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Optimization of microwave-assisted extraction of total flavonoid content from red betel leaf (*Piper crocatum* Ruiz and Pav) and its correlation with antioxidant and antibacterial activities using response surface methodology

Ririn Astyka[1], Poppy Anjelisa Zaitun Hasibuan[2], Sumaiyah Sumaiyah[3], Nur Aira Juwita[1], Muhammad Fauzan Lubis[1]*

[1]Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia.
[2]Department of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia.
[3]Department of Pharmaceutical Technology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia.

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ABSTRACT

The optimization of microwave-assisted extraction (MAE) was conducted using response surface methods to improve the extraction of flavonoids from *Piper crocatum* Ruiz and Pav leaf. The optimization process included a Box–Behnken experimental design (BBD), which involved three variables at three levels. The present study aimed to evaluate the impact of varying ethanol concentrations (50%, 75%, and 100%), microwave power levels (180, 300, and 450 W), and extraction durations (3, 8.5, and 14 minutes) on the respective responses. The experimental data was subjected to fitting using a second-order polynomial model. Subsequently, an analysis of variance (ANOVA) and multiple regression analysis were employed to assess the adequacy of the model and determine the ideal settings. Taking into account the highest concentration of extracted total flavonoids, as well as the antioxidant and antibacterial properties. The experimental results indicate that the optimum conditions for all the reactions under investigation were an ethanol concentration of 78.48%, a microwave power of 327.96 W, and an extraction duration of 8.60 minutes. Under the ideal conditions, the anticipated outcomes of the sample indicate a total flavonoid content (TFC) of 229.647 mg QE/g dry weight (DW), a 1,1-diphenyl-2-picryhydrazyl (DPPH) scavenging activity of 73.915%, and an inhibition zone measuring 18.621 mm. The implementation of a concurrent MAE methodology for the isolation of total flavonoids, as well as the evaluation of antioxidant and antibacterial properties from *P. crocatum*, signifies the recognition of the extract as a significant reservoir of bioactive substances.

INTRODUCTION

It has become widely recognized throughout the years that there is a strong connection between health benefits and nutrition [1]. As a result, consumers have increasingly been inclined to select food products that are filled with bioactive compounds, as these substances have been found to have a good impact on human health [2]. Plant materials have garnered significant attention due to their high concentration of bioactive chemicals [3]. Typically, most bioactive chemicals are situated within the cellular environment and necessitate liberation into the extracting solvent during extraction [4,5]. The conventional extraction methods, namely maceration, distillation, and soxhlet extraction, are frequently employed to extract bioactive compounds from plant materials [6].

Nevertheless, these methods are associated with drawbacks, including low extraction efficiency, excessive use of extracting solvents, high energy consumption, and prolonged extraction duration [7]. Hence, the concept of green

*Corresponding Author
Muhammad Fauzan Lubis, Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia.
E-mail: fauzan.lubis @ usu.ac.id

extraction has garnered considerable attention and has been put out by numerous scholars in the field [8]. Green extraction refers to a type of extraction that involves using solvents with reduced extraction capabilities, resulting in lower energy consumption and shorter process durations while increasing the yield of the extraction process [9]. The development of green extraction methods has been motivated by producing environmentally friendly and economically viable solutions in response to industrial issues. These approaches aim to minimize ecological and environmental impacts throughout the extraction process [10].

Microwave-assisted extraction (MAE) is a widely utilized sophisticated technique to extract phytochemicals from plant sources. This technique involves the utilization of microwaves to induce thermal energy, establishing a pressure gradient within the sample [11]. Consequently, phytochemicals present in the plant are released via diffusion, tissue bursting, or cell wall rupturing. The increase in temperature additionally facilitates the softening of plant tissue, enhances mass transfer, heat transfer, and solvent penetration within the sample, disrupts the chemical structure, and aids in the extraction of polyphenols into the solvent [12]. Multiple factors contribute to the phenomenon of MAE, potentially influencing both the quantity and quality of phytochemicals extracted from botanical substances [13]. The utilization of MAE in the extraction process has predominantly been documented for obtaining a flavonoid compound [14]. However, optimizing several factors, such as solvent concentration, microwave power level, and irradiation period, is necessary to achieve improved extraction outcomes [15].

Piper scrotum Ruiz and Pav, generally known as red betel, is frequently observed in several Southeast Asian nations, with a notable presence in Indonesia. The plant in question is a member of the *Piper*aceae family and is indigenous to the region [16]. In Indonesian society, the leaves of this plant have been traditionally used for medicinal purposes. Numerous research has documented the pharmacological properties exhibited by this particular plant, including antioxidant [17], antibacterial [18], anti-inflammatory [19], and anticancer activity [20]. *Piper crocatum* has been shown to possess many secondary metabolites, primarily steroids, tannins, saponins, alkaloids, and flavonoids. The *P. crocatum* plant possesses pharmacological properties attributed explicitly to its components, notably flavonoids [21]. As documented in scientific literature, there is a significant correlation between flavonoids and their antioxidant qualities and antibacterial activity [22]. Nevertheless, the significant accumulation of flavonoids might be a health advantage due to their antioxidant and antibacterial properties, making them suitable for utilization in the pharmaceutical and food sectors [23,24].

The food business has encountered challenges in improving process efficiency without a concurrent investment and resource use increase. Investigating to determine the optimal conditions for the system or the food optimization process is crucial in resolving this predicament [25]. Historically, the optimization process involved examining the impact of altering one parameter at a time on a given output while keeping other parameters constant. However, this approach failed to consider the interactive effects between specific parameters, resulting in a lack of comprehensive understanding of the combined effects of all factors on the response [26]. Moreover, this methodology necessitates further experimentation, escalating expenses, and time consumption. Hence, multivariate statistical methods have been employed to improve the process parameters in food applications [27]. Response surface methodology (RSM) is widely recognized as a prominent approach for analyzing multivariate statistical approaches [28]. The RSM is a statistical and mathematical technique that uses polynomial models to assess data compatibility [29]. Its primary objective is to reveal the underlying patterns and behaviors within the data, ultimately leading to the development of mathematical models to make predictions [30].

Currently, there is a lack of research investigating optimizing flavonoid content extraction from *P. cronatum* leaf utilizing MAE and RSM as well as the correlation with antioxidant and antibacterial activities. This approach establishes the ideal extraction conditions by considering independent factors such as ethanol concentration, microwave power, and extraction duration. The investigation of various parameters on the efficacy of MAE involved the determination of total flavonoid content (TFC) and the assessment of antioxidant and antibacterial activities. In addition, the relationship between TFC, and antioxidant and antibacterial activities will be examined to elucidate the extract's mechanism as an antioxidant and antibacterial agent.

MATERIALS AND METHODS

Plant material

The leaf of *P. crocatum* was cultivated inside the botanical areas of the Faculty of Pharmacy at Universitas Sumatera Utara, located in Indonesia. The leaf was identified as *P. crocatum* Ruiz and Pav by the Herbarium Medanese, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, with a voucher ID of 512/UN.5.1.1-HM/2023. The collected leaves were subjected to a drying process and stored at an ambient temperature. The dried leaves were pulverized using a household blender, and the resulting material's average particle size (0.450 mm) was determined using a set of sieves. The dried powder was stored at room temperature before its utilization in the extraction process.

Chemicals and media

Chemicals such as ethanol, methanol, 1,1-diphenyl-2-picryhydrazyl (DPPH), aluminum chloride, and quercetin were used in this research and are analytical and certified by Sigma-Aldrich, United Kingdom. The *Staphylococcus aureus* (ATCC 29737), deMann Rogosa and Sharpe Agar (MRSA), and then Nutrient Agar (NA) were obtained from the Microbiology Laboratory, Faculty of Pharmacy, Universitas Sumatera Utara, Indonesia.

MAE procedure

Single conditions of MAE were performed in a homemade setup consisting of the microwave with modification (Samsung ME731K, Seoul, South Korea). Seventeen experimental MAE runs on different ethanol

concentrations (X_1), microwave power (X_2), and extraction time (X_3), were performed according to the results of Design-expert v.13. In every iteration of the experiment, a mass of 10 g of the sample was combined with a volume of 100 ml of the extraction solvent. The flasks were subsequently positioned within the MAE device, and extractions were conducted at a predetermined frequency. Following the extraction process, the crude extracts were promptly subjected to filtration using filter paper with a pore size ranging from 4 to 12 µm, employing a vacuum system. The collected samples were placed into glass flasks and thereafter stored at a temperature of 4ºC until they were ready for additional examination [31].

Selection of factors

A significant number of characteristics may influence the researched extraction system. Consequently, it is crucial to choose the carefully most substantial impact elements. Several factors can potentially affect a given study's MAE. These factors include the nature of the solvent used, the ratio of solvent to solid material, the duration of the extraction process, the power level of the microwave used, the temperature at which the extraction is conducted, the properties of the sample being analyzed, and the number of extraction cycles performed [32]. Understanding the effects and combinations of these elements on the MAE process holds significant importance. The initial phase was the selection of the most appropriate solvent. Numerous findings indicate that ethanol is suitable for extracting diverse polyphenolic components from plant sources [33]. The selection of condition ranges for the MAE was informed by existing literature. The X_2 exhibited an influence ranging from 180 to 400 W, while the X_3 ranged from 3 to 14 minutes. The analytical findings are provided in Table 1.

Total flavonoids content

The TFC of extracts was obtained using spectrophotometry methods. Briefly, 2 ml of extracts in methanol was mixed with 0.10 ml of 10% $AlCl_3$, 0.10 ml of $NaC_2H_3O_2 \cdot 3H_2O$ (1 M), and 2.80 ml of distilled water. The samples were incubated for 40 minutes, and after that, the absorbance of samples was measured at 432 nm. To determine the TFC of the samples, a calibration curve was built utilizing quercetin as the standard compound. The flavonoid concentration is expressed as mg QE/g sample. The equation to determine TFC as can see below (Eq. 1) [34]

$$C\,(\mathrm{QE}) = \frac{C \times V}{M} \times F$$

Table 1. The experimental domain used in BBD.

Variables	Symbol	Levels		
		−1	0	+1
Ethanol concentration (%)	X_1	50	75	100
Microwave power (W)	X_2	180	300	450
Extraction time (min)	X_3	3	8.5	14

C (QE): Concentration of flavonoid as quercetin equivalent

C: Concentration determined from standard curve (µg/ml)

V: Volume used in the assay (ml)

M: Mass of the sample which used in the assay (g)

F: Dilution factor

Antioxidant activity

The measurement of free radical scavenging activity was conducted using the DPPH technique. A 0.2 mM solution of DPPH in methanol was produced. Subsequently, 100 µl of this solution was added to a solution containing extracts at a concentration of 100 µg/ml. After 60 minutes, the measurement of absorbance was conducted at a wavelength of 516 nm. The calculation of the percentage of inhibition was performed by comparing the absorbance values obtained from the control group with those obtained from the samples, as illustrated below (Eq. 2) [35].

$$\%\ \text{Inhibition} = \frac{\text{Absorbance control x Absorbance sample}}{\text{Absorbance control}}\ \text{x}100\%$$

Antibacterial activity

To determine the antibacterial activity of extracts, a standard disc diffusion method was performed. Briefly, the 100 mg/ml of each extract was used as a sample test against *S. aureus*. Sterile discs with a diameter of 6 mm were saturated with 25 µl of extract solution. Following a 15-minutes incubation period for optimal extract diffusion, the discs were placed onto a NA surface coated with 0.1 ml of a bacterial culture. The bacterial culture had been standardized to a concentration of 0.5 McFarland standards (106 CFU/ml). The plates were subjected to incubation at a temperature of 37ºC for 12–14 hours. The outcomes were documented by measuring the area of growth inhibition surrounding the discs [34].

Statistical analysis and experimental planning

The RSM was employed to investigate the extraction parameters' effects and optimize the conditions for different responses. The response pattern was established first using a Box–Behnken design (BBD) with three variables. The design, which had 17 combinations and five replicates at the central point, was carried out randomly. Ethanol concentration (X_1, 50%–100%), microwave power (X_2, 180–450 W), and extraction duration (X_3, 3–14 minutes) were the three independent variables employed in this investigation. Each coded variable was made to have a range from –1 to 1 to equalize the parameters. This made the answer more evenly affected, and the units of the parameters were, therefore, unimportant. The following second-order polynomial model, which can typically explain the relationship between the responses and the independent factors, was fitted to the response variables (Eq. 3) [36]:

where *Y* represents the response variable; *Xi* and *Xj* are the independent variables affecting the response; and A_0, *Ai*,

Aii, and *Aij* are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively.

Optimal extraction parameters were discovered for three separate responses: TFC, antioxidant, and antibacterial activity. Various responses were handled based on the desirability function, and the best conditions were chosen. Using Design-Expert v.13 (Stat-Ease, Minneapolis, MN, USA), multiple linear regression analysis and the experimental design were carried out. ANOVA was used to assess the results statistically, using 0.05 as the significance level. The coefficient of determination (R^2), coefficient of variance (CV), and *p*-values for the model and lack of fit testing were used to assess the models' suitability [36].

$$Y = A_0 \sum_{i=1}^{3} A_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{j=i+1}^{3} A_{ij} X_i X_j$$

RESULTS AND DISCUSSION

Model fitting analysis

The response surface approach was used to successfully optimize the MAE of flavonoid content from the *P. crocatum* leaf. Seventeen runs of the experimental design comprised streamlined experimental sets with five independent variable central values. Table 2 lists the BBD configuration and the observed responses for TFC, DPPH scavenging, and antibacterial activity. Using the second-order equation (Eq. 3), the R^2 of the model's intercept, linear, quadratic, and interaction terms were determined. ANOVA was used to determine the significance of the influence of the linear, quadratic, or interaction coefficients on the answer (Table 3). Each component's *p*-value indicates its significance (Table 3). The fitted model accurately represents the experimental data, which has high correlation values (R^2) ranging from 0.9339 to 0.9976 (Table 3). Table 3's ANOVA demonstrates that the regression models for TFC, DPPH scavenging activity, and antibacterial activity were statistically significant, with significance levels ranging from $p = 0.0001$, $p = 0.0023$, and $p = 0.0002$, respectively. The models also showed no statistically significant lack of fit with $p > 0.05$. This led to the establishment of successful well-fitting models for TFC, DPPH scavenging activity, and antibacterial activity [29].

The effect of independent variables on TFC

The TFC of *P. crocatum* leaf extracts varied from 152.94 to 231.57 mg QE/g DW, depending on different investigated parameter levels. The lowest yield of TFC was obtained on the lower concentration of ethanol (X_1, 50%), higher level of microwave power (X_2, 450 W), and middle level of extraction time (X_3, 8.5 minutes), while the higher TFC was obtained on the middle of X_1, X_2, and X_3 (75%, 300 W, and 8.5 minutes, respectively). According to *p* values of regression coefficients (Table 3), the TFC was the most significantly influenced by X_1 and quadratic of independent variables (X_1^2, X_2^2, and X_3^2) ($p < 0.0001$, Table 3). In addition, the other variables namely X_3 ($p < 0.0038$), interaction of ethanol concentration and microwave power (X_1X_2, $p < 0.0023$), interaction of ethanol concentration and extraction time (X_1X_3, $p < 0.0067$), and interaction of microwave power and extraction time (X_2X_3, $p < 0.0375$) statistically significantly affect the total

Table 2. BBD with natural and coded MAE conditions and experimentally obtained values of TPC (mg QE/g), antioxidant activity (% Scavenging activity), and antibacterial activity (mm).

Run	Independent variables			Responses		
	Ethanol concentration (%)	Microwave power (W)	Extraction time (min)	TFC (mg QE/g)	Antioxidant activity (%Scavenging)	Antibacterial activity (mm)
1.	50	300	14	157.21	60.09	13.00
2.	100	300	14	184.32	67.33	15.23
3.	75	450	3	205.63	70.51	16.20
4.	50	450	8.5	152.94	67.94	12.90
5.	100	450	8.5	182.33	65.92	15.90
6.	75	300	8.5	231.57	75.22	18.50
7.	75	300	8.5	226.21	73.93	17.43
8.	100	180	8.5	174.03	65.36	14.90
9.	75	450	14	192.45	69.3	15.90
10.	75	180	3	203.39	68.09	16.05
11.	50	180	8.5	163.56	58.93	12.50
12.	75	180	14	200.09	70.65	16.20
13.	100	300	3	180.03	64.66	14.50
14.	75	300	8.5	230.95	70.56	18.98
15.	75	300	8.5	228.35	74.01	19.05
16.	75	300	8.5	229.14	73.94	18.40
17.	50	300	3	168.32	59.43	12.15

Table 3. Corresponding *p*-values of linear, interaction, quadratic, and ANOVA of the fitted second-order polynomial model for TFC, antioxidant, and antibacterial activities.

Source	TFC (mg QE/g)		Antioxidant activity (%Scavenging)		Antibacterial activity (mm)	
	Coefficient	*p*-value	Coefficient	*p*-value	Coefficient	*p*-value
Model		<0.0001		0.0023		0.0002
Constant (A_0)	229.34		73.70		18.50	
X_1	10.10	<0.0001	1.99	0.0262	1.26	0.0005
X_2	−0.9650	0.2202	1.33	0.1014	0.1563	0.4755
X_3	−3.06	0.0038	0.5314	0.4771	0.1712	0.4373
X_1X_2	4.72	0.0023	−2.21	0.0616	0.1604	0.6001
X_1X_3	3.85	0.0067	+0.5025	0.6299	−0.0300	0.9213
X_2X_3	−2.59	0.0375	−0.9640	0.3647	−0.1357	0.6565
X_1^2	−44.47	<0.0001	−7.88	<0.0001	−3.39	<0.0001
X_2^2	−16.65	<0.0001	−1.28	0.2358	−1.06	0.0082
X_3^2	−12.30	<0.0001	−2.78	0.0245	−1.36	0.0021
R^2	0.9976		0.9339		0.9703	
Adjusted R^2	0.9945		0.8489		0.9322	
Lack of fit		0.5728		0.3032		0.6632
CV (%)	1.04		2.93		3.72	

Table 4. Pearson's correlation coefficient (*R*) and *p*-values for TFC, antioxidant and antibacterial activities.

Investigated response	TFC	Antioxidant activity	Antibacterial activity
TFC		0.863[a]	0.957[a]
		0.000[b]	0.000[b]
Antioxidant activity	0.863[a]		0.892[a]
	0.000[b]		0.000[b]
Antibacterial activity	0.957[a]	0.892[a]	
	0.000[b]	0.000[b]	

[a]Pearson's correlation coefficient (*R*).

[b]*p*-value ($p < 0.05$ significant).

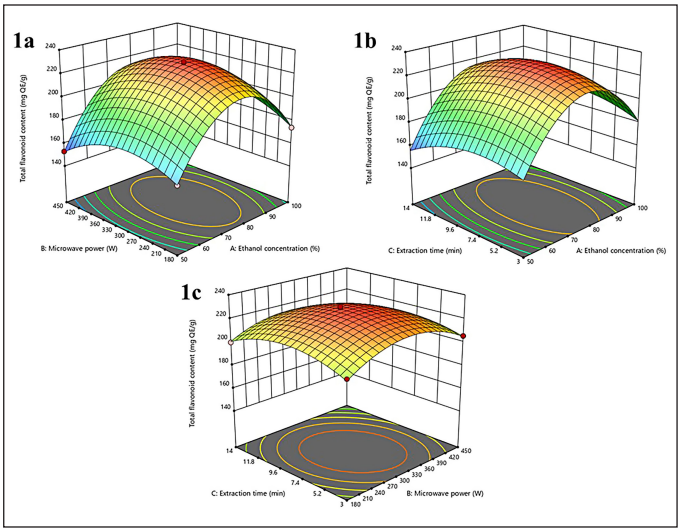

Figure 1. 3D plots of total flavonoids content (1a: interaction of X_1X_2, 1b: interaction of X_1X_3, 1c: interaction of X_2X_3.

flavonoids content. Linear term of X_1 and interaction terms of X_1X_2 and X_1X_3 exhibited positive influence. The positive effects of independent variables demonstrated that a rise in the response value can result from their positive modifications [37]. The following graph illustrates the second-order polynomial model that was used to represent the TFC as a function of independent variables:

Total flavonoids content (mg QE/g) = 229.34 + 10.10 X_1 – 0.9650 X_2 – 3.06 X_3 + 4.72 X_1X_2 + 3.85 X_1X_3 – 2.59 X_2X_3 – 44.47 X_1^2 – 16.65 X_2^2 – 12.30 X_3^2

Response surface plots (Fig. 1a and b) show the relation of X_1X_2 and X_1X_3 on the TFC of extracts. The TFC of extracts reached the maximum when the X_1 was around 75% with X_2 of 300 W. The TFC will decrease after the X_1 and X_2 increase. The interaction of X_1X_3 was described as a significant effect of X_1, the graphs in Figure 1b, also show that the TFC slightly increased with an increase of X_1 from 50% to 80%, and decreased after 80% although the X_3 in the lowest or highest conditions.

That was described as the effect of X_1 is significant on TFC [38]. In addition, the interaction of X_1X_2 and X_1X_3 was significantly proven through total flavonoid extraction ($p < 0.0023$ and $p < 0.0067$, respectively). In this research, ethanol was used as the solvent due to its high extraction efficacy, compatibility with human consumption, and environmental safety considerations [39]. The efficacy of flavonoid extraction is enhanced when alcohol is combined with water, as compared to using alcohol as a solvent alone [40]. This is attributed to

the fact that the extraction and separation of flavonoids are heavily influenced by the polarity of solvents and the chemical properties of the molecules involved [41]. The presence of a specific quantity of water is likely to induce the expansion of plant material, leading to an augmentation in the surface area of contact between the solvent and the plant matrix. This, in turn, directly influences the effectiveness of the extraction process [42]. Furthermore, to achieve optimal extraction of target compounds, it is advisable to conduct the extraction using a solvent mixture with a ratio determined based on the chemical composition and polarity of the target compound(s) [43]. The selection of a suitable solvent for MAE is contingent upon its capacity to absorb microwave energy and then convert it into heat. The dielectric characteristics of the solvent influence this conversion process [44].

Furthermore, Figure 1c, shows the significant interaction of X_2X_3 ($p < 0.0375$). This interaction has not depended on one factor. The X_2 and X_3 affect TFC and will increase if the X_2 and X_3 increase to 330 W and 10 minutes. The TFC has slightly decreased after the maximum point of X_2 and X_3. This phenomenon can be elucidated by the following observation: an elevation in microwave power resulted in a rise in system temperature, facilitating the extraction of nonflavonoid components [45]. Consequently, there was a proportional reduction in the overall concentration of flavonoids in the extracted samples. One notable advantage of utilizing MAE compared to traditional extraction methods is the reduction in the time necessary for extracting phytochemicals from plant sources [46]. The concentration of flavonoids in the extracts exhibited a decline when the duration of the extraction process exceeded 10 minutes, as depicted in Figure 1c. The extended duration of the sample's exposure to microwave irradiation and the solvent likely resulted in the extraction of chemical compounds from the extract, besides from flavonoids. These chemicals may include minerals and carbohydrates. Our findings were consistent with the outcomes reported in other studies [47].

The effect of independent variables on DPPH scavenging activity

This work aimed to assess the antioxidant activity of the leaf extracts of *P. crocatum* by in vitro experiments, specifically the DPPH radical scavenging assay. The DPPH radical scavenging assay involves the radicals' quenching by hydrogen atom transfer by antioxidants [48]. The DPPH scavenging activity of the extracts is quantified as the percentage of scavenging. The percentage of DPPH scavenging exhibited by the extracts obtained using MAE fell within a specific range from 58.93% to 75.22% (Table 2). The lowest % activity was observed on the lower level of X_1 (50%) and X_2 (180 W), but the middle level of X_3 (8.5 min). These results more highest than those reported by Alfarabi *et al.* [49] of 59.34% in 100 µg/ml conventional extract of *P. crocatum* leaf and ethanolic extract of *P. crocatum* leaf (33.00%) in 100 µg/ml reported by Fatmawaty *et al.* [17]. On the other hand, the antioxidant activity of this work is higher than that reported by Rahardjo *et al.* [50], and hot water extract of *P. crocatum* leaf reported by Kamaruzaman *et al.* [51] of 74.90% in 20 mg/ml. The antioxidant activity of the extract has a strong correlation with TFC which is $R = 0.863$ (Table 4). The increase in the total content of flavonoid extract has a significant impact on increasing its antioxidant activity. This result is similar to other studies that were reported by Do *et al.* [32] and Alide *et al.* [52].

Response surface plots (Fig. 2a) show the interaction of X_1 and X_2 is not significantly affected by % DPPH scavenging activity, but X_1 has significantly ($p < 0.0262$) affected the % DPPH scavenging activity. The increasing X_1 up to 85% was identified, leading to the % DPPH scavenging activity increase, and above 85%, the % DPPH scavenging activity will decrease. This phenomenon was identified in a range X_2 of 180–450 W. Similar to the previous factor, the interaction of X_1 X_3 and X_2 X_3 was described not significantly. The response of % DPPH scavenging activity with these factors' interaction can be seen in Figure 2b and c. The second-order polynomial

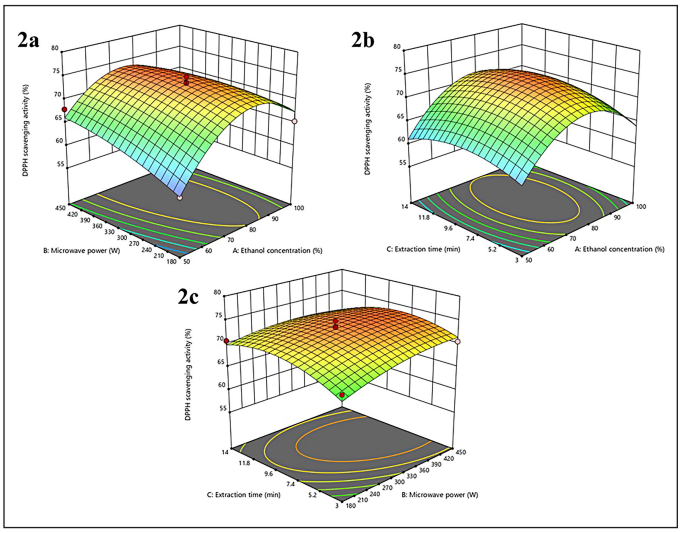

Figure 2. 3D plots of antioxidant activity (2a: interaction of X_1X_2, 2b: interaction of X_1X_3, 2c: interaction of X_2X_3.

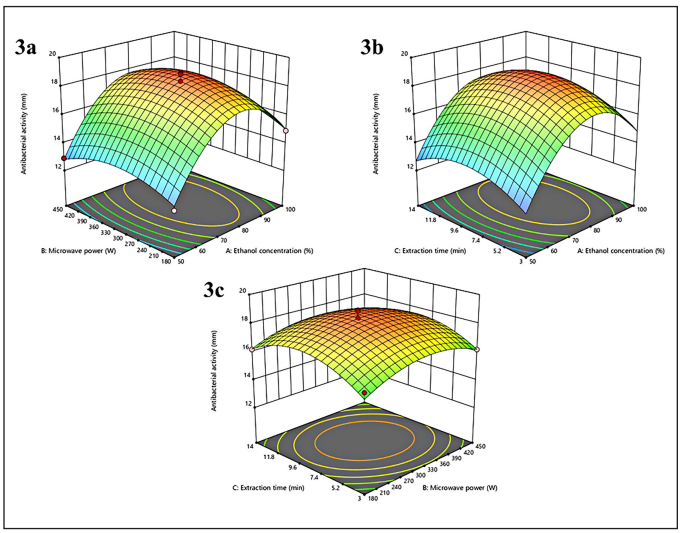

Figure 3. 3D plots of antibacterial activity (3a: interaction of X_1X_2, 3b: interaction of X_1X_3, 3c: interaction of X_2X_3.

model used to express the % DPPH scavenging activity as a function of independent variables (in terms of coded values) is shown below:

$$\%\ \text{DPPH scavenging activity} = 73.70 + 1.99\,X_1 + 1.33\,X_2 + 0.5314\,X_3 - 2.21\,X_1X_2 + 0.5025\,X_1X_3 - 0.9640\,X_2X_3 - 7.88\,X_1^2 - 1.28\,X_2^2 - 2.78\,X_3^2$$

The X_1 has a factor with a significant impact against % DPPH scavenging activity with a positive coefficient. Many studies have reported the impact of changing X_1 led % DPPH scavenging activity. Kaneria *et al.* [53] reported that the 75% methanolic extract is the best solvent for the highest % DPPH scavenging activity from *A. indica* and *M. zapota*. Mannoubi *et al.* [54] reported that 80% ethanol is the best solvent compared to 80% methanol and acetone to get the highest antioxidant activity using the DPPH method. In addition, Gonfa *et al.* [55] showed that the 80% ethanolic extract has a better DPPH scavenging activity than absolute ethanol and absolute methanol, although 80% methanol is the best solvent. Furthermore, ethanol is a solvent with a low toxicity risk, so it is better used in the extraction [56]. These reports described the significant impact of X_1 through % DPPH scavenging activity and all results are similar to this study.

The effect of independent variables on antibacterial activity

The antibacterial effect of the extract was carried out using the diffusion disc method against *Staphylococcus aureus* (ATCC 29737). The 100 µg/ml extracts were used to observe antibacterial activity through the inhibition zone (mm). The inhibition zone after the extracts treated can be seen in Table 2. The inhibition zone after treated extracts varies from 12.15 to 19.05 mm. The lowest inhibition zone was obtained from lower X_1 (50%), middle X_2 (300 W), and lower X_3 (3 minutes), while the highest inhibition zone from middle X_1, X_2, and X_3 of 75%, 300 W, and 8.5 minutes, respectively. This result shows better antibacterial activity against *S. aureus* than previous reports by Kusuma *et al.* [57]. Based on our observation, only X_1 has significantly affected the antibacterial effect from three linear variables with a positive coefficient ($p < 0.0005$). In addition, the quadratic variables X_1^2, X_2^2, and X_3^2 have significantly impacted the antibacterial activity with $p < 0.0001$, $p < 0.0082$, and $p < 0.0021$, respectively. In more detail, the second-order polynomial model to express the antibacterial activity as a function of independent variables (in terms of coded values) is shown below:

$$\text{Antibacterial activity (mm)} = 18.50 + 1.26\,X_1 + 0.1563\,X_2 + 0.1712\,X_3 + 0.1604\,X_1X_2 - 0.0300\,X_1X_3 - 0.1357\,X_2X_3 - 3.39\,X_1^2 - 1.06\,X_2^2 - 1.36\,X_3^2$$

Response surface plots (Fig. 3a and b) show the effect of X_1 against antibacterial activity in interaction with X_2 and X_3. The graphs describe the X_1 significantly impacts the antibacterial activity of extracts. The superior effect was identified in specific conditions of X_1, which is 75% to 85%, with increasing or decreasing X_2 and X_3. These are correct with the ANOVA analysis that is shown in Table 3. Figure 2c gives a different impact on antibacterial activity. It is not identified which one is more affected by the interaction between X_2 and X_3. The maximum antibacterial activity is described at a certain point from X_2 and X_3 of 300 W to 380 W and 7 to 10 minutes, respectively. This study shows that antibacterial activity has a strong correlation with TFC which is $R = 0.957$ (Table 4) and it is similar to previous studies by Yuan *et al.* [58] who mention that flavonoid compounds have antibacterial activity with correlation coefficients above 0.93. As reported by Jawhari *et al.* [59], the *Anacyclus pyrethrum* capitula extract has the highest flavonoid content than seeds extract and the strongest antibacterial activity against *S. Aureus*. Similar to reports by Bouchelaghem *et al.* [60], about the antibacterial activity of Hungarian propolis ethanol extract lead increase and with the TFC increase and Sartini *et al.* [61] described the phenolic content as correlated with antibacterial activity. Furthermore, the activity of flavonoid compounds as antibacterial was identified in many reports. Various processes explain flavones' antibacterial properties. Chen's study found that baicalein at 32 and 64 µg/ml reduced quorum-sensing system regulators agrA, RNAIII, and sarA and gene expression of intercellular adhesin (ica) in *S. aureus* biofilm producer cells [62]. The most effective antibacterial flavonoids are quercetin, myricetin, morin, galangin, entadanin, rutin, piliostigmol, and their derivatives. For instance, quercetin and its derivatives inhibited *S. aureus* and other germs [63]. Morin is reported to be efficient against Gram-positive bacteria [64]. Combining plant-derived flavonol with β-lactam antibiotics significantly increased MRSA sensitivity to oxacillin [58]. Conversely, flavonoid-rich plants can affect bacterial surface and cellular leakages [65].

Optimization of MAE and validation of the models

The primary aim of this study was to determine the optimal conditions for generating extracts with elevated levels of flavonoids, as well as enhanced antioxidant and antibacterial properties. Based on the analysis of the maximum content of extracted total flavonoids, the percentage of DPPH scavenging, and the inhibitory zone, it can be concluded that the ideal conditions for all three examined responses were as follows: X_1 at a level of 78.48%, X_2 at a level of 327.96 W, and X_3 at a level of 8.60 minutes. The TFC, % DPPH scavenging activity, and antibacterial activity are displayed in Table 5. The determination of optimal conditions, predicted value, and observed value are achieved by the utilization of a desirability function, which yielded a value of 0.944 for multiresponse optimization [66]. To validate the predictive mathematical model of the researched process, MAE was conducted on the estimated ideal conditions for all three examined responses.

This experiment showed the observed results in optimum conditions which are mentioned in Table 5 are not significantly different from the predicted results by RSM. The observed values of TFC, % DPPH scavenging activity, and antibacterial activity were 232.532 ± 1.05 mg GAE/g, 75.352% ± 0.85%, and 17.863 ± 0.92 mm, respectively. While, the predicted values of 229.647 mg GAE/g, 73.915%, and 18.621 mm, respectively. If we compared between observed and predicted values, the entire results were not significantly different even though the observed values of TFC and % DPPH scavenging activity showed a slight rise and the antibacterial activity showed a slight decrease than predicted values. The comparison between the observed experimental findings and the expected values revealed that all response variables fell within the 95% confidence interval of the predicted model. The strong connection seen in the results provides evidence for the

Table 5. Predicted and observed values of each response at optimal conditions.

Optimal conditions	Investigated response		
	TFC (mg GAE/g)	Antioxidant activity (%Scavenging)	Antibacterial activity (mm)
Ethanol concentration (78.48%)	Predicted 229.647	73.915	18.621
Microwave power (327.96 W)	Observe		
Extraction time (8.60)	232.532 ± 1.05	75.352 ± 0.85	17.863 ± 0.92

[a] Mean ± SD (standard deviation; $n = 3$)

appropriateness of the employed model and the effectiveness of RSM in optimizing the studied conditions for minimizing MAE.

CONCLUSION

RSM was used to find the best options for MAE of TFC, percentage of DPPH scavenging, and antibacterial activity of the extracts. The ANOVA showed that the second-order polynomial model was a good mathematical representation of the MAE linked with flavonoids with strong antioxidant and antibacterial properties. Taking into account all of the factors, it is clear that X_1 had a big effect on the MAE. In the end, the best settings for the three factors that were looked at were for X_1 to be 78.48%, X_2 to be 327.96 W, and X_3 to be 8.60 minutes. The results of this study show that a very effective natural extract can be made when all of the factors are met. Furthermore, the utilization of MAE as an environmentally friendly way to make flavonoid-rich extracts from *P. crocatum* has provided better antioxidant and antibacterial properties.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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