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Original Article

Novel approach extraction method to obtain optimum antioxidant and skin-lightening compound from *Rhodomyrtus tomentosa* (Aiton) Hassk. leaves

[Nuevo método de extracción para obtener un compuesto antioxidante y aclarador de la piel óptimo a partir de hojas de *Rhodomyrtus tomentosa* (Aiton) Hassk.]

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Abstract

Context: *Rhodomyrtus tomentosa* leaves have the potential to be developed as a raw material for traditional medicine or herbal cosmetics. In this study, the green extraction method and the selection of solvents to increase the extraction yield of active compounds and bioactivities from *R. tomentosa* leaves were carried out.

Aims: To develop the environmentally friendly extraction method for *R. tomentosa* leaves using pharmaceutical excipients and find out the best extraction solvent and conditions that can simultaneously extract the active compound and bioactivities.

Methods: *R. tomentosa* leaves were extracted using 14 different solvents including conventional solvents and pharmaceutical excipients. The ultrasound-assisted extraction method was chosen to promote the green extraction principles. All the extracts obtained were then evaluated for their phenolic, flavonoid content, antioxidant, and *in vitro* tyrosinase inhibition activities. Meanwhile, the optimization was carried out using Box Behnken response surface methodology.

Results: The ethanolic extract of *R. tomentosa* leaves has the highest ($p<0.05$) extraction yield, very strong antioxidant activity, and tyrosinase inhibitory activity compared to water, ethyl acetate, and chloroform. Among 10 excipients tested in this study, propylene glycol has the best ($p<0.05$) ability to extract *R. tomentosa* leaves. Propylene glycol was then used in the extraction optimization phase. Our result showed that the extraction conditions for optimizing phenolic compounds were a temperature of 30°C, an extraction time of 40 min, and a solid-to-liquid ratio of 0.05 g/mL.

Conclusions: The use of propylene glycol and the UAE method together showed good potential to be developed as a green extraction method for *R. tomentosa* leaves.

Keywords: flavonoid; green extraction; phenolic; response surface methodology; *Rhodomyrtus tomentosa*; tyrosinase.

Resumen

Contexto: Las hojas de *Rhodomyrtus tomentosa* tienen el potencial de ser desarrolladas como materia prima para la medicina tradicional o la cosmética herbal. En este estudio, se llevó a cabo el método de extracción verde y la selección de solventes para aumentar el rendimiento de extracción de compuestos activos y bioactividades de las hojas de *R. tomentosa*.

Objetivos: Desarrollar un método de extracción respetuoso con el medio ambiente para las hojas de *R. tomentosa* utilizando excipientes farmacéuticos y descubrir el mejor solvente de extracción y las mejores condiciones que puedan extraer simultáneamente el compuesto activo y las bioactividades.

Métodos: Las hojas de *R. tomentosa* se extrajeron utilizando 14 solventes diferentes, incluidos solventes convencionales y excipientes farmacéuticos. Se eligió el método de extracción asistida por ultrasonido para promover los principios de extracción verde. A continuación, se evaluaron todos los extractos obtenidos en cuanto a su contenido fenólico, flavonoides, antioxidantes y actividades de inhibición de la tirosinasa *in vitro*. Mientras tanto, se llevó a cabo la optimización utilizando la metodología de superficie de respuesta de Box Behnken.

Resultados: El extracto etanólico de hojas de *R. tomentosa* tiene el mayor rendimiento de extracción ($p<0,05$), una actividad antioxidante muy fuerte y una actividad inhibidora de la tirosinasa en comparación con el agua, el acetato de etilo y el cloroformo. Entre los 10 excipientes probados en este estudio, el propilenglicol tiene la mejor capacidad ($p<0,05$) para extraer hojas de R. tomentosa. Luego se utilizó propilenglicol en la fase de optimización de la extracción. Nuestro resultado mostró que las condiciones de extracción para optimizar los compuestos fenólicos fueron una temperatura de 30°C, un tiempo de extracción de 40 min y una relación sólido-líquido de 0,05 g/mL.

Conclusiones: El uso de propilenglicol y el método UAE juntos mostraron un buen potencial para ser desarrollados como un método de extracción verde para hojas de *R. tomentosa*.

Palabras Clave: extracción verde; fenólico; flavonoide; metodología de superficie de respuesta; *Rhodomyrtus tomentosa*; tirosinasa.

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INTRODUCTION

Rhodomyrtus tomentosa (W. Ait) Hassk.), known as rose myrtle or karamunting in Indonesia, belonging to the family *Myrtaceae*, is a native flowering plant from Asia, especially Indonesia. The flowers of *R. tomentosa* are widely used in aromatherapy, and its fruits are used for health purposes such as anti-diabetes, hepatoprotector, anti-diarrhea, wound healing, and prevention of post-partum hemorrhage (Mo et al., 2021; Umaternate, 2019; Vo and Ngo, 2019). Previous *in vitro* studies showed the ability of *R. tomentosa* as an anti-cancer and potential for the treatment of hypertension in pregnancy (Rumahorbo et al., 2023; Situmorang et al., 2022). Several studies have been conducted to explore its phytochemicals, and phenolic compounds have been identified as the major compounds in the leaves of *R. tomentosa* (Lai et al., 2013; Sinaga et al., 2019). On the other hand, our previous research reported the skin-lightening activity associated with the phenolic compound (Oktaviyanti et al., 2020; 2022). The skin-lightening mechanism of the phenolic compound is by inhibiting tyrosinase due to its structure, which is very similar to L-tyrosine so that it can compete with L-tyrosine to occupy the active site of the tyrosinase (An et al., 2008; Liang et al., 2014). Thus, *R. tomentosa* leaf extract is promising to be developed as an active ingredient in skin-lightening preparations.

Extraction solvent is one of the important factors that can affect the extraction efficiency of metabolites from plants. The solvent selection must be carried out to increase the extraction efficiency of the target compound (Ngo et al., 2017). In a study conducted by Hamid et al. (2017), the antioxidant and antiproliferative activities of *R. tomentosa* leaf extracts were compared using different organic solvents. Previous studies on *R. tomentosa* leaf extract are still limited to conventional extraction methods using organic solvents (Mitsuwan et al., 2020; Saising et al., 2011). Nowadays, public concern about environmental issues prompted researchers to explore more environmentally friendly extraction methods. Several approaches from the principle of green extraction are reducing the energy consumption, using alternative solvents to replace organic solvents, using renewable plant sources, using biodegradable materials, and reducing waste production by increasing byproducts (Bubalo et al., 2018; Chemat and Strube, 2015). In our recent study, the extraction was performed using pharmaceutical excipients, so it is very possible to formulate it directly into a dosage form without having to go through the extract evaporation step, which requires energy. In addition, the use of biodegradable excipients as extraction solvents is also more environmentally friendly compared to organic solvents.

The application of non-conventional extraction methods is often carried out for the undertaking of increasing biological activities and chemical compound yields. In addition, choosing an extraction method that can improve the extraction time can also be a strategy to reduce energy consumption in the implementation of the green extraction principle. ultrasound-assisted extraction (UAE) is an ultrasound wave-assisted extraction method that has been shown to improve the extraction efficiency of bioactive compounds (Dzah et al., 2020). Extraction efficiency was increased due to the microbubbles cavitation phenomenon, which causes a physical impact on the plant matrix, resulting in higher mass transfer into the solvent (Chemat et al., 2017).

Extraction optimization using the single-factor experimental method, where only one variable is changed, and all variables are held constant, has limitations because it cannot identify interactions between variables. In previous publications, response surface methodology (RSM) has been widely used to optimize the extraction conditions of numerous active compounds from plants. RSM is a statistical tool that can provide information about the correlation between variables with the number of effective experiments using a minimum number of samples. In addition, RSM is also accompanied by important mathematical and statistical techniques to evaluate the regression model fits.

To the best of our knowledge, the skin-lightening activity of *R. tomentosa* leaf extract has never been reported. Moreover, there is no research that has carried out the extraction of *R. tomentosa* leaves by applying the green extraction principle using cream preparation excipients. In brief, this research contributes to provide alternative extraction methods that are more environmentally friendly which increase the extraction yields of active compounds as antioxidants and skin lightening agents.

MATERIAL AND METHODS

Plant material

The plant material used in this research was *Rhodomyrtus tomentosa* leaves and fruits (Fig. 1), which were obtained from Palangkaraya, Central Kalimantan, Indonesia, and were verified by the Center for Traditional Medicine Information and Development, Faculty of Pharmacy, University of Surabaya. The fresh leaves and fruits were collected, cut into small pieces, and then dried under the shade. All samples

Figure 1. The plant material used in this study. **(A)** Leaves and **(B)** Fruits of *R. tomentosa.*

were powdered and stored in an airtight container for further processing.

Chemicals

All excipients used for the extraction in our recent study were pharmaceutical grade including isopropyl myristate, polyethylene glycol 400 (PEG 400), polisorbate 20 (Tween 20), polisorbate 60 (Tween 60), polisorbate 80 (Tween 80), glycerin, sorbitan laurate (Span 20), sorbitan oleate (Span 80), stearic acid, propylene glycol. Meanwhile, Folin-Ciocalteu reagent, chloroform, ethyl acetate, and ethanol were purchased from Merck, Germany. The gallic acid and quercetin standards; 2,2-diphenyl-1-picrylhydrazyl (DPPH); 2,2'-azinbis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); mushroom tyrosinase, and L-tyrosine were purchased from Sigma Aldrich, USA.

Preparation of the extract

In this study, the leaves of *R. tomentosa* were extracted using a variety of solvents and excipients. Table 1 provides a list of the solvents and excipients used for the extraction of compounds from *R. tomentosa* leaves and fruits, along with the sample code used in this study. Approximately 2.5 g of powdered *R. tomentosa* leaves and fruits were weighed and transferred to a beaker glass. Approximately 50 mL of each extraction solvent and excipient was added and the mixture was then sonicated for 15 min. Subsequently, the extract was filtered using nonbleached cloth into a volumetric flask, and the solvents were added until the total volume reached 50.0 mL. The extraction procedure was conducted in five replicates.

Determination of total phenolic compound (TPC)

The total phenolic compound was measured spectrophotometrically using Folin-Ciocalteu reagent and gallic acid as the standard according to the method described by Arrahman et al. (2018) with modifications. Briefly, 1.0 mL of the extract solution was pipetted and transferred to a volumetric flask, followed by the addition of 2.0 mL of Folin-Ciocalteu reagent, 0.2 mL sodium carbonate, and an aqueous methanol solution until the total volume reached 50.0 mL. After incubated for 10 min at ambient temperature, the mixture was measured for its absorbance at a wavelength of 750 nm using a UV-Vis spectrophotometer (UV-1900, Shimadzu Corp, Kyoto, Japan). Gallic acid was used as the reference compound standard, thus enabling the total phenolic compound to be expressed as milligrams of gallic acid equivalent (GAE) per gram of the dried samples (mg GAE/g dried samples). The analysis procedures were performed in quintuplicate.

Determination of total flavonoid compound (TFC)

The total flavonoid content of the sample was determined using a method originally described by Mun'im et al. (2017) with modifications. The initial step involved the mixing of 1.0 mL of the extract solution, 1.5 mL of 0.32% $AlCl_3$, and 1.5 mL of a 10% sodium acetate solution in a 10.0 mL volumetric flask. Subsequently, the mixture was diluted with ethanol until a volume of 10.0 ml was reached, after which it was homogenized and incubated for 30 minutes. The absorbance was subsequently analyzed at the maximum wavelength of 432.5 nm.

Table 1. A list of solvents and excipients used for the extraction of plant parts of *R. tomentosa* and their abbreviations as a code.

Extract code	Plant parts	Compound for extraction	
		Solvent	Excipient
FEt	Fruit	Ethanol 96%	-
LEt	Leaves	Ethanol 96%	-
LWa		Water	-
LCh		Chloroform	-
LEa		Ethyl acetate	-
LIm		-	Isopropyl myristate
LP400		-	Polyethylene glycol 400 (PEG 400)
LT20		-	Polysorbate 20 (Tween 20)
LT60		-	Polysorbate 60 (Tween 60)
LT80		-	Polysorbate 80 (Tween 80)
LGl		-	Glycerol
LS20		-	Sorbitan laurate (Span 20)
LS80		-	Sorbitan oleate (Span 80)
LSa		-	Stearic acid
LPg		-	Propylene glycol

FEt: ethanolic fruit extract; LEt: fruit extract using 96% ethanol; LWa: leaves extract using 96% ethanol; LCh: leaves extract using chloroform; LEa: leaves extract using ethyl acetate; LIm: leaves extract using isopropyl myristate; LP400: leaves extract using PEG 400; LT20: leaves extract using Tween 20; LT60: leaves extract using Tween 60; LT80: leaves extract using Tween 80; LGl: leaves extract using glycerol; LS20: leaves extract using Span 20; LS80: leaves extract using Span 80; LSa: leaves extract using stearic acid; LPg: leaves extract using propylene glycol.

The total flavonoid level was expressed as milligram quercetin equivalent (QE) per gram of the dried samples (mg QE/g dried samples). The analysis procedures were conducted in five replications.

Evaluation of DPPH scavenging activity

The DPPH free radical scavenging power was measured to determine the antioxidant activity of *R. tomentosa* leaf extracts according to the method described by Oktaviyanti et al. (2019) with minor modifications. The solution was prepared in a volumetric flask to a volume of 100.0 mL. The extracts of the various solvents were weighed equivalent to 1 g of dried leaves and added with deionized water to dilute into five different concentrations (equivalent to dried samples concentration). Each solution obtained was pipetted in 100 µL and mixed with 100 µL DPPH solution of concentration 40 ppm. The mixture solution was incubated for 30 minutes at room temperature, and the absorbance was read at a wavelength of 520 nm using a microplate reader (BMG Labtech, Germany). The percentage inhibition of DPPH radical was calculated using the following equation [1].

$$\% \text{ inhibition} = \frac{A - B}{A} \times 100\% \quad [1]$$

Where A is the absorbance of the free radicals solution; B is the absorbance of the sample after mixing and incubation with the free radical solution.

The antioxidant activity was calculated as the half-maximal inhibitory concentration (IC_{50}). The IC_{50} values were obtained from the linear regression equation, which was the result of the interpolation between the concentration (x-axis) and percentage inhibition (y-axis). The same methodology was employed with ascorbic acid as a positive control for the antioxidant activity assay. All analytical procedures were performed in triplicate.

Evaluation of ABTS scavenging activity

The ABTS scavenging activity was determined using the spectrophotometric method previously described by Jacob et al. (2018) and Oktaviyanti et al. (2022). First, 7.1 mg of ABTS and 3.5 mg of $K_2S_2O_8$ were weighed and subsequently mixed and dissolved in deionized water until the total volume of 25.0 mL was reached. Following a 16-hour incubation period, the mixture underwent a transformation, resulting in the formation of an ABTS free radical solution. The solution was further used to assess the antioxidant activity exhibited by the extracts.

All extracts were weighed and diluted into five different concentrations, each equal to the amount of

the dried leaves. Subsequently, 160 µL of each solution was pipetted, and 40 µL of the ABTS free radical solution was added. The mixture was incubated for five minutes at room temperature and then measured at 730 nm using a microplate reader (BMG Labtech, Germany). The percentage inhibition and IC_{50} were calculated in a way that was analogous to the procedure described above for the DPPH assay. Ascorbic acid was also used as a positive control, and the aforementioned procedures were carried out. All antioxidant activity assays were conducted in triplicate.

Evaluation of tyrosinase inhibitory activity

The tyrosinase inhibitory assays were conducted using the methodology previously established by our research group (Oktaviyanti et al., 2019) with slight modifications. Initially, the tyrosinase enzyme and L-tyrosine substrate solution were freshly prepared by weighing 1.73 mg of mushroom tyrosinase (500 U/mL) and 1.81 mg of L-tyrosine, respectively. The two solutions were each dissolved in 0.05 M phosphate buffer solution (pH 6.5) to a volume of 10.0 mL. All extracts were diluted into five different concentrations, equivalent to the concentration of the dried samples. The enzyme, substrate, and sample solution were mixed in specific volumes according to the specifications outlined in Table 2. All mixtures were incubated at 25°C for 30 minutes and then analyzed using a microplate reader at 475 nm.

The % inhibition of each extract towards tyrosinase was calculated using equation [2].

$$\%\ \text{inhibition of tyrosinase} = \frac{(\text{AbsA} - \text{AbsB}) - (\text{AbsC} - \text{AbsD})}{(\text{AbsA} - \text{AbsB})} \times 100\% \qquad [2]$$

Where Abs_A: absorbance of solution A, Abs_B: absorbance of solution B, Abs_C: absorbance of solution C, Abs_D: absorbance of solution D.

In this assay, kojic acid was selected as the reference compound. The IC_{50} values of all samples and the reference compound were obtained from the linear regression equation, and all the procedures were conducted in triplicate.

Experimental design for extraction optimization

In this study, a Box-Behnken design analysis was conducted using response surface methodology (RSM) was carried out to enhance the yield of phenolic compounds in *R. tomentosa* leaves. Extraction conditions were optimized with regard to three variables: temperature, extraction time, and solid-liquid ratio. Each of these variables was assigned three levels. RSM was performed using Design Expert software (version 13). All codes for the extraction variables used in this study, along with the levels to which they were assigned, are shown in Table 3. Once all variables and levels were entered into the software, the software produced approximately 15 runs of experimental design, which were then implemented in the laboratory.

Statistical analysis

In this study, all data are presented as mean values and followed by standard deviations (mean ± SD). The results of total phenolic and flavonoid levels are expressed in terms of average yields, which are equivalent to standard compounds. The antioxidant and tyrosinase inhibition activities are presented as a means of IC_{50} values.

A one-way analysis of variance (ANOVA) was conducted to analyze the data using SPSS software version 23 for Windows (IBM, New York, United States). The significance level was set at $p<0.05$. Meanwhile, the Design-Expert software version 13 (Stat-Ease Inc., Minneapolis, MN, USA) was employed to apply the response surface method for the purpose of extraction optimizing the extraction of phenolic compounds.

Table 2. The composition of the solution utilized in the tyrosinase inhibitor assay.

Solution	Composition of solution (µL)			
	A	B	C	D
Buffer	120	160	80	120
L-tyrosine	40	40	40	40
Extract	-	-	40	40
Mushroom tyrosinase	40	-	40	-
Total volume (µL)	200	200	200	200

Table 3. The code for variables and levels utilized for extraction optimization.

Variables	Code	Range and level		
		-1	0	1
Temperature (°C)	X_1	30	45	60
Extraction time (min)	X_3	20	40	60
Solid-to-liquid ratio (g/mL)	X_2	0.02	0.05	0.10

A one-way analysis of variance (ANOVA) was conducted to analyze the data using SPSS software version 23 for Windows (IBM, New York, United States). The significance level was set at $p<0.05$. Meanwhile, the Design-Expert software version 13 (Stat-Ease Inc., Minneapolis, MN, USA) was employed to apply the response surface method for the purpose of extraction optimizing the extraction of phenolic compounds.

RESULTS AND DISCUSSION

Extraction yields and bioactivity from different plant parts

The distribution of phytochemical compounds may vary between different parts of the plant (Russo et al., 2018). The fruits of *R. tomentosa* are the most frequently used as nutraceuticals and medicinal agents, among other plant parts. However, the use of fruit as raw material for large-scale production may present certain challenges, particularly with regard to long-term availability. The fruits exhibited limited growth throughout the season, rendering them an inadequate raw material source. In contrast, the leaves are a viable alternative, given their abundant availability within the plant. Fig. 2 presents a comparison of the extraction yield and the bioactivity between the fruit and leaves. The results show that there is no significant difference between the two samples. Previous research conducted by Teleszko and Wojdyo (2015) on the phenolic compounds and antioxidant activity of the leaves of various edible fruits indicated that the leaves possess similar potential to their respective fruits. This strengthens the underlying reason that the leaf extract of *R. tomentosa* can be an alternative for effective and high-quality raw material sources.

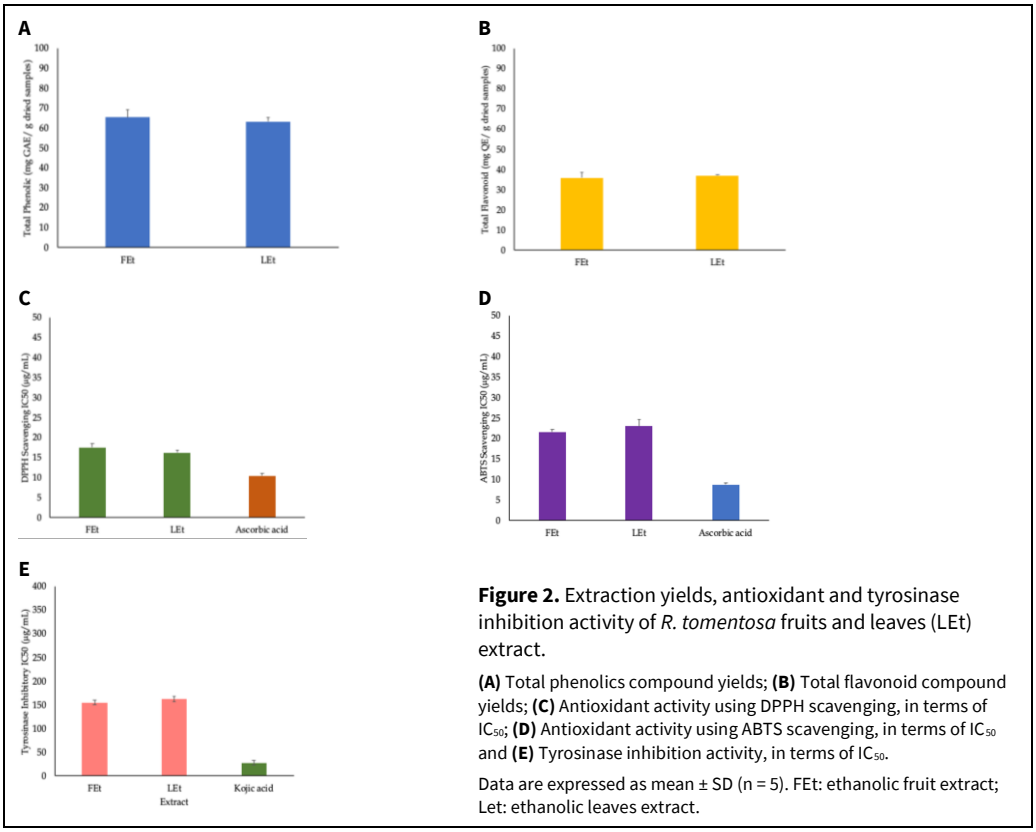

Figure 2. Extraction yields, antioxidant and tyrosinase inhibition activity of *R. tomentosa* fruits and leaves (LEt) extract.

(A) Total phenolics compound yields; **(B)** Total flavonoid compound yields; **(C)** Antioxidant activity using DPPH scavenging, in terms of IC_{50}; **(D)** Antioxidant activity using ABTS scavenging, in terms of IC_{50} and **(E)** Tyrosinase inhibition activity, in terms of IC_{50}.

Data are expressed as mean ± SD (n = 5). FEt: ethanolic fruit extract; Let: ethanolic leaves extract.

The impact of various solvents on the extraction of *R. tomentosa* leaves

The study demonstrated that the use of different extraction solvents on *R. tomentosa* leaves resulted in variations in the extraction yields, including phenolic compounds, flavonoids, antioxidant and tyrosinase inhibition activities.

Excessive exposure to solar radiation and free radicals has been demonstrated to induce melanogenesis in the skin, leading to hyperpigmentation and the development of age-related skin problems (Randhawa et al., 2015; Şöhretoğlu et al., 2018). Many studies have been done to develop strategies to prevent hyperpigmentation reactions. These studies have explored the potential of antioxidants and tyrosinase inhibitors to achieve this goal. Tyrosinase inhibitors are currently widely used in the formulation of skin-lightening cosmetics as melanogenesis inhibitors (Arung et al., 2006; Likhitwitayawuid, 2008). The accumulation of free radicals in the skin can induce damage to melanocytes due to oxidative stress. Furthermore, free radicals have the potential to inhibit the formation of collagen. The use of natural antioxidants derived from plants represents a promising strategy to overcome premature aging and melanogenesis caused by free radicals (Park et al., 2015; Szewczyk et al., 2021). Phenolic compounds, which represent one of the largest compounds in many plants, have been demonstrated to exhibit strong antioxidant activities (Alara et al., 2021). The phenolic compound is a natural compound group that is mostly known to have tyrosinase inhibitory activity. The activity of a phenolic compound as a tyrosinase inhibitor is facilitated by its ability to chelate copper at the active site of the enzyme and its molecular structure similarity (Panche et al., 2016; Parvez et al., 2007).

Figs. 3-4 showed that the ethanol solvent gave about 2 to 4 times higher extraction yield of phenolic and flavonoid compounds compared to other conventional solvents used in this study. Similar results were also shown in previous research conducted by Chen et al. (2022). This finding is related to the relative polarity of the ethanol (about 0.654) as an extraction solvent, which corresponds to the phenolic and flavonoid compounds in *R. tomentosa* leaves. The excellent ability of ethanol to extract compounds from *R. tomentosa* leaves followed by its antioxidant and tyrosinase inhibition activity. The ethanolic extract also has the lowest IC_{50} value for antioxidant activity of 16.2603 ± 0.6606 and 23.0383 ± 1.5701 µg/mL for the DPPH and ABTS methods, respectively. According to the IC_{50} value, it can be concluded that the ethanolic extract of *R. tomentosa* was very potent as an antioxidant agent (Figs. 5-7). Moreover, the tyrosinase inhibition assay also gave similar results concerning extraction yield and antioxidant activity. Thus, the ethanol solvent is the best conventional organic solvent to extract not only phenolic and flavonoid compounds simultaneously but also all the compounds that contribute to antioxidant and tyrosinase inhibition activity.

Based on our results, propylene glycol provided the highest total phenolic and flavonoid compounds. The extracts obtained from these excipients also have good activity as tyrosinase inhibitors and showed excellent ABTS and DPPH free radical scavenging activity. In addition, the extract using PEG400 and glycerol presented a high flavonoid compound and also showed good ability as an antioxidant agent. As mentioned above, the effectiveness of the solvent for extracting bioactive compounds is influenced by several parameters, such as its polarity. Among the 10 excipients used in this study, propylene glycol, glycerin, and PEG400 have the highest dielectric constant, so their polarity is higher than the other excipients. Meanwhile, phenolic compounds and flavonoids are polar and easily soluble in polar solvents. According to the principle of like dissolves like, it can explain the efficacy of propylene glycol, glycein, and PEG400 as effective alternative solvents (Babu et al., 2008).

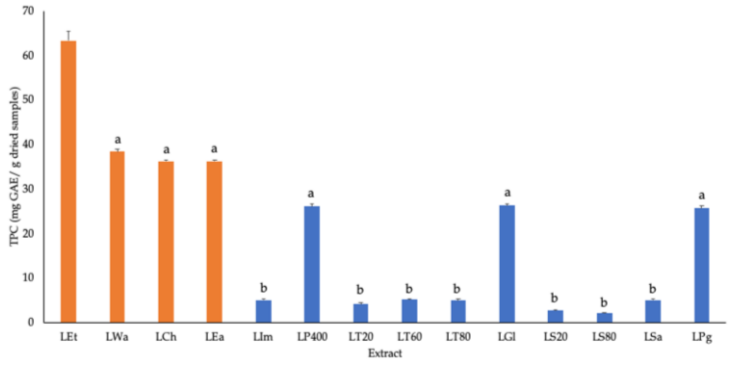

Figure 3. Comparison of total phenolic compound yields among the different solvent and excipient compounds for extracting *R. tomentosa* leaves.

Data are expressed as mean ± SD (n = 5). Different letters indicate statistically significant differences using Tukey post hoc test, LSD ($^{a}p<0.05$ vs. LEt; $^{b}p<0.05$ vs. LP400, LGl, and LPg). LEt: fruit extract using 96% ethanol; LWa: leaves extract using 96% ethanol; LCh: leaves extract using chloroform; LEa: leaves extract using ethyl acetate; LIm: leaves extract using isopropyl myristate; LP400: leaves extract using PEG 400; LT20: leaves extract using Tween 20; LT60: leaves extract using Tween 60; LT80: leaves extract using Tween 80; LGl: leaves extract using glycerol; LS20: leaves extract using Span 20; LS80: leaves extract using Span 80; LSa: leaves extract using stearic acid; LPg: leaves extract using propylene glycol.

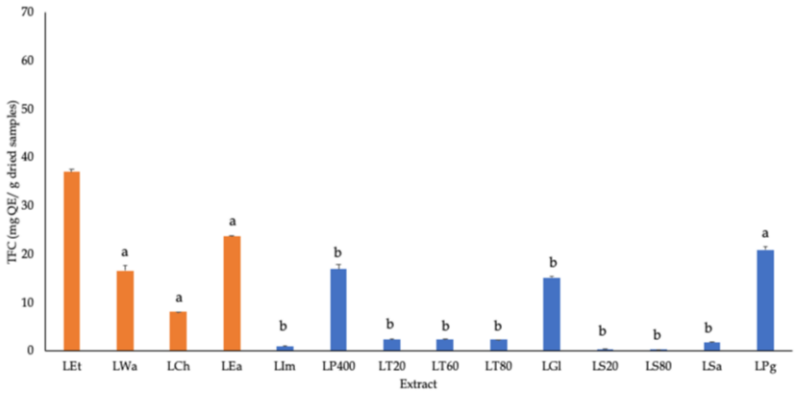

Figure 4. Comparison of total flavonoid compound yields among the different solvent and excipient compound for extracting *R. tomentosa* leaves.

Data are expressed as mean ± SD (n = 5). Different letters indicate statistically significant differences using Tukey post hoc test, LSD ($^{a}p<0.05$ vs. LEt; $^{b}p<0.05$ vs. LPg.). LEt: fruit extract using 96% ethanol; LWa: leaves extract using 96% ethanol; LCh: leaves extract using chloroform; LEa: leaves extract using ethyl acetate; LIm: leaves extract using isopropyl myristate; LP400: leaves extract using PEG 400; LT20: leaves extract using Tween 20; LT60: leaves extract using Tween 60; LT80: leaves extract using Tween 80; LGl: leaves extract using glycerol; LS20: leaves extract using Span 20; LS80: leaves extract using Span 80; LSa: leaves extract using stearic acid; LPg: leaves extract using propylene glycol.

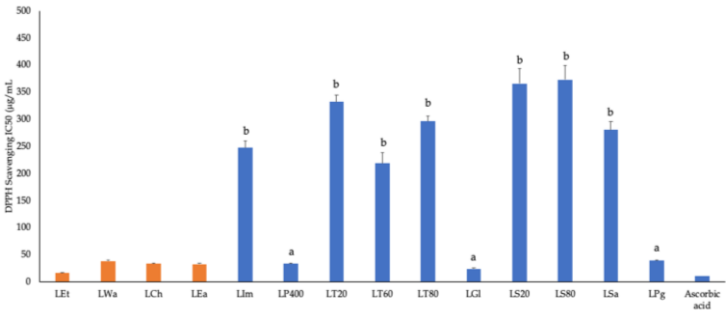

Figure 5. Comparison of antioxidant activity among the different solvent and excipient compounds for extracting *R. tomentosa* leaves using the DPPH scavenging method in terms of IC_{50}.

Data are expressed as mean ± SD (n = 3). Different letters indicate statistically significant differences using Tukey post hoc test, LSD ($^{a}p<0.05$ vs. LEt; $^{b}p<0.05$ vs. LP400, LGl, and LPg). LEt: fruit extract using 96% ethanol; LWa: leaves extract using 96% ethanol; LCh: leaves extract using chloroform; LEa: leaves extract using ethyl acetate; LIm: leaves extract using isopropyl myristate; LP400: leaves extract using PEG 400; LT20: leaves extract using Tween 20; LT60: leaves extract using Tween 60; LT80: leaves extract using Tween 80; LGl: leaves extract using glycerol; LS20: leaves extract using Span 20; LS80: leaves extract using Span 80; LSa: leaves extract using stearic acid; LPg: leaves extract using propylene glycol.

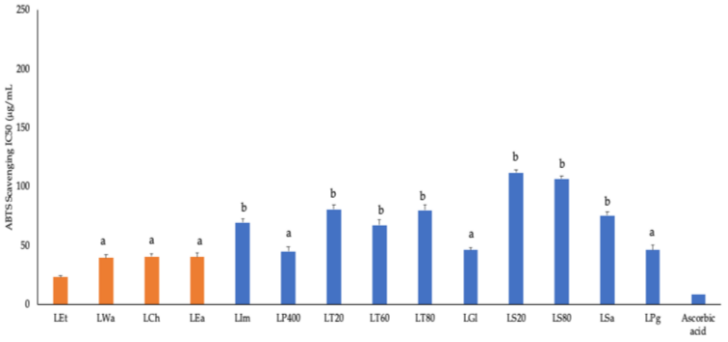

Figure 6. Comparison of antioxidant activity among the different solvent and excipient compounds for extracting *R. tomentosa* leaves using ABTS scavenging method, in terms of IC_{50}.

Data are expressed as mean ± SD (n = 3). Different letters indicate statistically significant differences using Tukey post hoc test, LSD ($^{a}p<0.05$ vs. LEt; $^{b}p<0.05$ vs. LP400, LGl, and LPg). LEt: fruit extract using 96% ethanol; LWa: leaves extract using 96% ethanol; LCh: leaves extract using chloroform; LEa: leaves extract using ethyl acetate; LIm: leaves extract using isopropyl myristate; LP400: leaves extract using PEG 400; LT20: leaves extract using Tween 20; LT60: leaves extract using Tween 60; LT80: leaves extract using Tween 80; LGl: leaves extract using glycerol; LS20: leaves extract using Span 20; LS80: leaves extract using Span 80; LSa: leaves extract using stearic acid; LPg: leaves extract using propylene glycol.

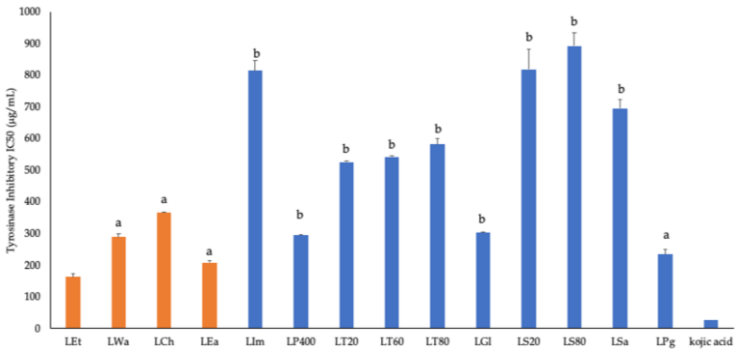

Figure 7. Comparison of tyrosinase inhibitor activity among the different solvent and excipient compounds for extracting *R. tomentosa* leaves, in terms of IC_{50}.

Data are expressed as mean ± SD (n = 3). Different letters indicate statistically significant differences using Tukey post hoc test, LSD ($^{a}p<0.05$ vs. LEt; $^{b}p<0.05$ vs. LPg). LEt: fruit extract using 96% ethanol; LWa: leaves extract using 96% ethanol; LCh: leaves extract using chloroform; LEa: leaves extract using ethyl acetate; LIm: leaves extract using isopropyl myristate; LP400: leaves extract using PEG 400; LT20: leaves extract using Tween 20; LT60: leaves extract using Tween 60; LT80: leaves extract using Tween 80; LGl: leaves extract using glycerol; LS20: leaves extract using Span 20; LS80: leaves extract using Span 80; LSa: leaves extract using stearic acid; LPg: leaves extract using propylene glycol.

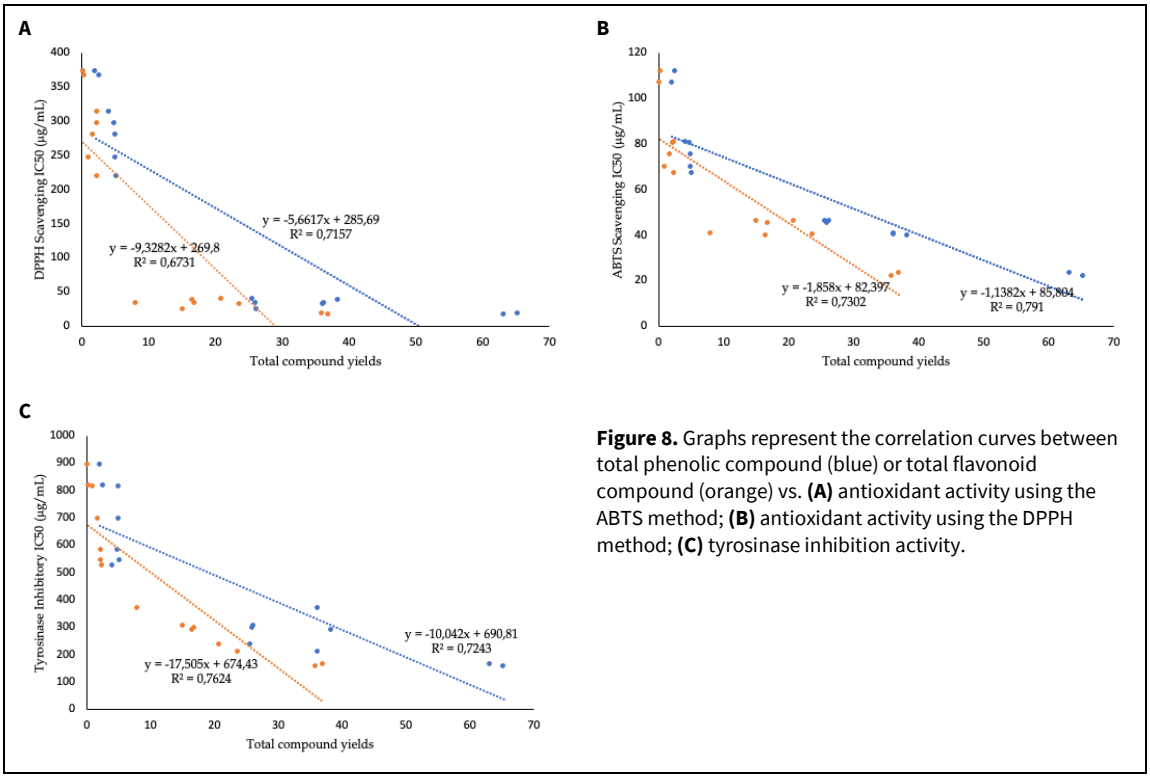

Figure 8. Graphs represent the correlation curves between total phenolic compound (blue) or total flavonoid compound (orange) vs. **(A)** antioxidant activity using the ABTS method; **(B)** antioxidant activity using the DPPH method; **(C)** tyrosinase inhibition activity.

$$y = 37.11 - 5.44x_1 - 0.17x_2 - 0.66x_3 + 1.47x_1x_2 + 0.55x_1x_3 - 1.05x_2x_3 - 4.18x_1^2 - 18.71x_2^2 - 15.98x_3^2 \quad [3]$$

The correlation coefficient (R^2 value) between compound and antioxidant activity can be used to predict whether the compounds are responsible for the activity (Aryal et al., 2019). Fig. 8 shows the correlation between total compound yields with the antioxidant activity and tyrosinase inhibitory activity. The R^2 value between total phenolics and DPPH scavenging, ABTS scavenging, and tyrosinase inhibitory activity was 0.7157, 0.7910, and 0.7243, while the R^2 value of the total flavonoid was 0.6731, 0.7302, and 0.7624, respectively. The correlation coefficient is quite high, especially for phenolic compounds. Based on the result of this study, it is known that phenolic compounds of *R. tomentosa* have a greater contribution to the activity than flavonoid compounds. However, there is still a possibility that the antioxidant activity and tyrosinase inhibition are caused by other compounds contained in *R. tomentosa* leaf extract. Our results also showed that there was a high correlation between antioxidant and tyrosinase inhibitory effects.

This phenomenon is very reasonable because tyrosinase is a type of polyphenol oxidase. Therefore, natural compounds with high antioxidant activity may have a good tyrosinase inhibitory effect (Riebel et al., 2015). Based on this positive correlation, the extraction conditions in this study optimized the phenolic compound as an antioxidant and skin-lightening agent. Furthermore, propylene glycol was selected as an alternative extraction solvent for the optimization of phenolic compounds from *R. tomentosa* leaf.

The optimum extraction condition

The extraction conditions were optimized using a three-variable Box-Behnken design (BBD). Approximately 15 experimental runs were conducted, and the phenolic compounds were measured as response variables. The total phenolic compound levels obtained from the 15 extraction runs are presented in Table 4. A mathematical model that shows the relationship between variables and the response was formulated in order to predict the optimum response as the equation [3].

In the aforementioned equation [3], the variables "X_1", "X_2" and "X_3" represent temperature, extraction time, and solid-to-liquid ratio, respectively.

The analysis of variance (ANOVA) was performed to ascertain the suitability of the model in explaining the data set (Table 5). To assess the sufficiency of the model, the R-squared value and the coefficient of variation (CV) were calculated. The R-squared value indicates the proportion of the observed variance in the responses that can be attributed to the model as opposed to random error. In theory, a well-fitting model should have an R-square value of at least 80%. The R-square value was found to be 0.9953, indicating

that 99.53% of the variability in the extraction yield can be explained by the model. Therefore, it can be concluded that the model is excellent for quantitative prediction. Moreover, the coefficient of variation (CV) is employed to quantify the extent of data dispersion. Our findings indicated that the CV was 9.06%, which is still within the acceptable range as it is less than 10%. The lack-of-fit represents a model's inability to adequately represent the experimental data at points excluded from the regression or variations in the models, which cannot be considered as a random error. Table 5 illustrates that the p-value of lack-of-fit was not significant, indicating that the models were sufficiently accurate to forecast the relevant responses (Feng, 2022; Koocheki et al., 2009; Suhaimi et al., 2019).

Table 4. The experimental design and total phenolic results.

Run	Independent variable			Response
	X_1 (temperature)	X_2 (extraction time)	X_3 (solid-to-liquid ratio)	Total phenolic (mg GAE/ g dried sample)
1	0	0	0	36.3630
2	1	1	0	10.9684
3	0	-1	1	4.5606
4	0	-1	-1	2.2379
5	1	-1	0	6.7484
6	1	0	-1	12.3075
7	-1	-1	0	20.4070
8	0	0	0	37.2008
9	0	1	-1	2.3938
10	-1	1	0	18.7546
11	0	1	1	0.5111
12	0	0	0	37.7552
13	1	0	1	10.5392
14	-1	0	1	20.4973
15	-1	0	-1	24.4555

The p-values for each factor in Table 5 were examined to gain insight into the pattern of mutual interactions between them. The total phenolic model indicates that temperature has a significant impact on both the linear and quadratic models. However, the solid-to-liquid ratio and time variables yielded disparate results. Although the linear effects of the time and solid-to-liquid ratio were not statistically significant, the quadratic effect coefficient was significant. The order of importance of the variables can be determined based on the sum of squares value, with temperature, solid-to-liquid ratio, and then extraction time being the most, second most, and third most important variables, respectively.

Fig. 9 depicts the response surface for the effect of the extraction variable in a three-dimensional graph. The interaction between variables can be observed by maintaining a variable at a constant value and varying the other two variables. Our findings indicate that temperature is a crucial factor in the extraction of phenolic compounds from *R. tomentosa* leaves. In several instances, an elevated temperature is necessary to enhance the solubility of the target compounds, thereby facilitating their extraction into the solvent (Shi et al., 2003). It is noteworthy that the elevated temperatures can lead to the oxidation and degradation of the targeted compounds, ultimately reducing the extraction yield (Sulaiman et al., 2017). Similarly, research conducted by Yim et al. (2013) demonstrated that the yield of phenolic compounds decreased when extraction was conducted at temperatures above 40.3°C. Moreover, the reduction in phenolic compound yield was accompanied by a decline in antioxidant activity. This is a notable finding, as it indicates that higher temperatures and energy consumption are not necessary to achieve high yields of phenolic compounds.

The findings of this study indicate that an extended extraction time resulted in an increased yield of phenolics from *R. tomentosa* leaves. This phenomenon can be attributed to an increase in cavitation bubble formation over time, as well as an enhanced opportunity for contact between the solid and the solvent (Lopeda-Correa et al., 2022). Nevertheless, an extended extraction time resulted in a decline in total phenolic yields. Suhaimi et al. (2019) additionally indicated that the phenolic compounds diminished when UAE exceeded 20 minutes, a consequence of compound degradation. The optimum yield of phenolic compounds was also reported by Baakili et al. (2023) to be at 40 minutes of extraction. As previously demonstrated in our own study (Oktaviyanti et al., 2020), flavonoid compound yields, which are included in the phenolic group, also exhibit a similar trend in relation to extraction time.

In the present study, a reduction in the solid-to-liquid ratio was observed to result in an initial increase in the yield of phenolic compounds, which reached a maximum point and subsequently declined. A lower solid-to-liquid ratio indicates a larger amount of solvent for extraction, which results in an excellent total phenolic compounds (TPC) value. This phenomenon could be explained by the increased solubility of the phenolic compounds due to the greater surface area contact with the substances along with the increasing amount of the solvent (Ez zoubi et al., 2021).

It was unexpected that a further reduction in the solid-to-liquid ratio resulted in a decrease in the yield of phenolic compounds. These findings are consistent with those of our previous study, suggesting that the dissolution of the phenolic compounds may be hindered by the presence of dissolved impurities when the solvent volume exceeds an optimal threshold (Oktaviyanti et al., 2020).

The highest total phenolic compound yield is predicted to be achieved when the temperature is maintained at 30°C, the extraction time is set at 40 minutes, and the solid-to-liquid ratio is set at 0.05 g/mL with a yield of 38.7279 mg GAE/g of dried samples. It is noteworthy that the current result is considerably higher than that of the previous study, which was conducted under suboptimal conditions and yielded 25.6298 mg GAE/g dried samples.

CONCLUSION

The present study demonstrated that the leaves of *R. tomentosa* are a valuable source of phenolic, flavonoid, and other compounds that are responsible for antioxidant and tyrosinase inhibition activity. The utilization of diverse excipients throughout the extraction process proved to be an effective strategy for the attainment of a high-quality extract in an environmentally friendly manner. The excipients that facilitate the most effective extraction yields and activity from *R. tomentosa* leaves are propylene glycol, glycerol, and PEG400. It is recommended that propylene glycol be used as an alternative solvent, as it has the potential to be a valuable resource for further extraction development. Furthermore, phenolic and flavonoid-rich extracts have been demonstrated to effectively decrease melanin by inhibiting tyrosinase, suggesting their potential use in the production of cream preparations for skin-lightening or anti-aging. The results showed that the optimal conditions for the extraction of phenolic compounds using the UAE method and propylene glycol were a temperature of 30°C, an extraction time of 40 minutes, and a solid-to-liquid ratio of 0.05 g/mL.

Table 5. The analysis of variance toward the prediction model.

Source	Sum of squares	Degrees of freedom	Mean square	*f*-value	p-value
Model	2342.63	9	260.29	118.22	< 0.0001
Linear					
X_1	237.08	1	237.08	107.68	0.0001
X_2	0.2198	1	0.2198	0.0998	0.7648
X_3	3.49	1	3.49	1.59	0.2634
Interaction					
$X_1 X_2$	8.62	1	8.62	3.92	0.1047
$X_1 X_3$	1.20	1	1.20	0.5445	0.4937
$X_2 X_3$	4.42	1	4.42	2.01	0.2156
Quadratic					
X_1^2	64.56	1	64.56	29.32	0.0029
X_2^2	1291.91	1	1291.91	586.77	< 0.0001
X_3^2	942.29	1	942.29	427.98	< 0.0001
Residual	11.01	5	2.20		
Lack-of-Fit	10.03	3	3.34	6.80	0.1308
Pure Error	0.9825	2	0.4913		
Cor total	2353.63	14			

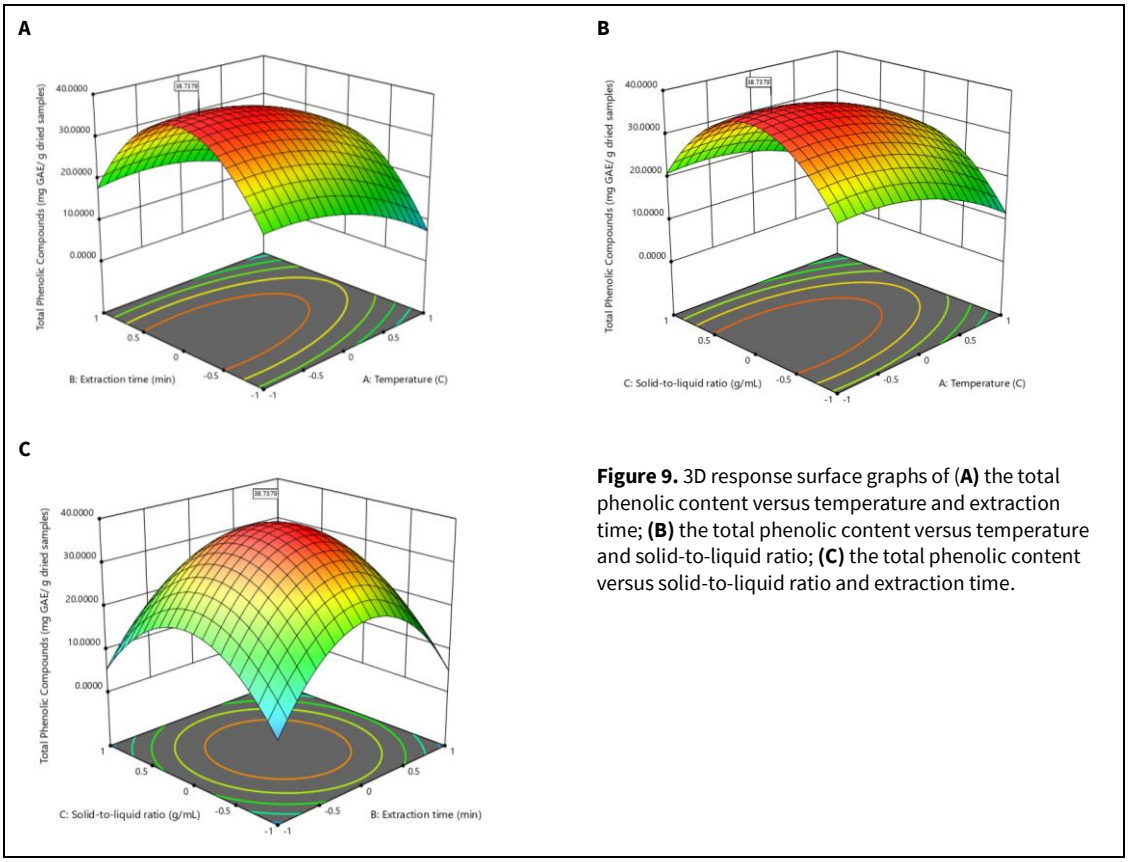

Figure 9. 3D response surface graphs of (**A**) the total phenolic content versus temperature and extraction time; **(B)** the total phenolic content versus temperature and solid-to-liquid ratio; **(C)** the total phenolic content versus solid-to-liquid ratio and extraction time.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Oktaviyanti ND	Kartini K	Setiawan F	Fitriani EW	Sukweenadhi J	Avanti C
Concepts or ideas	x	x	x	x	x	x
Design	x	x	x	x	x	x
Definition of intellectual content	x	x	x	x		x
Literature search	x	x	x	x		x
Experimental studies	x	x	x	x	x	x
Data acquisition	x	x	x		x	
Data analysis	x				x	x
Statistical analysis	x				x	x
Manuscript preparation	x		x	x		x
Manuscript editing	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x

Citation Format: Oktaviyanti ND, Kartini K, Setiawan F, Fitriani EW, Sukweenadhi J, Avanti C (2025) Novel approach extraction method to obtain optimum antioxidant and skin-lightening compound from *Rhodomyrtus tomentosa* (Aiton) Hassk. leaves. J Pharm Pharmacogn Res 13(3): 905–918. https://doi.org/10.56499/jppres23.1854_13.3.905