

FACTORIAL DESIGN OPTIMIZATION OF PHENOLIC AND ANTIOXIDANT EXTRACTION FROM *Justicia gendarussa* Burm. F.

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Abstract

Extraction is a pivotal initial step in the process of harnessing active constituents from plants. The extraction process aims to efficiently yield the highest concentration of target compounds. This study focused on optimizing the extraction of phenolic compounds and antioxidant capacity from *Justicia gendarussa* Burm.f., a plant esteemed for its medicinal properties. A two-level half-factorial design was utilized to systematically investigate the effects of the liquid-to-solid ratio, temperature, solvent concentration, time, and pH on the extraction yields. The study determined that the optimal extraction was achieved in run-13, where the total phenolic content reached 22.75 mg GAE/g, and the antioxidant capacities measured by DPPH and FRAP assays were 43.36 $\mu\text{mol TE/g}$ and 166.72 $\mu\text{mol TE/g}$, respectively. Temperature was found to be a significant factor influencing phenolic production, whereas the liquid-to-solid ratio and pH had significant effects on the antioxidant capacity of DPPH, according to statistical analysis. Similarly, the liquid-to-solid ratio is crucial for the antioxidant capacity of FRAP. Optimal extraction parameters were identified with a liquid-to-solid ratio of 93.7 mL/g, temperature of 43.4°C, methanol concentration of 60.6%, extraction time of 113 min, and pH of 3.45, leading to the most effective extraction process with a desirability level of 1. The results of this study provide a valuable methodology for enhancing the extraction efficiency of bioactive compounds from *J. gendarussa*, thus supporting its potential for pharmaceutical utilization.

Keywords: antioxidant; factorial; *Justicia gendarussa*; phenolic

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Introduction

Justicia gendarussa Burm. f., a member of the Acanthaceae family, is a versatile plant indigenous to various regions of Asia, including China, Malaysia, Indonesia, India, Philippines, Pakistan, Thailand, and Sri Lanka. Characterized by its vertical growth, reaching up to two meters (Putri *et al.*, 2020), *J. gendarussa* has been deeply rooted in traditional medicinal practices for centuries, thriving abundantly in forested areas (Akpriyanti *et al.*, 2017). *J. gendarussa* has been renowned for its therapeutic properties in treating a spectrum of ailments, including asthma, cough, joint and stomach pain, headaches, diarrhoea, fever, and as a diuretic (Kavitha *et al.*, 2014).

Recent studies have explored the phytochemical composition, quantity, and antioxidant potential of various extracts derived from *J. gendarussa*. With a rich array of bioactive substances, such as flavonoids, phenolics, alkaloids, saponins, polysaccharides, and terpenoids, *gendarussa* serves as a cornerstone in traditional medicine. Beyond their application as male contraceptives, these bioactive compounds exhibit diverse therapeutic properties including anti-inflammatory, cytotoxic, anticancer, antiangiogenic, and hepatoprotective effects. Naringenin, vitexin, a flavonoid belonging to the apigenin family, emerges as the primary active ingredient in this plant, recognized for its potent antioxidant properties and effectiveness against free radicals (Kuber, 2021).

Extraction is a pivotal initial step in the process of harnessing active constituents from plants. The extraction process aims to efficiently yield the highest concentration of target compounds (Pham *et al.*, 2019). This intricate process involves dissolving compounds within cells through solvents entering the cell matrix, followed by the extraction of dissolved substances diffused outside the cell matrix (Zhang *et al.*, 2018).

One disadvantage of comprehensively designed tests is the sheer number of trials that need to be conducted, which can be laborious and lead to the wasteful use of materials (Kobilinsky *et al.*, 2017). It is possible to drastically cut down on the materials, labor, and time needed to address problems by using factorial design. In addition, factorial design is thought to be a highly effective strategy that produces a large amount of data for data analysis and legitimate outcomes (Qamar *et al.*, 2022).

Marliani *et al.* (2022) have optimized a simplex centroid design model for *Gendarussa* plants using water, ethanol, and hexane. Similar research

was also conducted by Iqbal *et al.* (2022) using response surface methodology with three extraction factors: solvent concentration, temperature, and extraction time. In this research, our study employed a half-factorial design to comprehensively investigate the impact of independent variables based on the research of Wong *et al.* (2014), such as pH (2-6), methanol concentration (20-80%), temperature (30-80°C), time (30-300 min), and liquid-to-solid ratio (10-100 mL/g) on the recovery of antioxidant capacity and phenolic content in *J. gendarussa*. This study focused on optimizing the extraction of phenolic compounds and antioxidant capacity from *J. gendarussa* Burm. f., a plant esteemed for its medicinal properties. This research can serve as a reference for the extraction of secondary metabolite compounds from *J. gendarussa* Burm. f.

Materials and Methods

Chemicals and reagents

Analytical grade methanol was purchased from MERCK (Germany), Na₂CO₃, 2,2-diphenyl-1-picrylhydrazyl (DPPH) powder was purchased from Sigma-Aldrich (St. Louis, MO, USA), and Folin-Ciocalteu phenol reagent, 2,4,6-tripyridin-s-triazine (TPTZ), FeCl₃, and buffer acetate (pH 3.6) were purchased from Mallinckrodt plc (Cruiserath, Blanchardstown, Dublin 15, Ireland), 6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydrochromene-2-carboxylic acid (Trolox) standard was purchased from MERCK (Germany), and gallic acid (98.0%) was purchased from Sisco Research Laboratories Pvt. Ltd (India).

Plant Simplicia preparation and extraction

Preparation was performed according to the method described by Widodo *et al.* (2019). The Tropical Biopharmaca Research Center, Bogor Agricultural University, West Java, Indonesia (6°32'25.47" N, 106°42'53.22" E, 142.60 meters above sea level) collection was used to get *gendarussa* plant samples. To remove the dirt that adheres to the grass plant, *gendarussa* were picked and washed under running water. At 45°C, the cleaned sample was dried in an oven for two days and overnight. The dried sample was cleaned of any remaining dirt, blended to make it smooth, and then sieved through 60 mesh sieves. The extraction process was carried out in compliance with Wong *et al.* (2014) protocol. *Gendarussa* leaf powder (1 g) was dissolved in methanol. Extraction was

performed according to the design results obtained from the Design Expert application listed in Table 1.

Table 1. Experimental design of *J. gendarussa* extraction

Run	A	B	C	D	E
1	10	80	20	30	2
2	10	30	80	30	2
3	10	30	20	300	2
4	55	55	50	165	4
5	100	30	80	30	6
6	55	55	50	165	4
7	10	30	20	30	6
8	100	80	20	30	6
9	100	80	80	30	2
10	10	80	20	300	6
11	10	30	80	300	6
12	10	80	80	300	2
13	100	80	80	300	6
14	100	30	80	300	2
15	100	30	20	30	2
16	10	80	80	30	6
17	100	30	20	300	6
18	100	80	20	300	2
19	55	55	50	165	4

A: liquid/solid ratio (mL/g); B: temperature (°C); C: methanol concentration (%); D: time (min); E: pH

Determination of total polyphenol content

Total phenolic content (TPC) measurements

The determination of total phenolic levels refers to the procedure used by Nurcholis *et al.* (2022). 20 μ L of gendarussa extract is mixed with 120 μ L of 10% Follin-Ciocalteu reagent (v/v) into a 96-well clear polystyrene microplate and incubated for 5 minutes. Then, 10% Na_2CO_3 (80 μ L) was added and the mixture. The mixture was then incubated for 30 min at room temperature. Nano spectrophotometers were used to measure the absorption of the mixtures at 750 nm wavelengths.

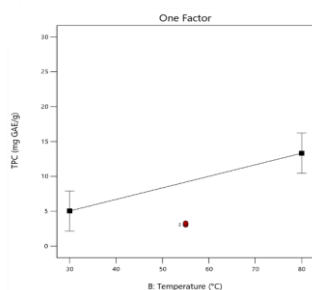


Figure 1. Effect of temperature on the recovery of total phenolics (mg GAE/g) from *J. gendarussa*. A greater slope indicates a greater effect of a given factor on the recovery of total phenolics using the TPC method

The production of standard curves using gallic acid at concentrations of 31.61, 63.21, 94.82, 126.42, 189.63; and 252.84 ppm. The final unit of analysis was given as mg gallic acid equivalent (GAE)/g.

Determination of antioxidant capacity

The antioxidant capacity of the extracts was analysed by nano-spectrophotometric calorimetry (SPECTROstar Nano BMG LABTECH) using the DPPH and FRAP techniques and the Trolox equivalent antioxidant capacity (TEAC) according to Nurcholis *et al.* (2022).

2,2-Diphenyl-1-picryl hydrazyl (DPPH) scavenging capacity method

The sample extract (100 μ L) and 125 μ M DPPH reagent (100 μ L) were added to a 96-well microplate. This was followed by 30 minutes of incubation in the dark. Trolox (50 mg) was dissolved in 50 mL of methanol p.a. to create a Trolox stock, which was diluted to concentrations of 6.32, 12.64, 18.96, 25.28, 37.93, and 44.25 ppm. Absorbance was measured at 515 nm with a spectrophotometer (SPECTROstar Nano BMG LABTECH).

Ferric reducing antioxidant power (FRAP) method

2,4,6-tripyridin-s-triazine (TPTZ), ferric chloride, and acetate buffer (pH 3.6) were combined in a 10:1:1 (v/v) ratio to form the ferric-reducing antioxidant assay reagent. Next, the mixture was incubated for thirty minutes. Trolox standards in different concentrations (126.42, 379.27, 505.69, 758.53, and 884.96 ppm) were developed. The test involved combining 10 μ L of sample or Trolox standard with 180 μ L of FRAP reagent and incubation for 30 minutes. A wavelength of 593 nm was used to measure the absorbance.

Statistical analysis

Polyphenol content and antioxidant capacity data are shown as mean $\pm$ standard error (SE) of three replicates. Software used for processing the results included SPSS version 26 and Design Expert Version 13.0 (Minneapolis, USA) for statistical analysis. Tukey's follow-up tests and analysis of variance (ANOVA) were used to assess the model's statistical significance (p-value < 0.05).

Results and Discussion

Total phenolic content

The outcomes of the runs are summarized in Tables 2, 3, and 4. Total phenolic levels, expressed as gallic acid equivalent (GAE) or mg GAE/g, were

measured at varying extraction temperatures. At 20°C, the total phenolic content was 5.05 mg GAE/g, exhibiting a notable increase to 13.33 mg GAE/g at 80°C (Figure 1). The statistical analysis showed that the temperature had a significant effect on the total phenolic content ($p < 0.05$), overshadowing the impact of the other four factors studied. The temperature factor, with a p -value of 0.0084, accounted for the largest percentage of total phenolic content at 31.43% (Tables 3 and 4).

Table 2. Total phytochemical content and antioxidant capacity of *J. gendarussa*

Run	TPC	DPPH	FRAP
1	7.61	13.58	37.67
2	7.78	9.07	44.21
3	6.53	13.38	35.94
4	3.04	9.69	29.83
5	4.53	23.94	112.07
6	3.06	10.14	32.07
7	2.99	4.66	29.14
8	10.67	28.93	98.07
9	20.69	23.04	149.73
10	7.63	52.70	37.11
11	5.19	4.82	22.34
12	21.62	5.93	53.08
13	28.15	54.19	218.73
14	7.16	46.6	141.07
15	7.64	29.17	121.73
16	6.95	7.33	35.41
17	7.67	24.45	129.73
18	12.41	55.42	152.07
19	3.32	7.60	29.77

TPC (mg GAE/g DW); DPPH ($\mu\text{mol TEAC/g}$); FRAP ($\mu\text{mol TEAC/g}$)

Table 3. P -value of responses against factors

Factors	P-value for responses		
	TPC	DPPH	FRAP
A	0.1506	<0.0001	<0.0001
B	0.0084	0.0628	0.1214
C	0.0914	0.3556	0.1478
D	0.2203	0.2249	0.0884
E	0.4231	0.0450	0.5562

A: liquid/solid ratio (mL/g); B: temperature ($^{\circ}\text{C}$); C: methanol concentration (%); D: time (min); E: pH

The observed temperature-dependent increase in total phenolic content aligns with the findings of Elez-Garofulić *et al.* (2013), who reported a similar enhancement in phenolic compound extraction with rising temperatures. This phenomenon can be attributed to the influence of the temperature on the extraction parameters. As noted by Moreira *et al.* (2017), higher temperatures during the extraction processes lead to augmented production of phenolic compounds. This effect is attributed to the increased hypodermal cell permeability and greater solubility of phenolic chemicals at elevated temperatures, facilitating more efficient extraction.

Table 4. Percentage contribution of factors on response variables

Factors	P-value for responses		
	TPC	DPPH	FRAP
A	7.62	66.25	69.82
B	31.43	4.38	2.15
C	10.85	0.96	1.85
D	5.42	1.70	2.67
E	2.23	5.22	0.28
AB	3.99	5.04	0.69
AC	0.20	2.47	1.12
AD	0.10	2.77	2.53
AE	4.09	1.21	0.17
BC	11.04	0.006	1.69
BD	2.94	0.04	1.42
BE	0.00	0.06	0.21
CD	2.03	0.01	0.07
CE	0.37	0.75	0.30
DE	2.72	0.07	1.11

A: liquid/solid ratio; B: temperature; C: methanol concentration; D: time; E: pH

Moreover, temperature fluctuations can affect the physical structure of the cell wall materials. Elevated temperatures are known to induce the opening of cellulose structures and expedited molecular exchange during the extraction process (Medina-Plaza *et al.*, 2019). Consequently, the observed temperature-dependent increase in total phenolic content may be attributed to a combination of enhanced solubility, improved permeability, and structural changes in the cell wall material. These findings underscore the importance of optimizing extraction temperatures to maximize the yield of phenolic compounds, which are crucial contributors to the antioxidant potential of *J. gendarussa*.

Antioxidant capacity

Two well-established procedures were used to assess antioxidant capacity: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the Ferric Reducing Antioxidant Power (FRAP) assays. DPPH involves the transformation of a free radical (diphenylpicrylhydrazine) into a non-radical form (diphenylpicrylhydrazine) by donating a hydrogen atom to a free electron on a radical molecule (Setiawan *et al.*, 2018). The FRAP method is based on the reduction of Fe^{3+} within a complex $\text{Fe}(\text{TPTZ})^{3+}$, resulting in Fe^{2+} and a distinctive blue color (Benzie and Devaki, 2017). Antioxidant capacities for both DPPH and FRAP were quantified in Trolox equivalent antioxidant capacity (TEAC) units ($\mu\text{mol TEAC/g}$).

The analyzed data are shown in Figures 2 and 3. The investigation revealed that, in terms of DPPH antioxidant capacity, the liquid-to-solid ratio and pH were significant factors, indicating the influence of the other three factors. The liquid-to-solid ratio and

pH yielded p-values <0.05, contributing the largest percentage to the antioxidant capacity of DPPH at 66.25% and 5.22%, respectively (Tables 3 and 4). Conversely, for the FRAP antioxidant capacity, only the liquid-to-solid ratio exhibited a significant effect ($p < 0.05$) (Table 3).

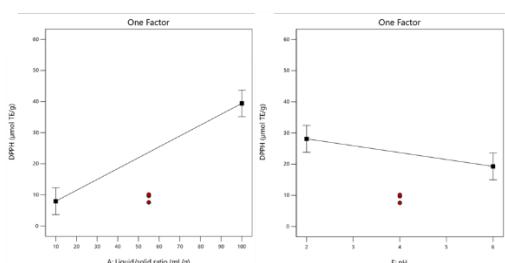


Figure 2. Effect of liquid/solid ratio and pH on the recovery of DPPH antioxidant capacity ($\mu\text{mol TEAC/g}$) of *J. gendarussa*. A greater slope indicates a greater effect of a given factor on the recovery of antioxidant capacity using the DPPH method

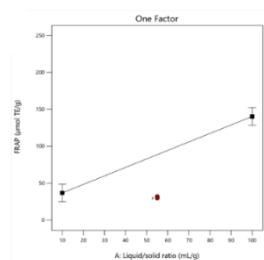


Figure 3. Effect of liquid/solid ratio on the recovery of FRAP antioxidant capacity ($\mu\text{mol TEAC/g}$) of *J. gendarussa*. A greater slope indicates a greater effect of a given factor on the recovery of antioxidant capacity using the FRAP method

The observed significance of the liquid-to-solid ratio and pH in determining DPPH antioxidant capacity is consistent with the results of previous studies. Castro-López *et al.* (2017) reported an increase in antioxidants when the liquid-to-solid ratio was elevated, supporting our findings. Additionally, the impact of pH on antioxidant activity was elucidated by Bayliak *et al.* (2016) and Ruenroengklin *et al.* (2008). The former demonstrated that antioxidant activity tends to be higher in acidic environments, which is consistent

with our results, and the latter showed the highest antioxidant activity at pH 3-4.

The need for various liquid-to-solid ratios in order to maximize the extraction of phenolic compounds, flavonoids, and antioxidant activity underlines the importance of this parameter. Higher liquid-to-solid ratios enhance the extraction efficiency by providing a greater quantity of soluble plant material components, facilitating increased contact with the solvent (Wang *et al.*, 2016; Dong *et al.*, 2010). The saturation phenomenon, wherein the extracted chemical amount diminishes with a lower liquid-to-solid ratio, aligns with the mass transfer theory, emphasizing the role of concentration gradients as the driving force during mass transfer. Furthermore, the influence of pH on antioxidant activity corresponds to the stability of phenolic compounds at acidic pH, as highlighted by Friedman and Jürgens (2000).

The vulnerability of phenolic compounds to damage at higher pH levels underscores the intricate interplay between environmental conditions and the preservation of antioxidant properties. This comprehensive understanding of the extraction parameters contributes to the optimization of processes aimed at obtaining the maximum yield of bioactive compounds from the Gandarusa plant.

Optimization and verification solution

The Design Expert 13.0 application provides an extraction optimization solution. The optimization solution is presented in Table 5. One method for optimizing the system components is the desirability function (Amdoun *et al.*, 2018). Desirability is founded on the notion that if numerous features of a product or process go beyond what is considered “desirable,” the quality of the product or process is unsatisfactory. The objective is to identify the appropriate experimental conditions that, when the process is used, can produce the best results for all the factors examined, including the major factors (Candioti *et al.*, 2014). The desirability values range from 0 to 1. The value 0 is given when factors give an unwanted response, while the value 1 indicates optimal performance for the factors being studied (Amdoun *et al.*, 2018).

Based on the two-level half-factorial design optimization results, the verification demonstrates

Table 5. Optimization solution verification results

	A	B	C	D	E	TPC	DPPH	FRAP	Desirability
Prediction	93.7	43.4	60.6	113	3.45	9.53	36.3	129	1.00
Verification	93.7	43.4	60.6	113	3.45	9.40	28.53	136.11	1.00
%RSE						1.36	21.40	5.51	

A: liquid/solid ratio; B: temperature; C: methanol concentration; D: time; E: pH.

that the best point solution is accurate in producing a response. The results of the verification analysis are listed in Table 5. TPC 0.054 (1.36%), DPPH 0.06 (21.40%), and FRAP 0.064 (5.51%) percentage RSE were found in this investigation. Residual standard error (RSE) is used to predict the real difference between actual and predicted values (Bahari *et al.*, 2020). A %RSE score of under 10% shows that there is no significant difference between the actual and predicted values. This indicates that the model used was appropriate (Salbi *et al.*, 2021). In this experiment, the phenolic and FRAP response values were found to be not significantly different from the predicted values. However, the DPPH response was found to exceed the 10% limit of RSE values. This indicates that more data may be needed to improve the accuracy of the model prediction. Optimal extraction parameters were identified with a liquid-to-solid ratio of 93.7 mL/g, temperature of 43.4°C, methanol concentration of 60.6%, extraction time of 113 min, and pH of 3.45.

Conclusions

This study successfully optimized the extraction of phenolic compounds and antioxidant capacity from *J. gendarussa*. Key extraction parameters, such as temperature, liquid-to-solid ratio, and pH, were meticulously evaluated. Temperature was found to be crucial for maximizing total phenolic content, while the liquid-to-solid ratio significantly influenced the DPPH antioxidant capacity and prominently affected the FRAP antioxidant capacity. Optimal extraction conditions were established to ensure high desirability and efficiency of the extraction process. These optimized parameters guarantee an enhanced yield of bioactive compounds, promising reproducibility and scalability for broader applications. This protocol provides a valuable method for extracting natural antioxidants from medicinal plants, advancing their potential for pharmaceutical innovation.

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