark bottle, wrapped in aluminum foil, and put in the refrigerator. After that, potassium persulfate was made by adding 66.289 mg K2S2O8 with aquabidest to a volume of 100 mL to produce 2.4 mM K2S2O8, then stored in a dark bottle, wrapped in aluminum foil, and put in the refrigerator. The ABTS reagent was made by mixing 7 mM ABTS and 2.4 mM K2S2O8 in a ratio of 2:1, then adding aquabidest. After that, the absorbance of the reagent was measured with a nano spectrophotometer with a wavelength of 734 nm to obtain A = 0.7 ± 0.02. Next, 20 µL of the sample was added with 180 µL of ABTS reagent and incubated for 6 minutes in the dark at room temperature. Absorbance was measured with a nano spectrophotometer at 734 nm. Antioxidant activity was expressed in the micromoles of Trolox equivalent per gram of extract (µmol TE/g).

4. Data Analysis (Nurcholis et al., 2022 with modifications)

Data from extraction optimization results were analyzed using Design Expert 13.0 software (Stat-Ease Inc, Minneapolis, USA). Significant differences were calculated in Ducan's test ($P < 0.05$) using SPSS 22.

RESULTS AND DISCUSSION

1. Sample Extraction Optimization

This research's sample extraction optimization process used the Simplex Centroid Design (SCD) method using Analysis of Variance (ANOVA) software. Experiments on the optimization process with SCD are based on the research results of Presenza et al. (2021) and carried out randomly to avoid division errors consisting of seven different formulations, namely solvents A, B, and C, which are solvents that correspond to pure components, including water solvent (A), ethanol solvent (B), and acetone solvent (C). The solvents AB (water : ethanol), AC (water : acetone), and BC (ethanol : acetone) are types of binary mixture solvents of two different pure component compounds. Lastly, ABC solvent (water : ethanol : acetone) is a type of ternary mixed solvent or a mixture of more than two different pure component compounds. The SCD results make it possible to identify the synergistic effects of the mixture as well as predict the model that will provide an answer to the results, such as high quality and low cost (Presenza et al., 2022).

In this study, sample extraction was conducted using three different solvents: water, ethanol, acetone, and their combination. The composition of each solvent can be seen in **Table 1**. The effect of different solvent systems on each response variable, namely TPC, FRAP, and ABTS, is also presented. The TPC response results ranged from 9 mg GAE/g to 63 mg GAE/g, with the maximum value produced by the solvent water : ethanol: acetone. Next, the antioxidant test was conducted using the FRAP and ABTS tests. In the FRAP test, the response produced ranged from 125 µmol TE/g to 386 µmol TE/g, with the maximum value produced by the water : ethanol solvent. Meanwhile, in the ABTS test, the response produced ranged from 59 µmol TE/g to 1014 µmol

TE/g, with the maximum value obtained from the solvent water : ethanol. Thus, it can be concluded that the solvent used for sample extraction has a different effect on the activity of TPC, FRAP, and ABTS.

2. Model Adjustment

Table 2 below shows the Analysis of Variance (ANOVA) to evaluate the mathematical model used in this research. The R^2 coefficient shows the suitability of the regression model. Natoen et al. (2018) explained that the coefficient of determination R^2 measures how far the model can explain variations in the dependent variables. If the R^2 value is close to one, then the model is getting better. This research produces a model that can explain the variability of TPC data by 74%, FRAP by 97%, and ABTS by 54% with a quadratic model.

In the TPC test, the F-value was 333.29. This value implies that the model is significant. There is only a 4.16% chance that an F-value this large could occur due to noise. A P-value less than 0.0500 indicates a significant model. In the FRAP test, an F-value of 8.07 was obtained, indicating that the model was insignificant relative to noise. There is a 26.08% possibility that an F-value of this size could occur due to noise. Then, in the FRAP test, the F-value was 0.2412, which implied the model was not significant relative to noise. There is a 90.27% possibility that an F-value of this size could occur due to noise.

Table 2. ANOVA results of *Psidium guajava* response variables

Values	TPC	FRAP	ABTS
	Quadratic	Quadratic	Quadratic
F-value	333.29	8.07	0.2412
p-value	0.0416	0.2608	0.9027
R^2	0.7467	0.9758	0.5466
Adjusted R^2	0.9964	0.8549	-1.7201

3. Effect of Solvent System on Response Variables

3.1 Total Phenolic Content (TPC)

The method that is widely used to determine the total phenolic content is Folin-Ciocalteu. The principle of this method is the oxidation of phenolic compounds by the Folin-Ciocalteu reagent. After oxidation, phenol is reduced to tungsten blue oxide (W8O23) and molybdenum (Mo8O23). The resulting blue color has a maximum absorption at 750 nm (Michiu et al., 2022). The equation obtained to show that the resulting quadratic model can determine the effect of TPC on the interaction of water (A), ethanol (B), and acetone (C) is as follows.

$$TPC = 32.32A + 39.23B + 9.84C + 94.42AB + 123.14AC + 111.63BC \quad ...(1)$$

In equation 1, all solvents gave a positive response in extracting phenolic compounds from *Psidium guajava* leaves. The highest yield was produced by the solvent water : acetone (123.14). Meanwhile, the lowest yield was produced by acetone solvent (9.84). According to the research results of Presenza et al. (2021), the distribution of seven different formulations in the results of the contour plot and 3D graph of *Psidium guajava* TPC content in **Figure 1** shows that points A, B, and C which are at the vertices of the triangle are the results of formulations using solvents that are suitable for pure components. Points AB, AC, and BC are binary mixtures of two different pure compounds and point ABC at the center of the triangle is a ternary mixture of three different pure compounds.

3.2 FRAP Antioxidant Content

Ferric Reduction Antioxidant Power (FRAP) is a method used to evaluate the antioxidant content in plants. The principle of this method is based on the ability of antioxidant compounds to reduce $Fe3+$ ions to $Fe2+$ so that the antioxidant power of compounds can be analogous to their reducing ability (Rahajo and Haryoto, 2019). The resulting model equation to determine the effect of FRAP on the interaction of water (A), ethanol (B), and acetone (C) is as follows.

$$FRAP = 215.20A + 189.20B + 129.03C + 686.68AB + 626.35AC + 335.02BC \quad ...(2)$$

Based on equation 2, all solvents gave a positive response in extracting the antioxidant compound FRAP from *Psidium guajava* leaves. The highest yield was produced by the solvent water : ethanol (686.68). Meanwhile, the lowest yield was produced by acetone solvent (129.03). **Figure 2** shows the results of the color plot and 3D graph of the *Psidium guajava* FRAP content, where points A, B, and C which are at the vertices of the triangle are the results of formulation using solvents that are suitable for pure components, then points AB, AC, and BC are a binary mixture, and point ABC which is in the center of the triangle is a ternary mixture.

(A) **(B)**

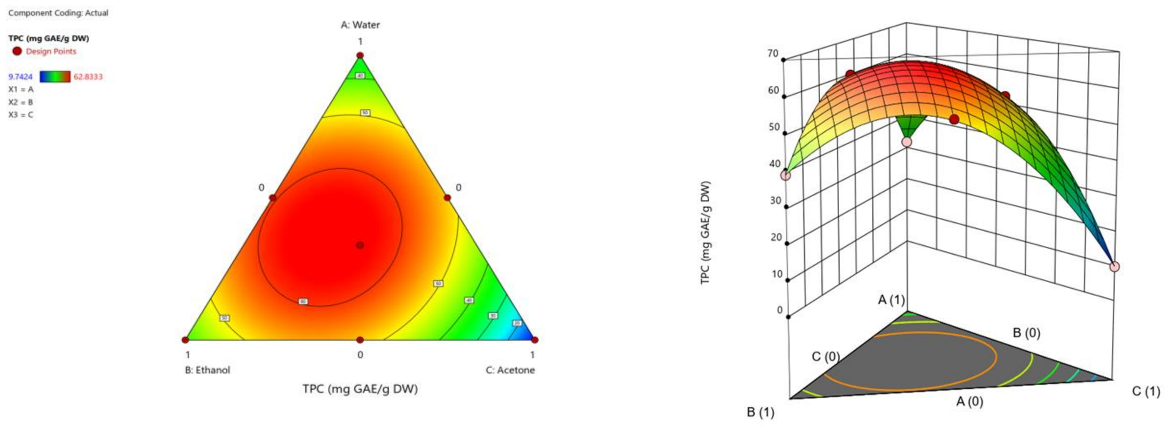

Figure 1. (A) Contour plot and (B) 3D graph of response surface analysis of the quadratic model of *Psidium guajava* TPC content with several solvents

(A) **(B)**

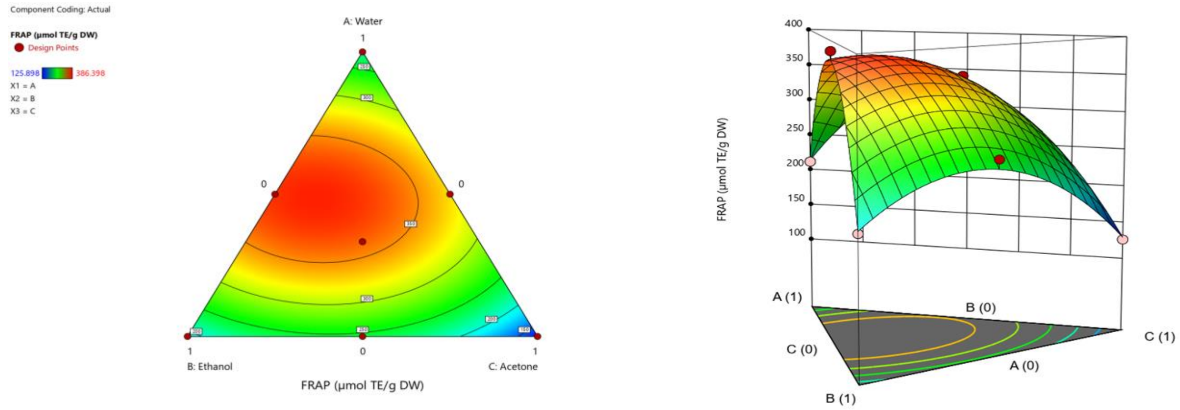

Figure 2. (A) Contour plot and (B) 3D graph of response surface analysis of the quadratic model of *Psidium guajava* FRAP content with several solvents

3.3 ABTS Antioxidant Content

The ABTS test is a method used to determine antioxidants in a sample. The principle of the ABTS test is a compound that stabilizes the color change of the ABTS radical cation (light blue) to become less colored (Fitriana et al., 2018). The resulting equation model to determine the effect of ABTS antioxidants on the interaction of water (A), ethanol (B), and acetone (C) is as follows.

$$ABTS = 487.15A + 582.17B + 104.84C + 1191.24AB + 1336.21AC + 309.05BC \quad ...(3)$$

Based on equation 3, all solvents also gave a positive response in extracting the antioxidant compound ABTS from *Psidium guajava* leaves. The highest yield was produced by the solvent water : acetone (1336.21). Meanwhile, the lowest yield was produced by acetone solvent (104.84). The following are the results of the turmeric plot and 3D graph of the ABTS content of *Psidium guajava* in **Figure 3** showing the results where points A, B, and C which are at the vertices of the triangle are the results of formulation using solvents that are suitable for pure components. Then points AB, AC, and BC are a binary mixture, and point ABC in the center of the triangle is a ternary mixture.

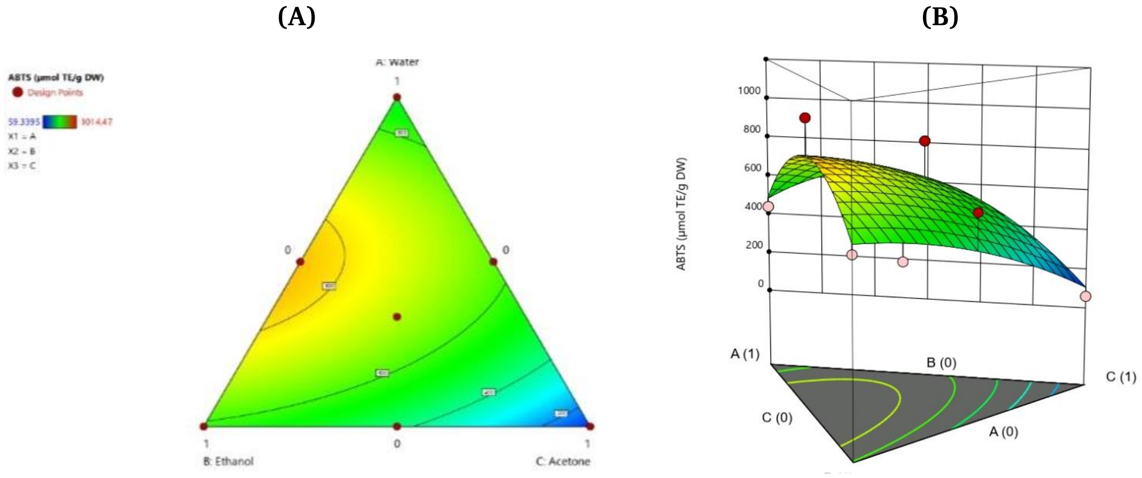

Figure 3. (A) Contour plot and (B) 3D graph of response surface analysis of the quadratic model of ABTS *Psidium guajava* content with several solvents

4. Optimization Solutions

After analyzing the influence of the solvent system, the next step is optimization using Design Expert. In this case, two optimization solutions were obtained, and then from these two solutions, the solution with the highest desirability value was selected. The optimization result chosen was a new run with water : ethanol : acetone ratio of 0.462:0.436:0.102 with the expected values of the TPC, FRAP, and ABTS response variables produced respectively of 62.833 mg GAE/g, 377.807 µmol TE/g, and 806.209 µmol TE/g. The optimization solution obtained can be seen in **Table 3** below.

Table 3. *Psidium guajava* extraction solvent optimization solution

No	A	B	C	TPC	FRAP	ABTS	*Desirability*	
1	0,462	0,436	0,102	62,833	377,807	806,209	0,911	*Selected*
2	0,488	0,512	0,000	59,454	373,442	833,463	0,897	

Note: A = water, B = ethanol, C = acetone

CONCLUSION

The total phenolic content and antioxidant activity test results on guava leaves analyzed in this study showed high results, where the highest total phenolic content (TPC) was obtained from the solvent water : ethanol: acetone with a value of 62.8333 mg GAE/g. Meanwhile, the antioxidant activity of both the FRAP and ABTS tests obtained the highest results for guava leaf extract using water : ethanol solvent with values of 386.3983 µmol TE/g and 1014.4688 µmol TE/g, respectively. The solvent optimization solution obtained was the use of water : ethanol: acetone with a ratio of 0.462:0.436:0.102 with a desirability of 0.911.

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