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Original Research Article

Optimization of Flavonoid Extraction and Tyrosinase Inhibitory Activity from *Rhodomyrtus tomentosa* Leaves Using NADES-Based Ultrasound-Assisted Extraction

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ABSTRACT

Flavonoid compounds have attracted considerable interest due to their promising potential applications cosmetic products, particularly as skin-lightening agents, while efficient and sustainable extraction remains a major challenge. This study aimed to optimize the extraction of flavonoid compounds and their tyrosinase inhibitory activity from *Rhodomyrtus tomentosa* leaves using ultrasound-assisted extraction (UAE) using natural deep eutectic solvents (NADESs). Initially, several choline chloride-based NADESs were evaluated for their extraction efficiency to identify the most effective solvent, which was subsequently used in the next phase involving the optimization of extraction conditions. The extraction variables, including extraction time, water addition to the NADESs, and the solid-to-liquid ratio were optimized using Response Surface Methodology (RSM) with a Box-Behnken design. Both total flavonoid content and tyrosinase inhibitory activity were determined spectrophotometrically. Choline chloride-propylene glycol (1:1) was identified as the optimal NADES compared to choline chloride-based NADESs combined with various other hydrogen bond donors. The developed models showed high R^2 value (0.9944 for flavonoids; 0.9998 for tyrosinase inhibition) with non-significant lack-of-fit values, indicating the excellent predictive performance of the response model. The optimal extraction conditions were obtained at an extraction time of 60 min, 20% water addition, and a solid-to-liquid ratio of 0.05 g/mL, resulting in a total flavonoid content of 14.3729 mg QE/ g dried leaves and tyrosinase inhibition of 83.56%. These findings highlight the potential of NADES-UAE as an efficient and environmentally friendly platform for producing bioactive extracts of *R. tomentosa* suitable for skin-lightening applications.

***Keywords*:** *Rhodomyrtus tomentosa*, Natural Deep Eutectic Solvent, Ultrasound-Assisted Extraction, Flavonoid, Tyrosinase Inhibition.

Introduction

Numerous plants are known for containing high abundances of flavonoids, which are responsible for their bioactivity, particularly for human skin, such as antioxidant, anti-inflammatory, skin-lightening, anti-aging, and photoprotective effects.[1,2] Recent studies have highlighted the increasing interest in flavonoids as multifunctional cosmetic ingredients due to their interactions with important skin-related enzymes and antioxidants activity.[3,4] Previous study demonstrated that flavonoids exhibit significant inhibition activity towards tyrosinase, a key enzyme involved in melanin biosynthesis, thereby contributing to their bioactivities as skin-lightening.[5,6] Tyrosinase is an enzyme that is important in melanin biosynthesis that further leads to the formation of melanin in human skin and also contributes to the enzymatic browning of food. For this reason, numerous investigations have been carried out to explore plant sources of flavonoids, which have great promise as tyrosinase inhibitors. *Rhodomyrtus tomentosa* as known as Karamunting in Indonesia is a plant belonging to the family of Myrtaceae which is known to have a high level of flavonoid.

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Flavonoid compounds are distributed in various parts of plants such as leaves, fruit and roots. *R. tomentosa* leaves are green with a glossy upper surface, while the flowers have a pink colour on the interior and white on the exterior. The green skinned unripe fruit has an astringent taste then turns into a purple-black colour with soft and tasty flesh when mature.[7] The fruits are most frequently used empirically and have been reported to exhibit various biological activities such as antipyretic, antidiarrheal, anti-dysenteric, antibacterial, anticancer, and antidiabetic.[8,9,10] However, this poses considerable risks to the sustainability of raw material availability, because fruit might be difficult to find at times. Our earlier study found no difference between the antioxidant and tyrosinase inhibition activity, flavonoid and phenolic component levels of fruit and leaves.[11] This reinforces the fundamental rationale for the potential of the *R. tomentosa* leaf as a substitute for high-quality and effective raw material sources.

There have been many attempts to extract high levels of flavonoids from plants because of their activity, both with conventional and non-conventional extraction methods. Non-conventional extraction methods are developed to enhance and implement more efficient and economical processes.[12] In addition, non-conventional extraction also supports the application of green technology principles in the extraction process, due to shorter extraction times and minimum energy consumption, such as ultrasound-assisted extraction (UAE). The UAE method, which is mediated by the cavitation phenomena, has more benefits than traditional solvent extraction methods since it requires less instruments, saves money, and extracts material quickly.[13,14] In the application of the green extraction principle, not only are non-conventional extraction methods used, but they are also combined with the use of environmentally friendly solvents, such as ionic liquids, natural deep eutectic solvents, supercritical fluids, etc.

Natural Deep Eutectic Solvents (NADESs) are an environmentally

friendly solvent that is currently used for extraction due to its biocompatible and biodegradable properties. NADES are formed by the presence of intermolecular hydrogen bonds between hydrogen bond donors (HBDs) and acceptors (HBAs) components.[15] The advantages of NADES solvents are their chemical properties, which include low melting points, low volatility, non-flammability, low vapor pressure, polarity, chemical and thermal stability, and miscible solubility.[16,17]
In the extraction process, various variables can affect quality.[18] In previous research, we optimized several variables in *R. tomentosa* leaf extraction, including extraction time, water addition, and solid-to-liquid ratio, to obtain the optimum extract. However, in order to optimize extraction efficiency, it is imperative to implement a systematic approach. The use of statistical methods such as response surface method (RSM) can be a solution to overcome the complexity of determining optimal conditions by using several types of variables at once. This method can reduce experimental trials, making it a widely preferred method in the optimization process.[19]Our previous study succeeded in optimizing the phenolic and antioxidant components in *R. tomentosa* NADES-UAE extract with the help of the statistical method of RSM, and the results were better than ethanol extract.[20] To the best of our knowledge, no research has been conducted to simultaneously optimize flavonoid compounds and tyrosinase inhibitory activity from *R. tomentosa* leaves, using NADES-based ultrasound-assisted extraction as a candidate for high-quality, environmentally friendly, and effective raw materials for skin care preparations. The objective of this study is to offer suggestions for the most effective extraction conditions for active compounds and skin-lightening activity from *R. tomentosa* leaf using the NADES-UAE method through the utilization of the RSM.

Materials and Methods

Plant materials
In this current study, *Rhodomyrtus tomentosa* leaves that were collected from Palangkaraya, Central Kalimantan, Indonesia (geographical coordinates: 2.21° S, 113.92° E) on 7 December 2023. The plant material was authenticated at the Center for Traditional Medicine Information and Development, Faculty of Pharmacy, University of Surabaya (voucher specimen No. 1532/D.T/IX/2023). Fresh leaves were washed, dried in a shaded area, pulverized, and sieved using a 30-mesh sieve. The leaf powder was stored in an airtight chamber at room temperature until further use.

Chemical materials
The chemicals used in this study included choline chloride (pharmaceutical grade) from Xi'an Rongsheng Biotechnology Co., Ltd. (China); propylene glycol, glycerol, sorbitol, and polyethylene glycol (pharmaceutical grade) from Jayarindo Pratama Laboratory (Indonesia); and quercetin, mushroom tyrosinase (T3824), L-tyrosine (T8566), and kojic acid (analytical grade) from Sigma Aldrich (Germany).

NADES-UAE Extraction Procedure
The NADES-UAE technique, combining of a green extraction solvent and method was employed for the extraction of *R. tomentosa* leaves. The extraction process in this study was divided into two phases. In the first phase, extractions were performed using combinations of choline chloride as HBAs and various HBDs to determine the most effective solvent system. (Table 1) presents the list of HBAs and HBDs used in this initial phase. All NADESs were prepared using the heating method as described by Sakurai et al.,[21] in which choline chloride was mixed with the corresponding HBDs at 80°C for 30 min under continuous stirring until a homogenous mixture was obtained. The prepared NADESs were required to remain homogenous, stable, and clear during storage at room temperature. For extraction, 1 g of dried *R. tomentosa* leaf powder was extracted with 10 mL of NADESs using ultrasonic bath (Powersonic 405, Hwashin, Seoul, South Korea) operating at a frequency of 40 kHz for 60 min at room temperature.
After determining the optimal solvent, extraction optimization was conducted. In this stage, a known amount of the dried sample was accurately weighed and mixed with the NADES solvent at different solid-to-liquid ratios (0.03, 0.05, or 0.10 g/mL). The selected NADES solvent was further modified by adding water at various concentrations (20%; 25%; or 30%). Extractions were then performed for predetermined durations (30; 40; or 60 min). All extraction procedures carried out in this study were replicated three times.

Table 1: List of HBA and HBD combination used

Groups	HBA	HBD	Molar ratio (HBA:HBD)	Reference
DES1	Choline chloride	Propylene glycol	1:1	[22]
DES2	Choline chloride	Glycerol	1:2	[23]
DES3	Choline chloride	Sorbitol	1:2	[24]
DES4	Choline chloride	Polyethylene glycol	1:2	[20]

Extraction Optimization using the Response Surface Method (RSM)
Extraction optimization to obtain a high level of total flavonoid and high tyrosinase inhibition activity was performed with the help of the RSM using the Box-Behnken design with three factors, each at three levels, and analyzed using Design-Expert software v.13 (Stat-Ease Inc., Minneapolis, MN, USA). Three extraction variables were selected as factors in this study, namely extraction time (min), water addition to NADES (%, v/v), and solid-to-liquid ratio (g/mL), which were coded as X_1, X_2, and X_3, respectively. The levels of each coded factor and their real values are shown in (Table 2).

Table 2: Coded and actual levels of optimized variables with experimental and predicted responses for all runs

Run	X_1 (T)[a]	X_2 (Wa)[b]	X_3 (Stl)[c]	TFC (mg QE/ g dried leaves)		Tyrosinase inhibition (%)	
				Experiment.	Pred.	Experiment.	Pred.
1	45(0)	30(1)	0.10(1)	7.7093	7.6847	28.42	28.54
2	45(0)	25(0)	0.05(0)	11.8427	10.7200	62.12	61.45
3	30(-1)	20(-1)	0.05(0)	13.6518	13.6420	65.36	65.16
4	30(-1)	25(0)	0.10(1)	10.5798	10.7391	6.13	5.79
5	45(0)	30(1)	0.03(-1)	11.0993	11.2469	23.22	22.68
6	60(1)	25(0)	0.03(-1)	13.4132	12.9445	29.04	29.37
7	45(0)	20(-1)	0.10(1)	12.6157	12.4447	35.88	36.40
8	60(1)	20(-1)	0.05(0)	14.3729	13.9096	83.56	83.34
9	45(0)	20(-1)	0.03(-1)	11.1476	11.1669	47.22	47.10
10	30(-1)	25(0)	0.03(-1)	11.0198	10.9977	7.08	7.39
11	60(1)	25(0)	0.10(1)	11.2059	11.2187	26.46	26.13
12	45(0)	25(0)	0.05(0)	11.7881	11.7200	62.61	62.45
13	30(-1)	30(1)	0.05(0)	10.9471	10.8064	45.85	46.04
14	60(1)	30(1)	0.05(0)	12.6594	11.6652	69.99	70.18
15	45(0)	25(0)	0.05(0)	11.5426	11.7200	62.63	62.45

[a] T is extraction time (min); [b] Wa is water addition to NADES (%, *v/v*); [c] Stl is solid-to-liquid ratio (g/mL)

The combination of factors obtained was subsequently tested in the laboratory to develop a response surface model represented by a quadratic polynomial regression (equation 1), as follows:

$$Y = \beta_0 + \sum_{k=1}^{3} \beta_k X_k + \sum_{k=1}^{3} \beta_{kk} X_k^2 + \sum_{k=1}^{2} \sum_{l=k+1}^{3} \beta_{kl} X_k X_l \quad equation\ 1$$

Where Y denotes the response variable (total flavonoid yields); β_0 is a constant and represents the intercept; and β_k, β_{kk}, and β_{kl}, correspond to the linear, quadratic, and interaction coefficients, respectively.

Flavonoid Content Determination

The total flavonoid content was determined using colorimetric method as previously described by Oktaviyanti et al.,[25] with minor modifications. About 0.2 mL of extract was pipetted, then added with 1.5 mL of 0.33% $AlCl_3$ and 1.5 mL of 10% acetic acid reagent. The mixture was then added with distilled water to a final volume of 10.0 mL and incubated for 30 min at room temperature. The absorbance of the solution was measured using a UV-Vis spectrophotometer (UV-1900, Shimadzu Corp., Kyoto) at a maximum wavelength of 435.6 nm, which has been previously determined. A calibration curve of quercetin was prepared using a concentration range of 5-30 ppm. The obtained absorbance values were substituted into the regression equation as y values to calculate the flavonoid content, expressed in milligrams quercetin equivalents per gram dried leaves (mg QE/ g dried leaves). All experiments were performed in triplicate and quercetin was used as the standard compound.

Evaluation of the Tyrosinase Inhibition Activity

Evaluation of tyrosinase inhibitors using the spectrophotometric method was carried out using a method that refers to Jung et al.[26] with slight modifications. In this study, the enzyme solution was freshly prepared by dissolving mushroom tyrosinase (T3824) in a phosphate buffer (pH 6.5) to obtain a final concentration of 500 U/mL. In addition, the substrate solution was prepared by dissolving 4.530 mg of L-tyrosine (T8566) in a phosphate buffer (pH 6.5) and diluting it to a final volume of 25.0 mL.

Furthermore, the control solution (A) was prepared by mixing 80 µL of the enzyme solution with 80 µL of the substrate solution, followed by the addition of phosphate buffer (pH 6.5). For the blank control (B), 80 µL of the substrate solution was added with phosphate buffer (pH 6.5), and its absorbance was measured at 476 nm using a UV-Vis spectrophotometer.

To evaluate the inhibitory activity of the extract, 40 µL of the sample extract sample was pipetted and mixed with 80 µL of the enzyme solution, 80 µL of the substrate solution, and phosphate buffer (pH 6.5) (C). As a blank sample (D), the absorbance of the extract in phosphate buffer (pH 6.5) was measured. All solutions (A, B, C, and D) were adjusted to a final volume of 200 µL with phosphate buffer (pH 6.5) and incubated at 25ºC for 30 min before the absorbance was measurement at 477 nm. Kojic acid at a concentration 40 ppm was prepared as a reference inhibitor, and all measurements were performed in triplicate.

The percentage inhibition value of each extract against tyrosinase was calculated based on the equation 2

$$\%\ \text{inhibition} = \frac{(\text{Abs}_A - \text{Abs}_B) - (\text{Abs}_C - \text{Abs}_D)}{(\text{Abs}_A - \text{Abs}_B)} \quad equation\ 2$$

Where Abs_A, Abs_B, Abs_C, and Abs_D are the absorbances of solutions A, B, C, and D, respectively.

Statistical Analysis

In this study, three replication procedures were used to obtain all of the results. All total flavonoid yields and tyrosinase inhibition data were presented as mean ± standard deviation (SD), where the SD values are represented as error bars in the figures. A one-way analysis of variance (ANOVA) was performed to evaluate the data on total flavonoid yield and tyrosinase inhibition (%), using a significance level of $p<0.05$ to indicate statistical significance (IBM SPPS Statistics, version 29.0; IBM Corp., New York, USA). Meanwhile, the analysis to determine the optimal extraction conditions was performed using the response surface methodology (RSM) (Design-Expert® software v13; Stat-Ease Inc., Minneapolis, MN, USA).

Results and Discussion

In this study, the principle of green extraction was applied by integrating an environmentally friendly extraction technique, ultrasound-assisted extraction (UAE), with natural deep eutectic solvents (NADESs) as sustainable alternatives to conventional organic solvents. NADESs offer not only low toxicity but also high extraction efficiency, with physicochemical properties that can be readily tailored to meet specific extraction requirements depending on the target compounds. These properties can be adjusted by selecting appropriate hydrogen bond acceptors (HBAs) and hydrogen bond donor (HBDs), as well as by adjusting their molar ratios.[27,28]

In this study, four types of NADES combinations were selected, each consisting of choline chloride as HBA and a polyalcohol compound as HBD. These compounds were chosen because of their ease of availability, affordability, safety, and extraction efficiency. Moreover, they are widely known for their applications in cosmetics formulations. Previous study has shown that the polyalcohol-based NADESs are superior to organic acids or sugars due to their stronger hydrogen bonding capacity, which enhances their ability to interact with the flavonoid compounds.[29,30]

As shown in (Figure 1) and (Figure 2), the type of HBDs employed in choline chloride-based NADESs plays an important role in the extraction efficiency for flavonoid compounds and tyrosinase inhibitory activity. The results show that the different types of HBD used led to statistically significant differences ($p<0.05$) in both flavonoid extraction and tyrosinase inhibition. Our findings indicate that total flavonoid compounds are best extracted using DES1. The viscosity of a solvent influences the diffusivity of the active compound, thereby impacts extraction efficiency. NADESs prepared from a combination of choline chloride and propylene glycol tend to have lower viscosities than other mixtures using glycerol, sorbitol, and polyethylene glycol. This phenomenon due to the ability of propylene glycol to reduce the viscosity of a solution by increasing the solvent's molecular dispersion capacity.[31]

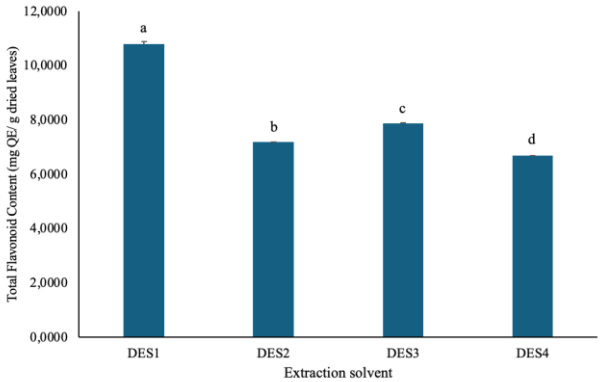

Figure 1: Effect of various NADESs on the total flavonoid yield. Data are expressed as mean±SD (n=3), different letters indicate *p<0.05*)

Similarly, the strongest tyrosinase inhibitory activity was also demonstrated by DES1. This effect can be attributed to the ability of flavonoids, which have been mechanistically proven to be able to bind and inhibit the tyrosinase through a competitive inhibition mechanism involving with the enzyme's active site. This can interfere the oxidation reaction of L-tyrosine by tyrosinase to form L-DOPA, which is subsequently become dopaquinone during the melanin biosynthesis process.[32]

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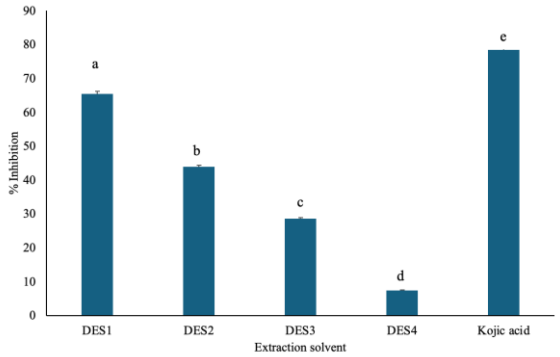

Figure 2: Effect of various NADESs on the tyrosinase inhibition. Data are expressed as mean±SD (n=3), different letters indicate *p<0.05*).

Furthermore, research conducted by Fan et al.[33] demonstrated the ability of quercetin, a flavonoid, to chelate the active site of enzymes, thereby preventing substrates from binding to the enzyme. The hydroxyl group in flavonoids can act as a chelating ligand, inhibiting the catalytic process. [34] Subsequently, entire of *R. tomentosa* leaf extraction procedure was carried out using a choline chloride-propylene glycol combination at a 1:1 molar ratio.

In the current investigation, a Box-Behnken design (BBD) was employed to optimize the extraction parameters using response surface methodology (RSM). All response values derived from the 15-run experiment and the predicted responses are presented in (Table 2). The strong correlation between the experimental and predicted values indicates the model's precision. The highest yield for both TFC and tyrosinase inhibition were observed in Run No. 8, with a TFC value of 14.3729 mg QE/ g dried leaves and an inhibition capacity of 83.56%. The experiment data were subjected to multiple regression analysis, and the regression equation for both responses were generated using Design expert software, illustrating the empirical relationship between the test variables and the corresponding responses (Table 3).

Table 3: Polynomial Regression Equations Describing the Effects of Process Variables on Each Response

Code	Response	Model Equation
Y_1	TFC (mg QE/ g dried leaves)	$Y_1 = 11.72 + 0.6816X_1 – 1.17X_2 – 0.5711X_3 + 0.2478X_1X_2 – 0.4418X_1X_3 – 1.21X_2X_3 + 1.05\ X_1^2 + 0.1358\ X_2^2 – 1.22\ X_3^2$
Y_2	Tyrosinase inhibition (%)	$Y_2 = 62.45 + 10.58X_1 – 8.07X_2 – 1.21X_3 + 1.49X_1X_2 – 0.4075X_1X_3 + 4.13X_2X_3 – 6.39\ X_1^2 + 10.12\ X_2^2 – 38.89\ X_3^2$

As shown as (Table 4) and (Table 5), the coefficient of variance (CV) and R-squared (R^2) value were calculated to evaluate the adequacy of the models. The R-squared quantifies the proportion of variability in the observed responses that is explained by the model. Theoretically, a model is considered to have a good fit when the R^2 value is at least 80%. Our result showed that the model explained 99.44% of the variability in total flavonoid yield and 99.98% of the variability in tyrosinase inhibition, respectively. The excellent results of the R^2 values indicate that the model provides a strong explanation of the variation between the data.[35]

Table 4: ANOVA summary of regression models to predict total flavonoid yield

Source	Degrees of freedom	Sum of Squares	Mean square	*f*-value	*p*-value
Model	9	34.62	3.85	98.24	< 0.0001
X_1	1	3.72	3.72	94.91	0.0002
X_2	1	10.98	10.98	280.43	< 0.0001
X_3	1	2.61	2.61	66.64	0.0004
X_1X_2	1	0.2456	0.2456	6.27	0.0542
X_1X_3	1	0.7808	0.7808	19.94	0.0066
X_2X_3	1	5.90	5.90	150.67	< 0.0001
X_1^2	1	4.05	4.05	103.46	0.0002
X_2^2	1	0.0681	0.0681	1.74	0.2444
X_3^2	1	5.47	5.47	139.72	< 0.0001
Residual	5	34.62	0.0392		
Lack of fit	3	0.1447	0.0482	1.89	0.3647
Pure error	2	0.0511	0.0256		

$R^2 = 0.9944$; Adjusted $R^2 = 0.9843$; Predicted $R^2 = 0.9302$; C.V % = 1.69

Table 5: ANOVA summary of regression models to predict tyrosinase inhibitory activity

Source	Degrees of freedom	Sum of Squares	Mean square	*f*-value	*p*-value
Model	9	7798.76	866.53	3217.13	< 0.0001
X_1	1	895.28	895.28	3323.87	< 0.0001
X_2	1	520.68	520.68	1933.10	< 0.0001
X_3	1	11.69	11.69	43.40	0.0012
X_1X_2	1	8.82	8.82	32.75	0.0023
X_1X_3	1	0.6642	0.6642	2.47	0.1771
X_2X_3	1	68.39	68.39	253.92	< 0.0001
X_1^2	1	150.55	150.55	558.94	< 0.0001
X_2^2	1	378.30	378.30	1404.51	< 0.0001
X_3^2	1	5584.48	5584.48	20733.32	< 0.0001
Residual	5	1.35	0.2693		
Lack of fit	3	1.18	0.3933	4.71	0.1800
Pure error	2	0.1669	0.0834		

$R^2 = 0.9998$; Adjusted $R^2 = 0.9995$; Predicted $R^2 = 0.9975$; C.V % = 1.19

The quadratic regression for total flavonoid presented adjusted R^2 values (0.9843) and predicted R^2 (0.9302), with a difference of less than 0.2. Similar results were also shown in quadratic regression for tyrosinase inhibitory activity, which were 0.9995 and 0.9975, respectively. This similarity and agreement between the adjusted R^2 and predicted R^2 values indicates that the model can be considered reliable for prediction.[36]

The additionally, the CV was used to assess the level of data variability, proving the accuracy of the model's predictions. Our findings demonstrated that the CV values of both responses were less than 10% (1.69 and 1.19), which is confirming the accuracy of the models. The inadequate representation of the experimental data by the model at specific places is shown by the lack-of-fit value. It is also clear that the

models were successful in forecasting the responses because the lack-of-fit value were not statistically significant ($p > 0.05$).[37,38]
According to the ANOVA result, the *p*-value obtained for both models indicated that the model was highly statistically significant for the responses ($p < 0.05$). The extraction time (X_1, min), water addition to NADES (X_2, %*v/v*), and solid-to-liquid ratio (X_3, g/mL) provide a significant *p*-value on total flavonoid yield and tyrosinase inhibitory activity. In order to find out the interaction among the variables, the three-dimensional surface plot graphs were created, with one independent variable kept constant while the other two were altered (Figure 3 and Figure 4).

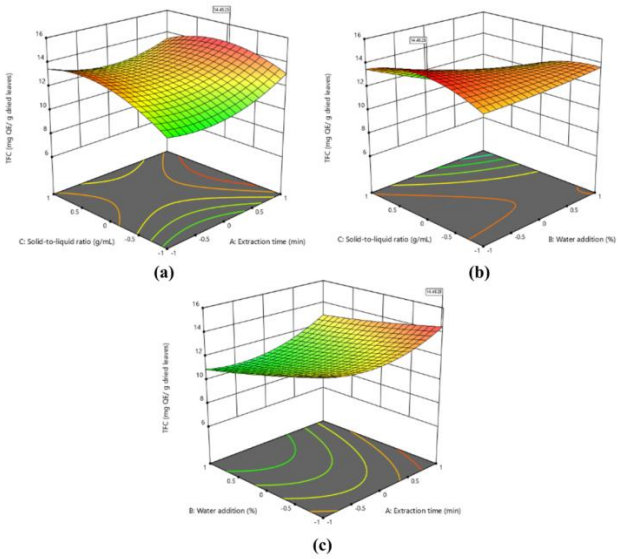

Figure 3: Predicted three-dimensional surface plot of total flavonoid extraction as a function of (a) extraction time and solid-to-liquid ratio; (b) water addition to NADES and solid-to-liquid ratio; (c) extraction time and water addition to NADES

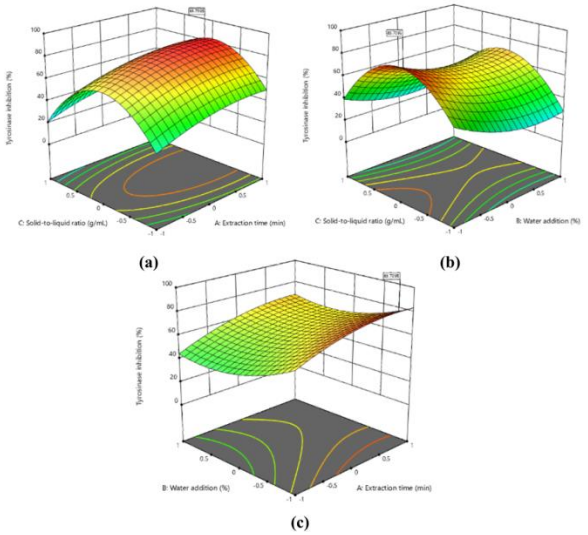

Figure 4: Predicted three-dimensional surface plot of tyrosinase inhibition activity as a function of (a) extraction time and solid-to-liquid ratio; (b) water addition to NADES and solid-to-liquid ratio; (c) extraction time and water addition to NADES

Both figures indicate that both responses increase as extraction time increases. A longer extraction time provides a greater opportunity for the compounds to come into contact with the extraction solvent. Extended extraction durations will also allow more time for the compound diffusion, the longer the duration, the more compounds are able to diffuse.[39]
NADESs have a notable drawback, namely their relatively high viscosity. The addition of water to NADESs can reduce this viscosity problem, thereby facilitating the diffusion of target compounds. Our results show that excessive water to NADESs decreases extraction efficiency. This is attributed to the potential weakening of intermolecular interactions due to the reconstruction of the hydrogen-bond structure between HBA and HBD, as well as with the target compound.[40] Moreover, water can drastically alter the polarity of NADESs, increasing it to a level that no longer aligns with the polarity of the target bioactive compounds, particularly flavonoids, which are relatively semipolar.[41]
As our expected, the solid-to-liquid ratio exhibited a quadratic effect on flavonoid yield and tyrosinase inhibitory activity. A lower solid-to-liquid ratio initially increased both flavonoid extraction yield and tyrosinase inhibition activity, likely due to enhanced solubility associated with a larger solvent volume[42]. The yield reached its maximum at 0.05 g/mL and subsequently decreased. Since diffusion of compounds during the extraction process depends on the concentration gradient, further decreases in the solid-to-liquid ratio will reduce this gradient between the plant matrix and the solvent, thereby lowering extraction efficiency.[43]
Our findings showed that flavonoid compounds and antioxidant activity were optimally obtained by extracting *R. tomentosa* leaves using NADESs consisting of a choline chloride-propylene glycol combination at a 1:1 molar ratio with the addition of 20% water (X_2), employing the UAE method for an extraction time of 60 min (X_1) and a solid-to-liquid ratio of 0.05 g/mL (X_3).
Limitations of this study include the lack of identification of specific flavonoid compounds responsible for the skin-lightening activity. Furthermore, the stability and efficacy of the extract obtained under optimum extraction conditions must be proven in the formulation of cosmetic products. Therefore, further studies focusing on specific compound identification, extract stability testing, and formulation development are needed to strengthen the findings of this study and to support the application of NADES-based *R. tomentosa* extract as a raw material for cosmetic products.

Conclusion

In general, this study succeeded in generating a sustainable extraction protocol that increased the yields of active flavonoid compounds and the potential of *R. tomentosa* leaves for skin lightening through tyrosinase inhibition activity, providing a scientific basis for further development in cosmetic and functional food applications. The optimum conditions using choline chloride–propylene glycol (1:1 molar ratio) with 20% water addition, an extraction duration of 60 min, and a solid-liquid ratio of 0.05 g/mL, successfully produced an extract with a high flavonoid as bioactive compound content and strong tyrosinase inhibition activity. These findings provide a scientific basis that strengthens the feasibility of *R. tomentosa* leaves as a reliable raw material in the future for cosmetic product development. Building on these findings, future research may focus on more comprehensive phytochemical profiling to identify specific flavonoid compounds responsible for skin-lightening activity, thereby strengthening the findings of this study and enhancing its potential applications. In addition, further investigations into extract stability and formulation development for facilitating the development of NADES-based *R. tomentosa* extracts for cosmetic use.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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