Journal of Research in Pharmacy

Research Article

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Optimization of phenolic and flavonoid content from *Graptophyllum pictum* (L.) Griff. leaf under maceration extraction methods using response surface methodology and its antioxidant activity

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Received: 5 June 2024 / Revised: 2 August 2024 / Accepted: 2 October 2024

ABSTRACT: *Graptophyllum pictum* (L.) Griff. is a plant with many pharmacological effects and contains secondary metabolites which are phenolic and flavonoid compounds. This study aims to determine the optimum phenolic and flavonoid extraction conditions using a Box-Behnken design (BBD) and the correlation of optimized extract with antioxidant activity. The experimental design was conducted based on 3 factors and 3 levels: solvent concentration (X_1; 50, 70, and 90% ethanol in water), extraction time (X_2; 1, 2, and 3 days), and solid-to-liquid ratio (X_3; 1: 5 – 1: 15). The phenolic and flavonoid compounds of the optimized extract were identified using LC-HRMS. Meanwhile, the antioxidant activity of the optimized extract was determined using DPPH radical scavenging activity. Statistical analysis results suggest that the model used is quadratic (p-value < 0.05) for the entire response. The R^2 was obtained at a high figure of 97.96%, 98.59%, and 91.13%, respectively. The optimum yield, TPC, and TFC with the application of response surface methodology were obtained at 68.33% (X_1), 2.10 days (X_2), and 1: 10.36 (X_3) and the experiment value of yield, TPC, and TFC was 48.712 ± 2.896 g, 99.937 ± 3.672 mg GAE/ g, and 37.562 ± 2.984 mg QE/ g, respectively. The optimized extract's phytochemical compounds included phenolics of p-coumaric acid, ferulic acid, cinnamic acid, and anacardic acid. Moreover, the optimized extract has the strongest antioxidant activity in radical DPPH inhibition with a dependent concentration manner. In summary, this method was successfully applied to optimize yield, TPC, and TPC in *G. pictum* leaves following the highest antioxidant activity. The optimized extraction method is reproducible and could be applied in the pharmaceutical industry to develop a product containing *G. pictum* extract.

KEYWORDS: *Graptophyllum pictum* (L.); response surface methodology; maceration; phenolic content; flavonoid; antioxidant

1. INTRODUCTION

Graptophyllum pictum (L.) Griff., also referred to as the caricature plant, is a medicinal shrub that belongs to the Acanthaceae family [1]. It is believed to have originated in New Guinea and subsequently expanded far to India, Mexico, United States, Ghana, Bolivia, and Asia. Presently, it is readily encountered in certain areas of Indonesia and is commonly referred to as Daun Ungu or Daun Wungu [2]. *G. pictum* has been extensively utilized for the treatment of several ailments including wound healing [3], hemorrhoids [4], fever reduction [5], pain relief, and menstruation issues [6]. The significant phytochemical constituents include flavonoids (rutin, heperoside, and quercetin), steroids, glycosides, tannins, saponins, chlorophyll, and non-toxic alkaloids [7]. Furthermore, the minimum quantity of essential oil was 0.4% and the presence of vomifoliol, a triterpenoid molecule, serves as a chemical marker [8]. Tahseen et al. discovered that *G. pictum* also possesses chemicals such as hexadecanoic acid, ethyl ester, 9-Tricosene, Squalene, Tocopherol, Stigmasterol, and Beta-sitosterol [9].

The extraction procedure is the initial step in obtaining the bioactive compounds from the samples [10]. The extraction has specific conditions and processes to achieve the highest possible recovery of phenolic

How to cite this article: Yuliasmi S, Keliat JM, Lubis LD, Lubis MF. Optimization of phenolic and flavonoid content from Graptophyllum pictum (L.) Griff. leaf under maceration extraction methods using response surface methodology and its antioxidant activity. J Res P harm. 2025; 29(5): 2077-2090.

ISSN: 2630-6344

https://doi.org/10.12991/jrespharm.1766338

compounds, as each plant possesses unique properties in terms of its phenolic contents [11]. Various parameters have been empirically demonstrated to have a substantial impact on the extraction yield [12]. These factors include the extraction techniques employed, the size of the particles, the types of solvents used, the concentrations of the solvents [13], the ratios of solvents to solids [14], the temperatures at which the extraction is carried out [15], the durations of the extraction process [16], and the pH levels of the system [17]. The single-factor becomes a conventional technique for analyzing optimization conditions in extraction by altering a particular factor at a time, meanwhile, the others will kept constant [18]. Nevertheless, this methodology is laborious and necessitates a substantial quantity of trials and resources [19]. Furthermore, the interactive effect between the components under investigation cannot be ascertained. Experimental design of extraction process has been employed to address these issues [20].

Experimental design is a methodical strategy that uses statistical approaches to conduct experiments in academic research and industry [21]. Response surface methodology (RSM) is the predominant statistical technique employed for process optimization [22]. RSM is a comprehensive statistical and mathematical approach used to establish the correlation between independent variables and responses, relying on experimental design [23]. In other words, RSM has emerged as a crucial technique used to model and determine the impact of multiple elements on a given process [24]. The Box and Wilson approach initially devised to optimize chemical processes, has since been widely implemented in diverse fields such as engineering, life sciences, agricultural, and pharmacy industry [25]. The RSM has several advantages, such as reducing the number of experimental trials, enabling the calculation of complex interactions between independent variables, facilitating analysis and optimization, and enhancing current designs [26]. Therefore, the RSM is crucial to optimize the extraction process for identifying the appropriate conditions for isolating bioactive chemicals, particularly from various food matrices [27]. In addition, the RSM could be predicting the best condition for phenolic and flavonoid extraction which is crucial to produce significant antioxidant properties of extract [28]. The previous study by Martinez et al (2022) revealed the increasing phenolic and flavonoid contents of *Castanea sativa* caused the antioxidant activity of the extract is turn up [29]. It is similar to the report by Kefayati et al (2017), which showed that Euphorbia splendida's antioxidant activity improved as phenolic and flavonoid contents increased [30]. Eventually, the present study was conducted to optimize extraction conditions under maceration using RSM for higher yield, total phenolic, and flavonoid recovery and to determine the antioxidant activity of the optimized extract from *G. pictum* in DPPH radical inhibition.

2. RESULTS AND DISCUSSION

2.1. Preliminary study of single-factor experiments for maceration extraction methods

2.1.1. Effect of solvent concentration

The solvent's characteristics play a crucial role in selectively extracting molecules, requiring a strong affinity and a high capacity for dissolution [31]. The polar phenolic compounds in plants mostly will be extracted using a polar solvent such as ethanol [32]. In this study, Figure 1A explains the effect of ethanol concentrations from 50% to 100% on yield, TPC, and TFC. The ethanol concentrations significantly impacted yield, TPC, and TFC with $p < 0.0002$; < 0.0002; and 0.0022. The highest yield, TPC, and TFC were obtained using 70% ethanol of 4.971 ± 0.072 g, 96.262 ± 0.635 mg GAE/g, and 37.561 ± 0.625 mg QE/ g, respectively. Afterward, the responses decreased after increasing and decreasing the ethanol concentration. This result describes the ethanol concentrations present an important role in improving a response extraction. A similar report by Dirar et al (2019) revealed that 70% ethanol is the best solvent for phenolic and flavonoid extraction of *Blepharis linariifolia* and *Guiera senegalensis* [33]. In addition, Vongsak et al (2013) explained that 70% ethanol is the best solvent for phenolic and flavonoid extraction of *Moringa oleifera* leaf [34]. Unfortunately, the report by Alara et al (2020) described 60% ethanol as the solvent for phenolic and flavonoid extraction of *Vernonia amygdalina* leaf [35]. This is not in accordance with what was obtained, so optimization of the ethanol concentration on the extract response is important.

2.1.2. Effect of extraction time

Minimizing the extraction time is crucial in order to reduce both the energy consumption and the cost of the extraction process [36]. The extraction time in this study has varied between 12 and 72 hours. Figure 1B revealed the yield, TPC, and TFC lead increased around the rise of extraction time from 12 to 48 hours. The responses have decreased in an extension of extraction time. The data showed a significant difference with p

< 0.0001, < 0.0001, and < 0.0004, respectively. The highest yield, TPC, and TFC were determined in extraction time of 48 hours which are 8.428 ± 0.416 g, 52.086 ± 0.651 mg GAE/ g, and 19.982 ± 0.639 mg QE/g, respectively. The previous study by Masjedi et al (2022) showed that 48 hours is the best extraction time for extracting the phenolic and flavonoid of *Ganoderma lucidum* [37]. Whereas, Jovanoic et al (2017) revealed the extension of extraction time from 5 to 90 minutes lead to increased phenolic content of *Thymus serpyllum* but no significantly difference [38]. Therefore, optimization of extraction time needs to be considered to reduce time and costs in the extraction process.

2.1.3. Influence of liquid-to-solid ratio

The result of the influence of the liquid-to-solid ratio on the yield, TPC, and TFC are shown in Figure 1C. The various ratios of 1:5 to 1:30 have been used in this experiment. Similar to previous factors, the increasing of ratio from 1:5 to 1:10 caused the yield, TPC, and TFC lead to rise. Therefore, the extension of the ratio caused the yield, TPC, and TFC to be reduced. The 1:10 ratio is the best factor for gaining the highest yield, TPC, and TFC of 8.268 ± 0.007 g, 71.821 ± 2.132 mg GAE/g. and 20.165 ± 0.945 mg QE/ g, respectively. The entire data showed a significant difference with p < 0.0001, < 0.0001, and < 0.0004, respectively. The study conducted by Wong et al. (2014) demonstrated that the liquid-to-solid ratio has the most significant impact on antioxidant capacity, total phenolic, and flavonoid content [36]. This phenomenon occurs because a more substantial number of solvents are able to penetrate cells, facilitating the permeation of a more extensive variety of compounds. These compounds can then diffuse into the solvent when the liquid-to-solid ratio is increased [37]. Unfortunately, the data in Figure 1c led to a reduction after the solvent increased. In summary, this result described the efficiency of solvent addition in the extraction process. The use of not too much solvent was able to increase the yield, TPC, and TFC. Therefore, an optimization process needs to be carried out for this factor.

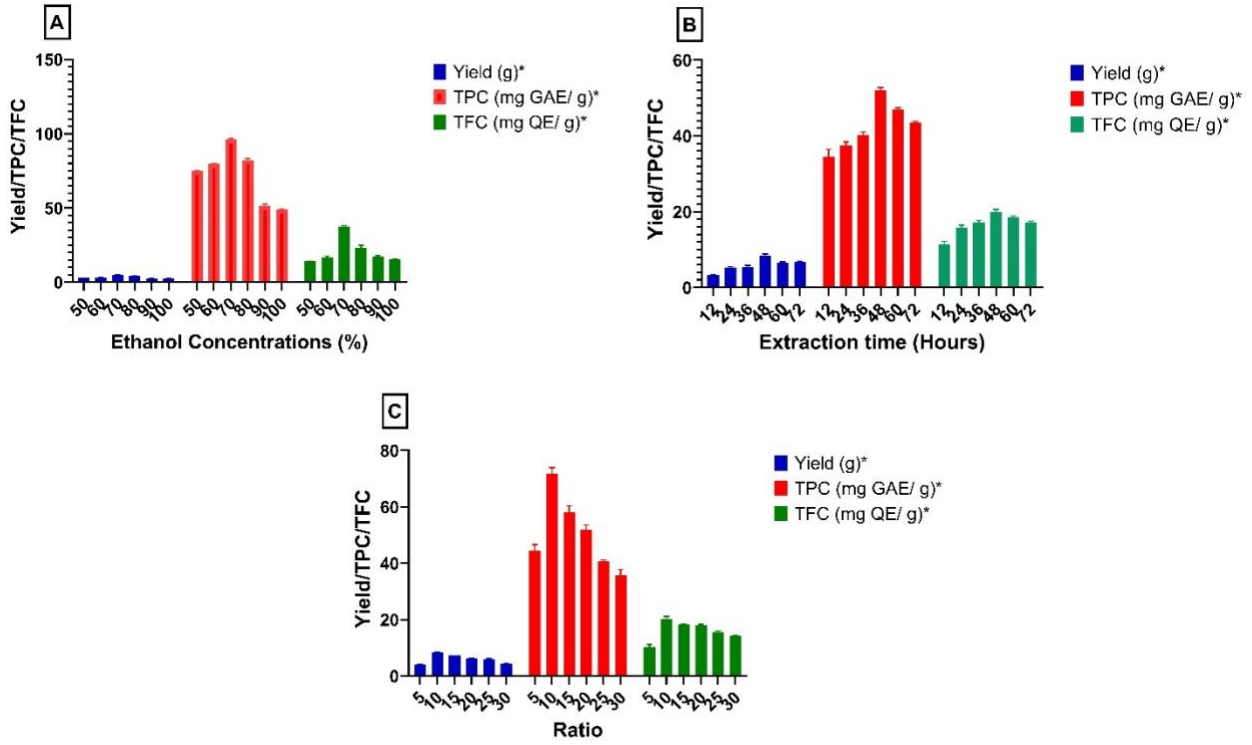


Figure 1. A single-factor effect in *G. pictum* extraction on yield, TPC, and TFC. (A) The effect of ethanol concentration, (B) The effect of extraction time, (C) The effect of ratio. The entire data showed n = 3 ± SD and Character * described the data as significantly different with p < 0.05.

2.2. Analysis of experimental design responses

2.2.1. Influence of the independent variables on the yield

The responses were analyzed according to the design model and the result was presented in Table 1. Thirteen experimental designs were conducted by Design Expert v. 13 and given a different result between

the entire design. The variation result of yield was described as around 0.763 ± 0.230 g to 4.894 ± 0.906 g. In this experiment, ANOVA was established to analyze the p-value of the model to explain the best conditions for the yield. Table 2 revealed that the model of yield was significant at $p < 0.0001$. with a p-value of the lack of fit test was not significant at 0.0753. According to this model, the X_1, X_2, and X_3 significantly affected the yield. In contrast, only X_2X_3 has been identified to affect the yield significantly. Therefore, entire quadratic variables showed a significant impact on the yield.

In this study, two factors were interacted while another factor was kept constant, as shown in Figure 2A-C. The effect of X_1X_2 on the yield is presented in Figure 2A. When X_1 is increased to a certain point while X_2 is kept, the response will increase and then decrease when X_1 continues to increase. A similar thing happens to explain the influence of X_2 with constant X_1 on the response. It was observed in the previous study by Sulaiman et al (2017), that X_1 and X_2 significantly affected the yield [38]. In this study, the optimum yield was obtained when X_1X_2 was at 65% and 2.3 days, respectively. Meanwhile, the interaction of X_1X_3 is presented in Figure 2B. Similar to the previous interaction, the optimum yield was achieved when each factor reached a definite value and it is similar to the study by Lima et al (2023) [39]. The present study showed the yield reaches a maximum if the X_1 was increased to 65% and X_2 was kept constant. This issue still occurred if the X_1 was kept constant and X_3 was increased gradually reaching 1:10 and going to decrease. Subsequently, the effect of X_2X_3 on the yield was established and reflected in Figure 2C. If the X_2 achieved 2 days and the X_3 was kept constant it reached the maximum yield. The maximum yield was obtained if the X_3 increased gradually to 1:10 and the X_2 was kept unchanged. The report by Ryu et al (2019) exhibited the extraction time significant impact on anthocyanin extraction. If the extraction time increases, the anthocyanin levels will be turned down [40].

Table 1. Box-Behnken design and the results of experiments for quantified responses with *G. pictum*

Run	Factor			Response		
	X_1 (%)	X_2 (Days)	X_3 (Ratio)	Yield (g)	TPC (mg GAE/g)	TFC (mg QE/g)
1	50	1	10	3.196 ± 0.867	75.205 ± 1.650	14.966 ± 2.581
2	70	2	10	4.932 ± 0.965	95.091 ± 3.361	37.251 ± 3.672
3	90	3	10	2.428 ± 0.634	59.351 ± 1.126	16.297 ± 2.811
4	90	2	5	1.295 ± 0.522	48.473 ± 1.051	23.821 ± 2.962
5	70	2	10	4.894 ± 0.906	95.353 ± 3.201	32.472 ± 3.429
6	50	2	15	2.436 ± 0.587	70.638 ± 1.451	12.650 ± 2.103
7	50	3	10	3.136 ± 0.783	70.216 ± 1.408	19.499 ± 2.901
8	70	3	5	1.381 ± 0.513	80.125 ± 2.180	14.989 ± 2.573
9	70	2	10	4.613 ± 0.910	97.311 ± 3.396	36.318 ± 3.582
10	90	2	15	1.991 ± 0.490	47.423 ± 1.025	25.011 ± 2.952
11	70	3	15	2.672 ± 0.592	80.982 ± 2.287	22.212 ± 2.893
12	50	2	5	1.670 ± 0.535	73.462 ± 1.474	12.605 ± 2.019
13	70	1	5	1.266 ± 0.501	70.984 ± 1.316	13.978 ± 2.215
14	70	1	15	0.763 ± 0.230	70.125 ± 1.251	21.363 ± 2.753
15	70	2	10	4.532 ± 0.814	94.357 ± 3.179	39.081 ± 3.724
16	90	1	10	1.486 ± 0.552	50.742 ± 1.231	16.399 ± 2.911
17	70	2	10	4.577 ± 0.919	98.126 ± 3.681	35.982 ± 3.305

Figure 2A-C revealed the independent variables have the strongest effect on yield. As reported by Nawas et al (2020), solvent polarity has a significant effect on extraction efficiency. The yield of the extract was seen to be greater in water, a polar solvent, compared to a nonpolar solvent [41]. The elevated yield in polar solvent suggests the existence of a greater amount of polar and water-soluble constituents in *G. pictum* leaf in comparison to non-polar constituents. Furthermore, extraction time describes the length of contact of the solvent with the dry powder. Solvent contact with the sample affects the efficiency of the solvent in penetrating and dissolving phytochemical compounds thereby increasing the yield [42]. The earlier work conducted by Vuong et al (2013) demonstrated the yield of extract was increased in an extension of extraction time from 5 to 20 min. Beyond this time, the yield was slightly reduced [43]. In addition, the yield was affected by another factor which is solid-to-liquid ratio. The solid-to-liquid ratio has a direct impact on the yield. When the ratio approaches 1:10, the yield increases, but decreases gradually when the ratio is reduced. According to Prasad et al (2009), the amount of extract obtained rose as the solid-to-liquid ratio increased [44]. This phenomenon is likely a result of increased permeability of the cells, allowing for more solvent penetration and extraction of the phytochemical substances [45].

2.2.2. Influence of the independent variables on the phenolic content

This experiment effectively assessed the impact of independent variables on the TPC. The result reflected that the TPC experiences differences in quantity when the extraction factors vary, ranging from 47.423 ± 1.025 mg GAE/ g to 98.126 ± 3.681 mg GAE/ g (Table 1). The model revealed a significant difference with $p < 0.0001$ and a lack of fit < 0.005. This model showed that X_1 and X_2 significantly affected the TPC with $p < 0.0001$ and 0.0295, respectively. Whereas the X_3 showed no significant effect on the TPC with a p of 0.6468. The entire quadratic factors revealed a significant impact on TPC with a $p < 0.0001$; 0.0009; and < 0.0001, respectively.

The relation of the two factors on TPC was revealed in Figure 3A-C. Figure 3A illustrates the impact of X_1X_2 on the yield. The TPC was increased when the X_1 rose from 50% to 65% with increasing or decreasing the X_2. This explanation revealed that the X_1 has the strongest effect on TPC although the X_2 was not constant. Furthermore, Figure 3B presents the relation of X_1X_3 on TPC. The interaction effect of X_1X_3 on TPC can be identified as the previous factors. The TPC was increased with increasing of X_1 although the X_3 was not constant. The maximum TPC was obtained when the X_1 reached 65%, while the X_3 was around 1:10. Meanwhile, the changes from X_3 don't have much of an impact if X_1 is kept constant. It was similar to the report by Metrouh-Amir et al (2015), that the solvent concentration has a crucial impact on phenolic content [46]. A slight difference can be seen when discussing the X_2X_3 interaction, where the TPC will increase and reach a maximum when X_2 and X_3 increase and are at a certain point (Figure 3C). TPC will experience another decline when X_2 and X_3 start to decline.

Table 2. The ANOVA analysis for the response surface model. TPC is expressed as total phenolic content. Meanwhile, TFC is expressed as total flavonoid content.

Source	Yield	TPC	TFC
p-value (Model)	< 0.0001	< 0.0001	0.0060
p-value (X_1)	0.0079	< 0.0001	0.1130
p-value (X_2)	0.0132	0.0295	0.6175
p-value (X_3)	0.0380	0.6468	0.2296
p-value (X_1X_2)	0.1520	0.0619	0.6030
p-value (X_1X_3)	0.9122	0.7484	0.8967
p-value (X_2X_3)	0.0238	0.7876	0.9854
p-value (X_1^2)	0.0005	< 0.0001	0.0025
p-value (X_2^2)	< 0.0001	0.0009	0.0020
p-value (X_3^2)	< 0.0001	< 0.0001	0.0056
R^2	0.9796	0.9859	0.9113
Adjusted R^2	0.9534	0.9678	0.7973
Adeq Precision	15.553	20.822	7.382
C.V.%	11.21	4.08	18.33

As reported by Lohvina et al (2021), the TPC increased with increasing the amount of water in ethanol reaching 30% and the maximum TPC was obtained using 70% ethanol [47]. This experiment demonstrated that solvents with high polarity, such as water and water-ethanol solutions with a higher water content, are capable of extracting a broader range of chemicals, including phenolic compounds [48]. Furthermore, the duration of the extraction process plays a vital role in solvent extraction for phenolic compounds. This is because the equilibrium concentrations of phenolic compounds are achieved before their apparent reduction occurs [49]. According to Bakar et al (2020), the TPC (total phenolic content) of *Euphorbia hirta* L. increased significantly when the solid-to-liquid ratio was increased from 1:15 to 1:20, along with an increase in extraction time [50]. This scenario demonstrated that the mass transfer theory is applicable in explaining the impact of the solid-to-liquid ratio on extraction time [51].

2.2.3. Influence of the independent variables on the flavonoid content

The effect of independent variables on TFC was carried out and presented in Figure 4A-C. The design model in Table 1 described that the model is significantly different with a p of 0.0060 and a lack of fit of 0.0596. The TFC expressed a similar trend with the yield and TFC which is a different of extraction conditions given various of TFC. The TFC was determined in a range of 12.605 ± 2.019 mg QE/g to 39.081 ± 3.724 mg QE/g. The independent variables expressed no significant affected on TFC with $p > 0.005$, respectively. Meanwhile,

the quadratic model of variables showed a significant affected on TFC with a $p < 0.0001$; 0.0009; and < 0.0001, respectively.

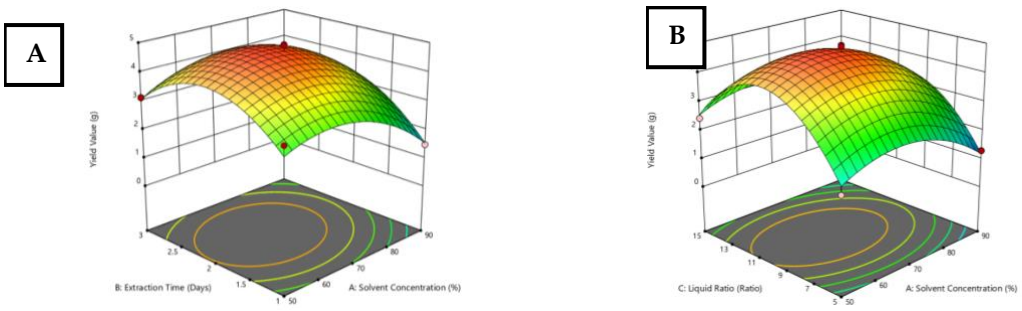
The interaction of each variable on TFC was established in this study. Figure 4A showed an interaction of X_1X_2 on TFC, that the TFC increased with increasing of X_1 and X_2 in a particular point. The maximum TFC was obtained with X_1 reached 75% and X_2 came near to 2.5 days. The TFC will be reduced with a decrease of X_1X_2. It was reported by Do et al (2014), that the solvent concentration and extraction time are a crucial factor to gained optimum flavonoid content [52]. This scenario is not similar to the explanation of TPC which is an X_2 was not significantly affected. Additionally, a higher X_1 is required to achieve maximum TFC. As reported by Sun et al (2015), the TFC tended to increase with increasing of ethanol concentrations [53]. On the other side, the effect of X_1X_3 on TFC was reflected in Figure 4B. Figure 4B describes the interaction effect of X_1X_3 on TFC is similar to the interaction of X_1X_2. It was revealed that the maximum TFC was obtained when the X_1X_3 was around a certain point which is 75% and 1:10. Afterwards, the TFC will be reduced with the increasing and decreasing of X_1X_3 passed 75% and 1:10. The amount of solvent used in extraction was proven to increase the efficiency of the solvent in attracting flavonoids [54]. As reported by Vo et al (2022), the increasing solid-to-liquid ratio from 1:10 to 1:30 significantly affected the TFC of *Cirtullus lanatus* [55]. However, increasing the solid-to-liquid ratio does not have a good impact on obtaining TFC because of the saturation period. The relation of X_2X_3 was determined in this study and presented in Figure 4C. The maximum TFC was obtained with an increase in the X_2X_3 reach to 2 days and 1:10. Observation results show that TFC will decrease when these factors decrease. This illustrates that the maximum TFC will be arrived at a certain point from X_2X_3.

2.3. Optimizing the maceration conditions and validating the models

The main objective of this study was to identify the most favorable circumstances for producing extracts with the highest amounts of yield, phenolics, and flavonoids. Based on the analysis of the maximum responses, it can be concluded that the ideal conditions for all three examined responses were as follows: 68.33% (X_1), 2.10 days (X_2), and 1 : 10.36 (X_3). The predicted values of yield, TPC, and TFC were 47.235 g, 96.356 mg GAE/g, and 35.896 mg QE/g, respectively. Meanwhile, the experimental values of each response were 48.712 ± 2.896 g, 99.937 ± 3.672 mg GAE/g, and 37.562 ± 2.984 mg QE/g, respectively (Table 3). The determination of optimal conditions and actual values is achieved by the utilization of a desirability function, which yielded a value of 0.963 for multi-response optimization. The comparison between the observed experimental findings and The calculated values showed that all response variables were within the 95% confidence interval of the anticipated model. The robust correlation seen in the findings offers proof of the suitability of the utilized model and the efficacy of RSM in improving the investigated parameters for maceration.

2.4. Antioxidant activity of the optimized extract

The improved extract's antioxidant activity was assessed by evaluating its ability to scavenge a DPPH radical. The antioxidant activity was quantified by calculating the percentage suppression of the DPPH radical and is seen in Figure 5. In this experiment, several concentrations were conducted to explain the effect of concentration on % inhibition of DPPH radical. This result revealed that the antioxidant effect of the optimized extract depended on concentration. The rising concentrations significantly affected the % inhibition of DPPH radical ($p < 0.0001$). In this study, the highest % inhibition of DPPH radical was obtained at 500.00 µg/mL which was 93.16 ± 0.32%. This value was significantly decreased to 79.53 ± 0.60% with a decrease in concentration by half ($p < 0.0001$). The lowest % inhibition of DPPH radical was identified in the lowest concentration at 15.625 µg/mL, which was 25.76 ± 0.94%.



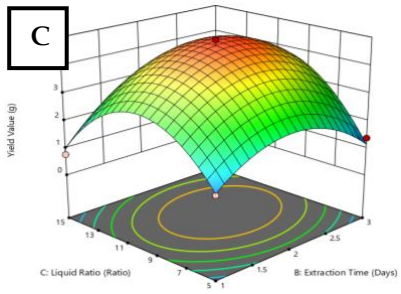


Figure 2. 3D response surface plots reflected the interactive effects of independent variables on the yield. A: The interaction of solvent concentration (X_1) with extraction time (X_2), B: The interaction of solvent concentration (X_1) with solid-to-liquid ratio (X_3), C: The interaction of extraction time (X_2) with solid-to-liquid ratio (X_3)

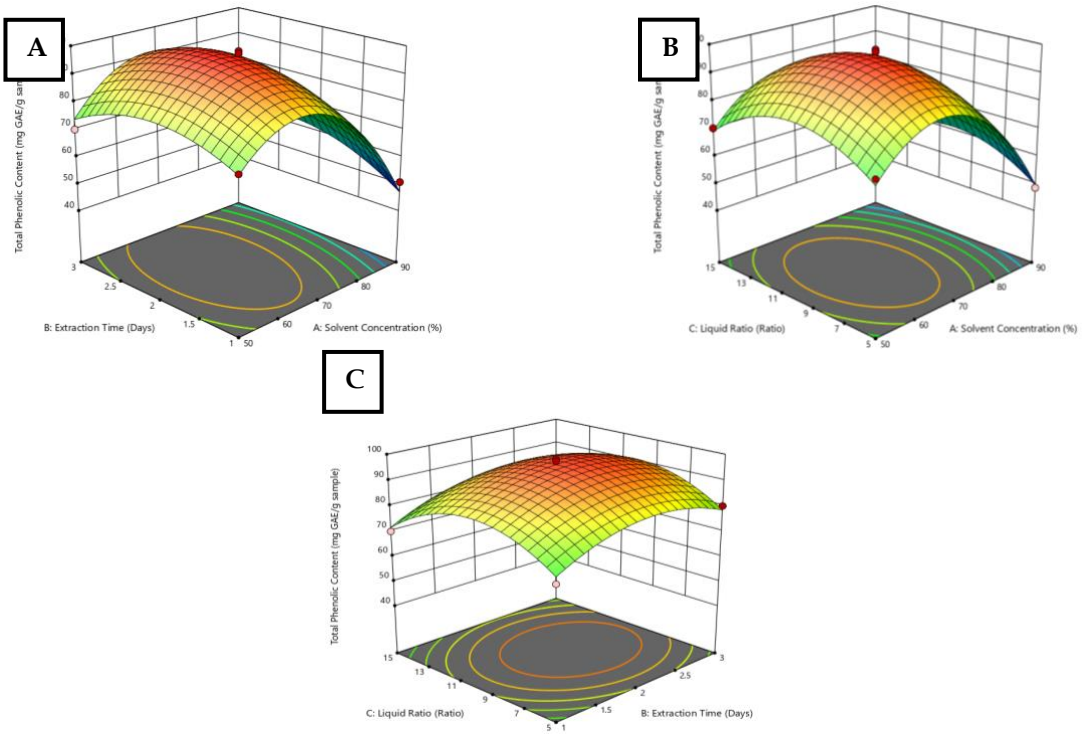


Figure 3. 3D response surface plots reflected the interactive effects of independent variables on the TPC. A: The interaction of solvent concentration (X_1) with extraction time (X_2), B: The interaction of solvent concentration (X_1) with solid-to-liquid ratio (X_3), C: The interaction of extraction time (X_2) with solid-to-liquid ratio (X_3)

This study revealed the antioxidant of the optimized extract was successfully obtained. This could be due to the content of hydrophilic polyphenolic compounds in the extract. Based on the observation using LC-MS/MS presented in Table 4, the optimized extract contains several polyphenol compounds of p-coumaric acid, ferulic acid, cinnamic acid, and anacardic acid. As reported by Makkiyah et al (2024), these compounds were verified to be contained in the *G. pictum* leaves [56]. Polyphenol was reported to have the strongest activity as an antioxidant [57]. As reported by Masek et al (2018), p-coumaric acid showed the strongest activity in DPPH and ABTS radical inhibition [58]. Meanwhile, Zdunska et al (2018) elucidated the antioxidant properties of ferulic acid, demonstrating its ability to suppress the generation of reactive oxygen species (ROS) or nitrogen, neutralize free radicals like DPPH radical, and chelate protonated metal ions such as Cu(II) or Fe(II) [59]. Furthermore, polyphenols have been identified as antioxidants through many mechanisms of action [60].

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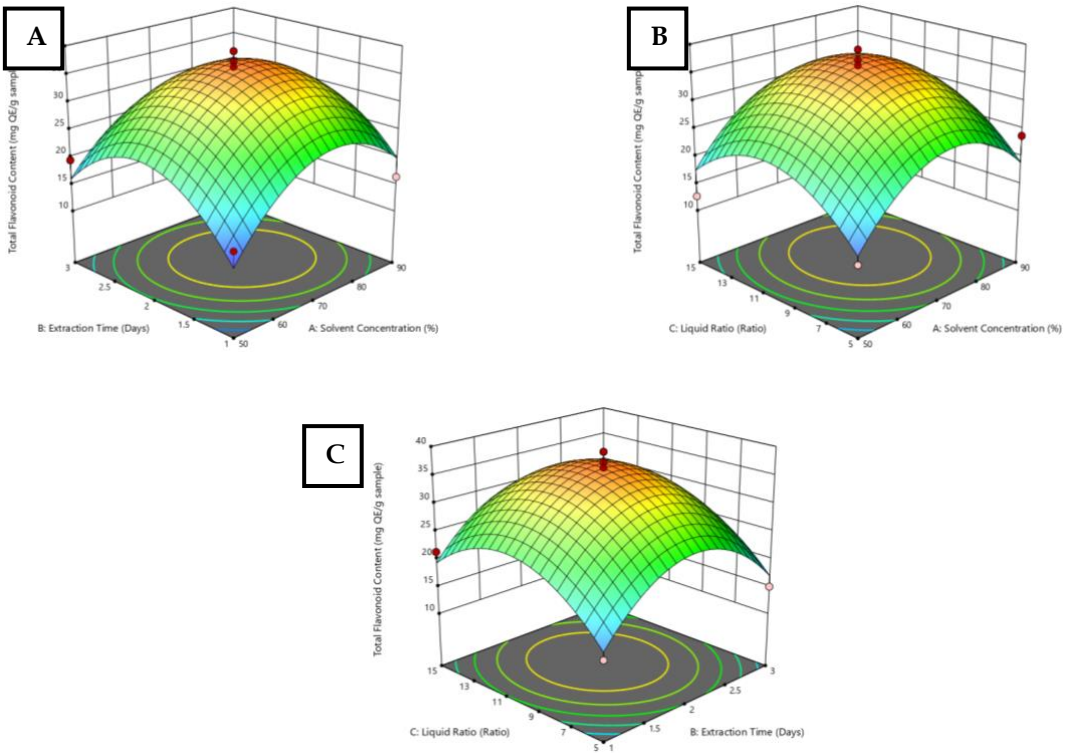

Figure 4. 3D response surface plots reflected the interactive effects of independent variables on the TFC. A: The interaction of solvent concentration (X_1) with extraction time (X_2), B: The interaction of solvent concentration (X_1) with solid-to-liquid ratio (X_3), C: The interaction of extraction time (X_2) with solid-to-liquid ratio (X_3).

Table 3. Actual and predicted values of yield, TPC, and TFC at the optimal conditions in the maceration

The independent variables			Responses	Predicted values	Experimental values
X_1 (%)	X_2 (Days)	X_3 (Ratio)			
68.33	2.10	1: 10.36	Yield (g)	47.235	48.712 ± 2.896
			TPC (mg GAE/ g)	96.356	99.937 ± 3.672
			TFC (mg QE/ g)	35.896	37.562 ± 2.984

3. CONCLUSION

Response Surface Methodology (RSM) was used to find the best options for maceration of yield, TPC, and TFC of the extracts. The analysis of variance (ANOVA) showed that the second-order polynomial model was a good mathematical representation of the maceration linked with yield, TPC, and TFC. Taking into account all of the factors, it is clear that X_1 had a big effect on the maceration. In the end, the best settings for the three factors that were looked at were for X_1 to be 68.33%, X_2 to be 2.10 days, and X_3 to be 1 : 10.36. The results of this study show that a very effective natural extract can be made when all of the factors are met. Also, using this conditions, the optimized extract was showed the highest DPPH radical scavenging. Exploration of antioxidant properties from the optimized extract of *G. pictum* leaf will be suitable to check in

vivo in future research. Finally, these conditions can be applied in the pharmaceutical industry to produce a *G. pictum* extract because increases the yield, TPC, and TFC, as well as antioxidant properties.

4. MATERIALS AND METHODS

4.1. Collection, identification, and preparation of the plant material

G. pictum leaves were collected in October 2023, from the Berastagi subdistrict, Karo regency, North Sumatera, Indonesia (3°11'11.8"N and 98°30'18.2"E). A voucher specimen of *G. pictum* was acquired from the Herbarium Medanese, which is part of the Faculty of Mathematics and Natural Sciences at Universitas Sumatera Utara in Indonesia. The voucher specimen number is 210/MEDA/2024. We have exclusively collected undamaged leaves at various elevations and promptly transferred them to our laboratory. On the same day, the leaves were pulverized using an electric mill and then kept in a dry and dark location until they were examined.

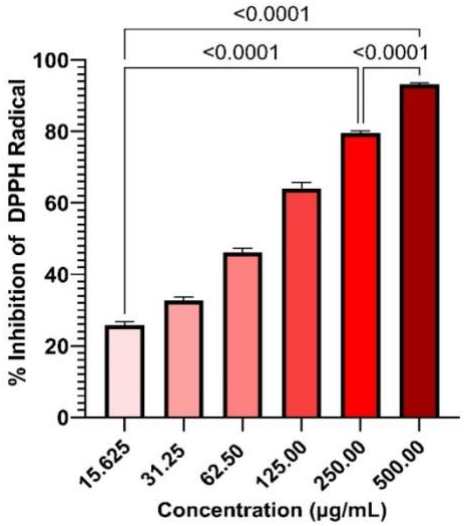

Figure 5. The effect of the optimized extract on DPPH radical in several concentrations ranges from 15.525 to 500.00 µg/mL

Table 4. Phenolic compounds of the optimized extract using LC-MS/MS

No	Compounds	Class	Molecular Formula	Molecular Weight	Retention Time
1	p-Coumaric acid	Phenolic	$C_9H_8O_3$	164.05	2.15
2	Ferulic acid	Phenolic	$C_{10}H_{10}O_4$	194.06	3.86
3	Cinnamic acid	Phenolic	$C_9H_8O_2$	148.05	3.86
4	Anacardic acid	Phenolic	$C_{22}H_{36}O_3$	348.27	19.29

4.2. Extract preparation

4.2.1. Single-factor experimental design

The single-factor variables for the extraction process were conducted to screen X_1, X_2, and X_3 with the best comprehensive extraction. Subsequently. X_1 (50 – 100%), X_2 (12 – 72 hours), and X_3 (1 : 5 – 1 : 30) were identified as single-factor variables. When the extraction is done, each variable will be variated according to previous statements, with the other variables kept constant. The yield, TPC, and TFC were established to express the best conditions for extraction [61].

4.2.2. Extraction process

Briefly, 20 grams of *G. pictum* leaf powders were extracted by maceration. The extraction process was carried out considering several factors from single-factor design which are X_1, X_2, and X_3 (Table 1). The extraction process was carried out with a magnetic laboratory shaker TTSSMS-200, TT-DMS series, operating at a constant speed of 1800 rpm [62].

4.3. Experimental design

The study employed RSM to examine the impact of three independent variables on the yield, TPC, and TFC content in *G. pictum* leaf extracts. The independent variables chosen for optimization in the extraction process were the solvent concentration (expressed as a %, X_1), extraction time (measured in days, X_2), and solid-to-liquid ratio (expressed as a ratio, X_3). The samples were stored at an ambient temperature to prevent the deterioration of thermolabile chemicals. The study conducted trials using the BBD in Design Expert v.13 (Stat-Ease, Minneapolis, United States). The experimental factors' level values can be seen in Tables 1 and 2. [63].

4.4. Quantification of overall phenolic content (TPC) and overall flavonoid content (TFC)

The quantification of the TPC was performed using the Folin–Ciocalteu technique with a UV-visible spectrophotometer (Beckman DU 800). The TPC was quantified by employing a calibration curve based on gallic acid and expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight (DW). In addition, the measurement of the overall flavonoid content was performed using the approach outlined by Lubis et al (2022) [64]. The total flavonoid content was quantified by employing a standard curve of quercetin and presenting the findings as milligrams of quercetin equivalent (QE) per gram of dry weight (DW) [64].

4.5. Antioxidant activity of the optimized extract

The assessment of the capacity to remove free radicals was performed using the DPPH method. A solution of DPPH in methanol with a concentration of 0.2 mM was prepared. Afterwards, 100 µL of this solution was introduced into a solution containing extracts with a concentration of 100 µg/mL. The absorbance measurement was performed at a wavelength of 516 nm after a time period of 60 minutes. The percentage of inhibition was calculated by comparing the absorbance values obtained from the control group with those obtained from the samples, as demonstrated in the following illustration [63-65]:

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

4.6. Phytochemical analysis of the optimized extract using LC-MS/MS

To summarize, a 10 mg portion of the sample was dissolved in 5 ml of methanol and then subjected to homogenization using an ultrasonicator for a duration of 30 minutes. The solution obtained was passed through a 0.2 µm PTFE membrane using a filtration process. Afterwards, a syringe was used to inject 2.0 µL of the sample into the LC-MS/MS. The mobile phase consisted of a mixture of H_2O with 0.1% formic acid (A) and 0.1% formic acid (B), flowing at a rate of 0.2 mL/minute. The gradient method used was as follows: from 0 to 1 minute, the mobile phase included 5% B; from 1 to 25 minutes, the percentage of B increased from 5% to 95%; from 25 to 28 minutes, the mobile phase contained 95% B; and from 28 to 30 minutes, the percentage of B decreased back to 5%. The immobile phase used in the experiment was accucore C_{18}, with dimensions of 100 × 2.1 mm and a particle size of 1.5 µm. The experiment was conducted at a temperature of 30°C. The apparatus was set up to detect mass sizes within the range of 100 to 1,500 m/z using the positive ionization method. The LC-MS/MS analytical results were analyzed using Compound Discoverer 3.2 software. The MS_1 and MS_2 spectra were compared with online databases (mzCloud, ChemSpider, and PubChem) to estimate the chemical structures of the metabolites [66-68].

4.7. Statistical analysis and experimental planning

The RSM was utilized to examine the impact of extraction parameters and optimize the conditions for various responses. A BBD with three variables was initially employed to establish the response pattern. The design, which had 17 combinations and five replicates at the central point, was carried out randomly. X_1 (50–100%), X_2 (1 – 3 days), and X_3 (1 : 5 – 1 : 15) 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. 1) [63]:

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

Where Y represents the response variable; X_i and X_j are the independent variables affecting the response; and A_0, A_i, A_{ii}, A_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively.

Optimal extraction parameters were discovered for three separate responses: yield, TPC, and TFC. Various responses were handled based on the desirability function, and the best circumstances were chosen. Using Design-Expert v.13 (Stat-Ease, Minneapolis, MN, USA), multiple linear regression analysis and the experimental design were carried out. Analysis of variance (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 [63].

Acknowledgments: We are grateful to Universitas Sumatera Utara for his support in funding this research via the TALENTA scheme 2023 with contract number: No. 53/UN5.2.3.1/PPM/KP-TALENTA/R/2023.
Author contributions: Concept – S.Y.; Design – S.Y., M.F.L.; Supervision – S.Y.; Resources – J.M.K., L.D.L.; Materials – S.Y., M.F.L.; Data Collection and/or Processing – J.M.K., M.F.L., L.D.L.; Analysis and/or Interpretation – S.Y., J.M.K., M.F.L., L.D.L.; Literature Search – S.Y., J.M.K.,; Writing – S.Y., J.M.K., M.F.L., L.D.L.; Critical Reviews – S.Y., J.M.K., M.F.L., L.D.L.
Conflict of interest statement: The authors declared no conflict of interest.

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