Trop J Nat Prod Res, October 2025; 9(10): 4749 - 4758
ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Optimization of Extraction Process of Flavonoid-Rich Extract From *Glochidion Littorale* Leaves With Anti-Inflammatory and Cytotoxic Activities Against Selected Cancer Cell Lines

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ARTICLE INFO

Article history:
Received 23 July 2025
Revised 12 September 2025
Accepted 22 September 2025
Published online 01 November 2025

ABSTRACT

The extraction conditions for a flavonoid-rich extract derived from the leaves of *Glochidion littorale*, evaluated for its anti-inflammatory potential and cytotoxicity against selected cancer cell lines, were optimized using response surface methodology (RSM) with the Box-Behnken design. The optimal conditions included an ethanol concentration of 68%, an ultrasonic extraction duration of 25 minutes, and a temperature of 60 °C. The total flavonoid content (TFC) obtained from the prepared extract was 198.18 ± 1.13 mg quercetin equivalents (QE)/g of extract, which closely aligned with the model-predicted value of 197.38 mg QE/g. Anti-inflammatory activity was assessed based on nitric oxide (NO) scavenging capacity—NO being a signaling molecule associated with inflammation. Results showed a dose-dependent reduction in NO levels, with the lowest NO concentration (31.39 ± 0.95 µM, corresponding to approximately 61% inhibition) observed at a sample concentration of 200 µg/mL. Regarding cytotoxic activity, half-maximal inhibitory concentration (IC_{50}) values were determined to be 133.48 ± 4.61 µg/mL for SK-LU-1 (lung cancer cells), 102.14 ± 0.35 µg/mL for HepG2 (liver cancer cells), and 144.20 ± 5.41 µg/mL for MKN7 (gastric cancer cells). The study concludes that the optimized flavonoid-rich extract of *Glochidion littorale* exhibited potent anti-inflammatory and cytotoxic potentials against lung, liver, and gastric cancer cell lines *in vitro*.

Keywords: *Glochidion littorale* Blume, flavonoid content, anti-inflammatory activity, cytotoxicity, cancer cell lines.

Introduction

Inflammation is a complex and critical defense mechanism of the human body, often triggered by microbial infections or resulting from tissue damage and trauma unrelated to pathogenic agents, also known as sterile inflammation.[1] Although fundamentally protective, unresolved inflammatory responses may lead to chronic inflammation and subsequent organ fibrosis.[2] These chronic inflammatory disorders contribute significantly to the pathogenesis of various non-communicable diseases, such as arthritis, diabetes, cardiovascular diseases, and cancer. According to GLOBOCAN, a global cancer statistics database, there were an estimated 20 million new cancer cases and 9.7 million cancer-related deaths worldwide in 2022. Modern biomedical research, therefore, aims to both prevent cancer-causing agents and develop therapeutic strategies to inhibit cancer initiation. Newman and Cragg reviewed drug discovery data from 1981 to 2019 and concluded that approximately 50% of approved drugs for cancer and other diseases are either directly or indirectly derived from natural products. The authors emphasize that, despite advances in synthetic chemistry, nature remains an essential source of inspiration for novel drug development.[3]

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Citation: Thuy P T L, Huy Q N, Phong H N, Mai V D. Optimization Of Extraction Process Of Flavonoid-Rich Extract From *Glochidion Littorale* Leaves With Anti-Inflammatory And Cytotoxic Activities Against Selected Cancer Cell Lines.Trop J Nat Prod Res. 2025; 9(10): 4749 – 4758
https://doi.org/10.26538/tjnpr/v9i10.8

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Consequently, the search for bioactive natural compounds has become increasingly vital and attracts significant interest from the scientific community. Among these, flavonoids, secondary polyphenolic metabolites characterized by a C6-C3-C6 backbone, are widely found in flowers, leaves, and fruits.[4] In recent years, flavonoids have gained considerable attention in both herbal medicine and modern pharmacology due to their potent antioxidant effects, anti-inflammatory properties, and selective cytotoxicity against cancer cells.[5] Compounds such as quercetin, apigenin, luteolin, and kaempferol have been shown to inhibit pro-inflammatory enzymes like cyclooxygenase (COX) and lipoxygenase (LOX), suppress pro-inflammatory cytokines such as TNF-α and IL-6, and induce apoptosis in various cancer cell lines.[6,7]

The genus *Glochidion* belongs to the Euphorbiaceae family and comprises approximately 300 species distributed from Madagascar to the Pacific Islands, with primary presence in tropical Asia.[8] Several species in this genus have been reported to contain diverse phytochemicals, including flavonoids, tannins, and triterpenoids.[9] *Glochidion littorale* Blume, also known in Vietnam as “Bọt ech bien” or “Tram bot”, is a species in the Euphorbiaceae family.[10] This plant grows wild throughout Vietnam, especially in the central coastal and southern regions. Traditionally, it is used as a medicinal plant due to its anti-inflammatory, antibacterial, antioxidant, and anticancer properties.

Despite its promising pharmacological potential, research on the extraction process and bioactivity evaluation of flavonoid-rich extracts from this plant remains limited. Variability in phytochemical content and biological activity due to differences in species, growth conditions, and extraction methods poses challenges for standardization and application. The extraction procedure plays a pivotal role in obtaining bioactive compounds from herbal materials and directly affects the yield, purity, and efficacy of the final product.[11] Therefore, optimizing the extraction process is crucial to

improve the therapeutic potential of flavonoid-containing extracts. The methanol extract of the *G. littorale* stem bark was separated by liquid-liquid extraction, followed by different chromatographic techniques to purify the chemical composition. The result showed that seven terpenoids, including lupeol, 3-epi-lupeol, betulin, lup-20 (29)-ene-1ß, 3ß-diol, lupenone, glochidonol, and glochidone, were isolated from the methanol extract.[12] A recent study in 2023 by Wannachai Chantan confirmed the high concentrations of flavonoids and phenolic derivatives in *G. littorale* leaves and displayed high antioxidant activity with an IC_{50} value of 67.18 µg/mL by DPPH assay.[13] Response surface methodology (RSM) with Box-Behnken design was employed for optimization in this study, as it efficiently models the interactions between extraction variables while minimizing the number of experiments required. This study represents one of the first attempts to optimize the extraction of flavonoid-rich extracts from *G. littorale* leaves using RSM and to evaluate their anti-inflammatory and cytotoxic activities against specific cancer cell lines, addressing a gap in the literature on this understudied species. This research aims to optimize the extraction process of flavonoid-rich extracts from the leaves of *Glochidion littorale* and evaluate their potential applications in modern medicine based on their biological activities.

Materials and Methods

Plant Material

Leaves of *Glochidion littorale* Blume were collected from Group 8, Thoi Hoa Area, Thoi An Dong Ward, Binh Thuy District, Can Tho City, Vietnam (GPS coordinates: latitude 10.07° N, longitude 105.74° E) on February 24, 2024. The botanical identification was conducted based on morphological characteristics described in the *Dictionary of Vietnamese Medicinal Plants* by Vo Van Chi (2021). The morphology of *Glochidion littorale* is illustrated in Figure 1.

Chemicals and Reagents

Dimethyl sulfoxide (DMSO, Merck, USA); phosphomolybdic acid (PMA, Sigma Aldrich, Germany); Griess reagent (FUJIFILM-Wako, Japan); sodium nitroprusside (Merck, USA); sodium nitrite (Xilong, China); organic solvents including n-hexane, ethyl acetate (EA), methanol (MeOH), ethanol (Chemsol, Vietnam), chloroform (Chemsol, Vietnam); LPS (lipopolysaccharide, FUJIFILM-Wako, Japan); Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Switzerland); fetal bovine serum (Gibco, UK); penicillin-streptomycin (Sigma-Aldrich, Switzerland); Cell Counting Kit-8 (CCK-8, Dojindo, Japan); N-Nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich, Switzerland); curcumin (Sigma-Aldrich, Switzerland); phosphate-buffered saline (PBS) pH 7.4 (Biosharp, China).

Experimental Design Using the Box-Behnken Model

Effect of Ethanol Concentration on Flavonoid Extraction

Powdered leaves (0.50 g) of *G. littorale* were extracted using ethanol at concentrations of 40%, 50%, 60%, 70%, 80%, 90%, and 99%. Other extraction parameters were kept constant. Each condition was performed in triplicate. Flavonoid content in the extracts was used as the primary evaluation metric.

Effect of Temperature on Flavonoid Extraction

Extraction was conducted at varying temperatures (30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, and 90 °C) under otherwise identical conditions. Each trial was performed in triplicate, and flavonoid content was measured.

Effect of Ultrasonic Extraction Time

Extraction was performed using ultrasonic treatment for 5, 10, 15, 20, 25, 30, and 35 minutes. All other factors remained constant, and each test was conducted in triplicate. The flavonoid yield was determined for each condition.

Effect of Solid-to-Solvent Ratio

The solid-to-solvent ratios (w/v) tested were 1/10, 1/15, 1/20, 1/25, 1/30, 1/35, and 1/40. All other parameters remained unchanged. Each extraction was performed in triplicate, and the flavonoid content was assessed.

Optimization of Flavonoid Extraction Process

After investigating the effects of individual parameters, the most influential variables (ethanol concentration, extraction temperature, and ultrasonic duration) were selected to construct an optimized extraction protocol. Experimental design and statistical analysis were carried out using Design-Expert software version 12.0 based on response surface methodology (RSM). Optimal conditions were determined to maximize flavonoid yield.

Anti-Inflammatory Activity Assay

The anti-inflammatory activity of the extracts was evaluated by measuring nitric oxide (NO) production inhibition in LPS-stimulated RAW 264.7 macrophages. Cells were treated with the extract in the presence or absence of LPS and incubated for 18 hours. Subsequently, 100 µL of culture supernatant was reacted with 100 µL of Griess reagent and incubated at room temperature for 10 minutes. Absorbance was measured at 540 nm. N-Nitro-L-arginine methyl ester (Sigma-Aldrich, Switzerland)(100 µM) was used as a positive control.[14]

Cytotoxicity Assay on Cancer Cell Lines

The cytotoxic activity was assessed using the *in vitro* protocol standardized by the U.S. National Cancer Institute (NCI), developed initially by Monks *et al.*.[15] The method involves quantifying cellular protein content based on optical density (OD) following sulforhodamine B (SRB) staining. Trypsinized cells were seeded into 96-well plates and treated with extract concentrations of 10, 40, 80, 120, 160, and 200 µg/mL. Incubation was performed at 37 °C for 48 hours. Cells in control wells (without treatment) were fixed with trichloroacetic acid (TCA) after 1 hour. After 48 hours, all cells were fixed, stained with SRB for 30 minutes, washed with acetic acid, and dried. Bound SRB was solubilized with 10 mM unbuffered Tris base, and absorbance was measured at 515 nm using an ELISA plate reader (Bio-Rad). Ellipticine was used as a positive control, and 10% DMSO as a negative control. The percentage inhibition was estimated based on equation 1

$$\text{Inhibition efficiency (\%)} = \frac{1-OD_{sample}}{OD_{control}} \times 100 \qquad (1)$$

Data Processing and Statistical Analysis

Data from single-factor experiments were expressed as mean ± standard deviation (SD), analyzed using ANOVA with Tukey's post hoc test in Minitab 21.1.0 (Minitab, LLC, State College, PA, USA, 2021). Graphs were generated using Microsoft Excel 2021 (Microsoft Corporation, Redmond, WA, USA). Optimization model data and predicted values were processed with Design-Expert 12.0 (Stat-Ease, Inc., Minneapolis, MN, USA, 2019).

Results and Discussion

In this study, the effects of single factors on the flavonoid content of *Glochidion littorale* leaf extracts were studied. Results of the study showed that ethanol concentration significantly influenced the flavonoid extraction yield from *G. littorale* leaves. As ethanol concentration increased from 40% to 70%, the flavonoid content increased, peaking at 128.58 ± 5.41 mg QE/g extract at 70% ethanol, approximately 1.7 times higher than that obtained with 40% ethanol. However, flavonoid yield declined as ethanol concentration increased beyond 80%, reaching a low of 89.17 ± 2.17 mg QE/g at 99.5%. The optimal performance at 70% ethanol concentration likely reflects a favorable ethanol-water polarity balance, enhancing the solubility of both polar and moderately polar flavonoids. Ethanol disrupts cell membranes to release bioactive compounds, while water facilitates the dissolution of polar constituents.

Similarly, temperature was another key factor affecting flavonoid extraction. When the temperature increased from 30 °C to 60 °C, flavonoid content rose markedly from 90.07 ± 4.73 to 153.36 ± 2.17 mg QE/g. Beyond 60 °C, however, a decline was observed, with only 110.56 ± 3.40 mg QE/g at 90 °C. The rise in flavonoid content up to 60 °C may be due to improved solvent penetration and diffusion efficiency. Nevertheless, because flavonoids are heat-sensitive and prone to oxidation, excessive temperatures may degrade the compounds, reducing yield and biological activity.

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The effect of Ultrasonic extraction time on flavonoid content was also evaluated. Ultrasonic treatment significantly affected extraction efficiency. Flavonoid content increased with sonication duration up to

Figure 1: The morphology of *Glochidion littorale*

Table 1: Predicted and experimental flavonoid yields from *G. littorale* leaf extract

Axiom	Independent Variable			Flavonoid content (mg QE/g extract)	
	A (%)	B (ºC)	C (minute)	Predicted	Experimental
1	50	60	25	109.30	108.31^{f} ±2.95
2	70	60	25	117.58	118.00^{e} ± 4.07
3	50	80	25	69.77	69.35^{g} ± 3.72
4	70	80	25	58.00	58.99^{h} ± 2.44
5	50	70	20	143.39	144.35^{b} ± 3.96
6	70	70	20	133.31	132.86^{c} ± 2.17
7	50	70	30	142.32	142.77^{b} ± 2.44
8	70	70	30	148.91	147.95^{b} ± 7.83
9	60	60	20	116.39	116.42^{e} ± 4.43
10	60	80	20	69.22	68.67^{g} ± 2.73
11	60	60	30	126.02	126.55^{d} ± 3.58
12	60	80	30	74.10	74.08^{g} ± 3.47
13	60	70	25	195.20	198.85^{a} ± 3.38
14	60	70	25	195.20	192.77^{a} ± 5.85
15	60	70	25	195.20	195.92^{a} ± 1.70
16	60	70	25	195.20	196.82^{a} ± 4.43
17	60	70	25	195.20	191.64^{a} ± 9.61

Means followed by different letters indicate statistically significant differences ($p < 0.05$). A denotes ethanol concentration, B denotes temperature, and C denotes ultrasonic time.

ISSN 2616-0684 (Print)
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Table 2: ANOVA results for the flavonoid content model of *Glochidion littorale* leaf extract

Source	Sum of Squares	Df	Mean Square	F-value	P-value
Model	38046.15	9	4227.35	736.53	< 0.0001
A-temperature	6.10	1	6.10	1.06	0.3371
B-Ethanol	4910.34	1	4910.34	855.53	< 0.0001
C-Time	105.49	1	105.49	18.38	0.0036
AB	100.54	1	100.54	17.52	0.0041
AC	69.47	1	69.47	12.10	0.0103
BC	5.59	1	5.59	0.9744	0.3565
A^2	3915.15	1	3915.15	682.14	< 0.0001
B^2	24349.44	1	24349.44	4242.41	< 0.0001
C^2	2174.39	1	2174.39	378.84	< 0.0001
Residual	40.18	7	5.74		
Lack of Fit	5.12	3	1.71	0.1947	0.8950
Pure Error	35.06	4	8.76		
Cor Total	38086.33	16			

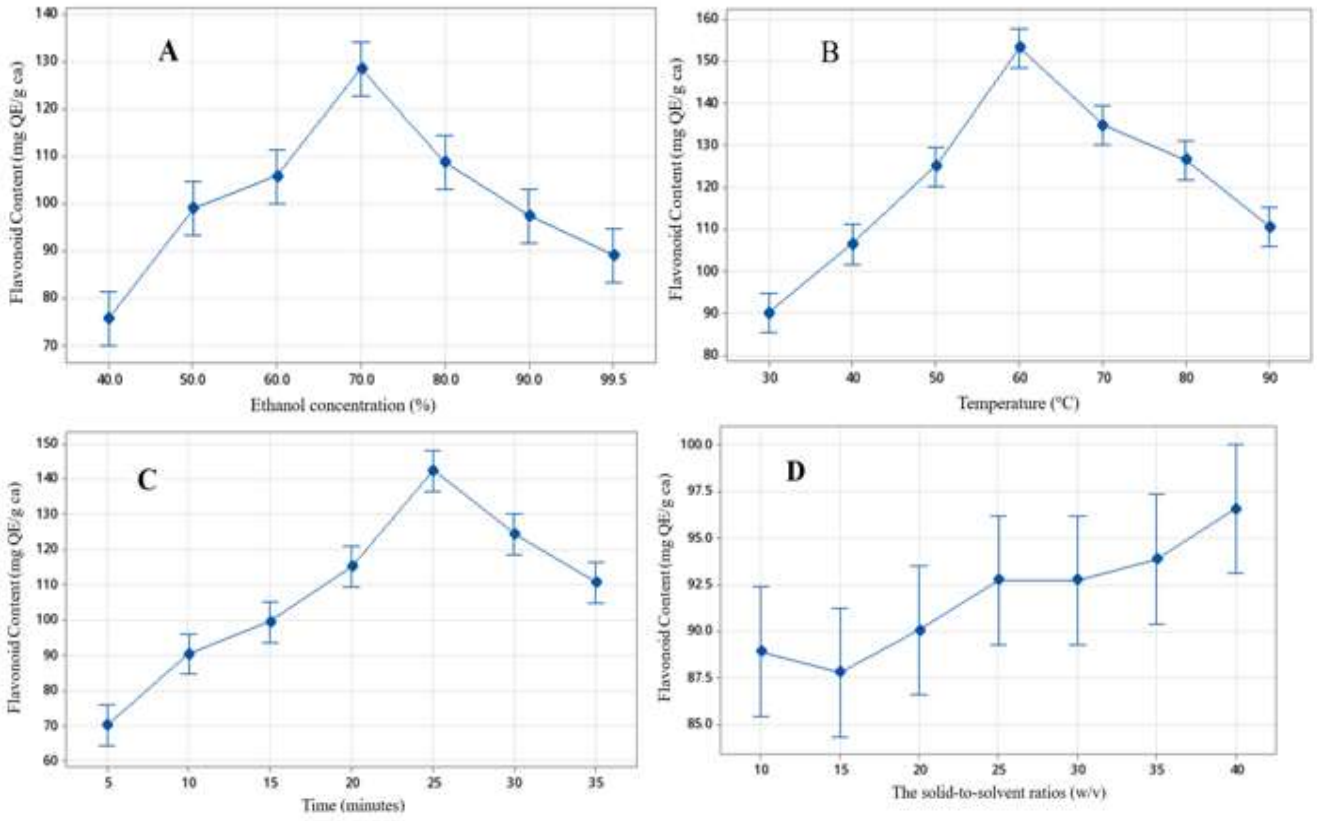

Figure 2: Single-factor analysis of extraction conditions

Note: A – Effect of ethanol concentration; B – Effect of temperature; C – Effect of ultrasonic duration; D – Effect of solid-to-solvent ratio on flavonoid content.

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Table 3: IC_{50} values for inhibition of SK-LU-1 lung cancer cells

Cell Line	Sample Type	IC_{50} value (µg/mL)
SK-LU-1	Conventional flavonoid extract	207.53 ± 0.98
	Flavonoid-rich extract	133.48 ± 4.61
	Ellipticine	0.67 ± 0.03

25 minutes, peaking at 142.55 ± 7.16 mg QE/g, approximately 2.03 times greater than the yield after 5 minutes. However, a prolonged duration beyond 25 minutes resulted in decreased flavonoid levels, suggesting potential degradation or structural alteration of flavonoids under prolonged ultrasonic energy exposure. The initial increase is attributed to cavitation effects that disrupt plant cell walls and enhance compound diffusion.

The effect of solid-to-solvent ratio on flavonoid content increased as the solid-to-solvent ratio decreased (i.e., more solvent volume), peaking at 96.60 ± 3.19 mg QE/g at a 1:40 ratio. The lowest yield, 87.82 ± 1.41 mg QE/g, occurred at 1:15. However, no statistically significant differences were observed between ratios of 1:25 and 1:40 ($p > 0.05$), suggesting diminishing returns in extraction efficiency beyond a certain solvent threshold.

The optimization of flavonoid extraction using Box–Behnken Design (BBD) was based on the single-factor results, and ethanol concentration, extraction temperature, and ultrasonic duration were selected as independent variables for optimization. Seventeen experimental runs were conducted, including five center points. Table 1 summarizes the predicted and experimental flavonoid contents under different combinations of these variables. The results confirmed the reliability of the 17 experimental runs within the model, as the expected flavonoid content closely matched the experimental values, with discrepancies falling within the acceptable error margin. The highest flavonoid contents were observed in the central point runs (experiments 13, 14, 15, 16, and 17). The highest recorded value was 198.85 ± 3.38 mg QE/g extract, obtained in run 13 under the following conditions: 60% ethanol concentration, 70 °C extraction temperature, and 25 minutes of ultrasonic treatment. Other central runs yielded experimental values ranging from 191.64 ± 9.61 to 198.85 ± 3.38 mg QE/g extract, which were consistent with the model's predicted optimal values.

Table 4: IC_{50} values for inhibition of HepG2 liver cancer cells

Cell Line	Sample Type	IC_{50} value (µg/mL)
HepG2	Conventional flavonoid extract	198.09 ± 6.34
	Flavonoid-rich extract	102.14 ± 0.35
	Ellipticine	0.74 ± 0.04

To establish a reliable basis for identifying the optimal conditions for maximizing flavonoid content, a second-order polynomial regression equation was constructed using the response surface model. This equation predicts flavonoid yield based on the independent variables: ethanol concentration (A), temperature (B), and ultrasonic duration (C):

$$Y_{flavonoid} = \begin{pmatrix} -5163.35486 + 37.93043 \times B + 107.58614 \times A + 42.83041 \times C \\ -0.050134 \times A \times B + 0.083349 \times B \times C - 0.023649 \times A \times C \\ -0.304934 \times B^2 - 0.760460 \times A^2 - 0.908994 \times C^2 \end{pmatrix}$$

Where: A = ethanol concentration (%), B = temperature (°C), C = ultrasonic time (min)

Following this, data from all 17 experimental runs were further analyzed using the Design-Expert 12.0 software via ANOVA to statistically validate the model and determine the significance of each factor. The results demonstrated that the constructed model was statistically significant and exhibited a good fit, as indicated by the validation parameters. Specifically, the model yielded a p-value of < 0.0001, a coefficient of determination (R^2) of 0.9989, and an adjusted R^2 of 0.9976, indicating that the model accounted for over 99% of the variability in the experimental data. The p-value < 0.0001 further confirmed that the model was highly statistically significant (confidence level > 99.99%). Additionally, the lack-of-fit test yielded a p-value of 0.8950 (> 0.05), suggesting that the discrepancy between the model and the experimental data was not statistically significant. Moreover, the standard deviation (Std. Dev. = 2.40) and the coefficient of variation (CV% = 1.78%) were relatively low, reflecting the precision and high reproducibility of the experiments. The adequate precision value of 74.6692 far exceeded the minimum threshold of 4, indicating that the model possessed high sensitivity and was suitable for process optimization and control.

Table 5: IC_{50} values for inhibition of MKN7 gastric cancer cells

Cell Line	Sample Type	IC_{50} value (µg/mL)
MKN7	Conventional flavonoid extract	185.03 ± 0.74
	Flavonoid-rich extract	144.20 ± 5.41
	Ellipticine	0.72 ± 0.02

Based on the regression equation, three-dimensional (3D) response surface plots were generated using Response Surface Methodology (RSM). These 3D graphs illustrated the interactions between pairs of independent variables and their combined effects on the response variable, while holding the third factor constant. As shown in Figure 3, all three 3D response surface plots reveal pronounced interaction effects among the investigated variables in the optimization of the flavonoid extraction process from *Glochidion littorale* leaves. Plot A illustrates the interaction between ethanol concentration and temperature, where flavonoid content reaches its optimum in the range of 60 °C to 65 °C and at an ethanol concentration of approximately 70%. A slight decline in flavonoid yield beyond this range indicates the presence of optimal extraction conditions. Plot B demonstrates the interaction between ultrasonic time and temperature, with the highest flavonoid yield observed at 26 to 28 minutes and temperatures around 60 °C to 65 °C. Plot C shows the combined effect of ultrasonic time and ethanol concentration, with maximum flavonoid content recorded at 26 to 28 minutes and ethanol concentrations between 70% and 75%. These results collectively indicate that the optimal strategy for extracting flavonoids from *Glochidion littorale* leaf extract involves finely tuning the key parameters within these identified ranges.

The practical extraction was carried out under the optimal conditions predicted by the model: 68% ethanol concentration, extraction temperature of 60 °C, and ultrasonic time of 25 minutes. The resulting flavonoid content was 198.18 ± 1.13 mg QE/g of extract, which closely matched the predicted value, demonstrating a high degree of reliability and consistency between experimental and modeled results.

Anti-inflammatory activity of the flavonoid-rich extract was evaluated using the Nitric oxide (NO) inhibitory method. NO is an inflammatory mediator produced by immune cells upon stimulation with lipopolysaccharides (LPS). Therefore, the concentration of NO is commonly used as an indicator to assess the anti-inflammatory potential of test samples. NO levels significantly increased upon LPS stimulation, with the average concentrations observed in the control group (treated with LPS only) reaching approximately 76.25 ± 2.02 µM for the conventional extract and 80.81 ± 2.39 µM for the flavonoid-rich extract. In contrast, the untreated control group (without LPS stimulation) exhibited only ~18–19 µM NO, indicating a robust inflammatory response was successfully induced. Upon treatment with the *Glochidion littorale* leaf extracts, both types of extracts exhibited dose-dependent inhibition of NO production. At a

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concentration of 200 µg/mL, the conventional extract reduced NO levels to an average of 67.41 ± 1.90 µM. In comparison, the flavonoid-rich extract

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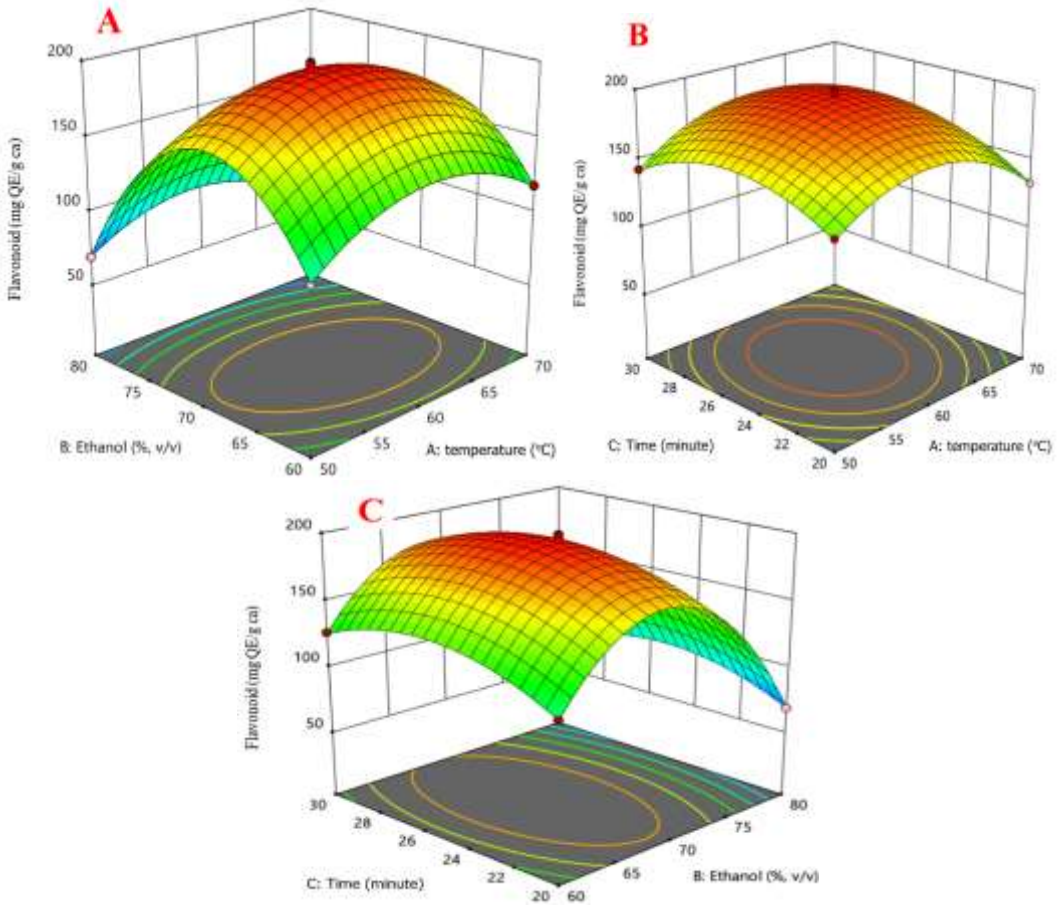

Figure 3: Response surface plots of flavonoid content in extracts from *Glochidion littorale* leaves

: Interaction model between ethanol concentration and temperature; **B:** Interaction model between ultrasonic time and temperature; **C:** Interaction model between ultrasonic time and ethanol concentration

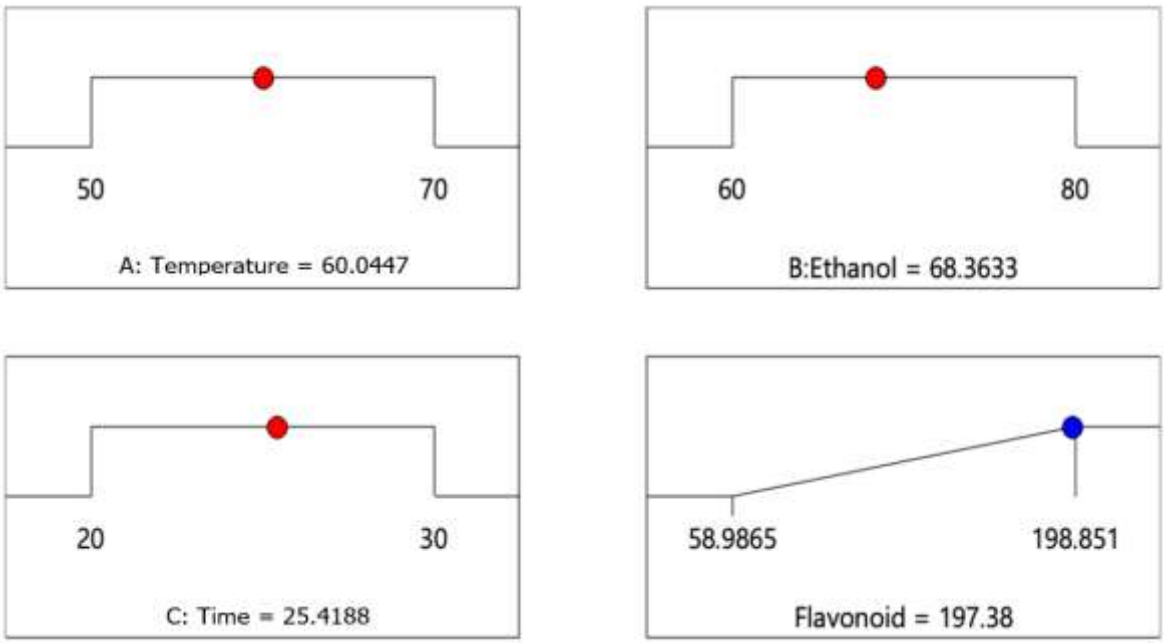

Figure 4: Desirability function and optimal conditions for flavonoid extraction from *Glochidion littorale* leaves

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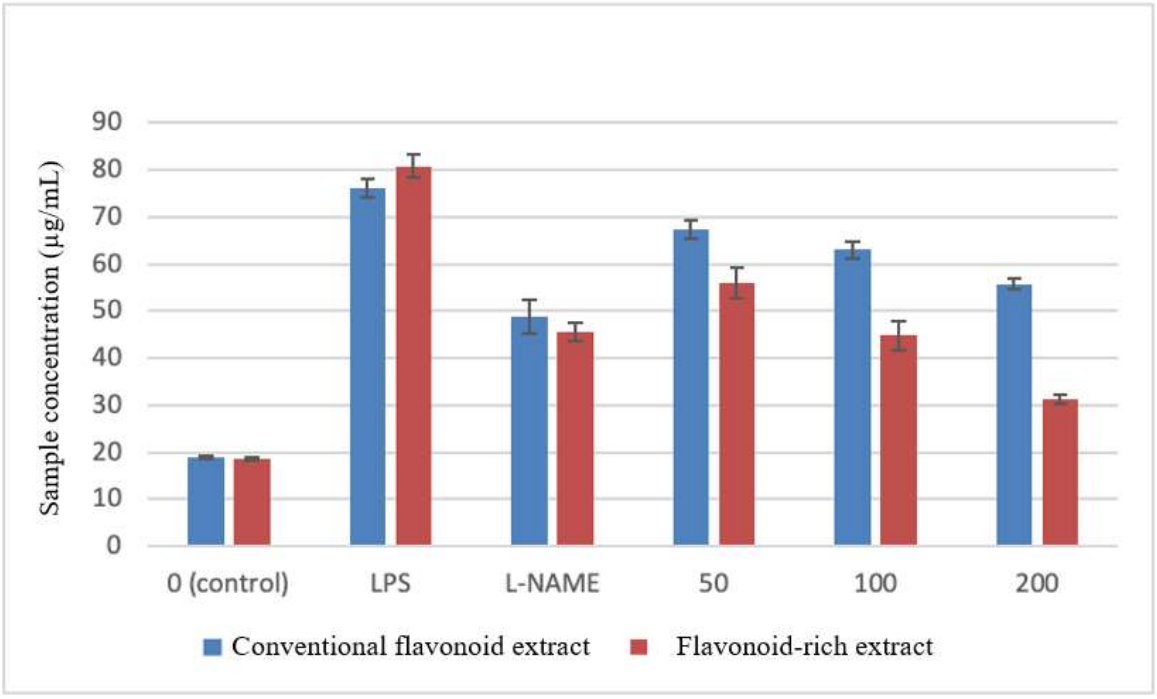

Figure 5: Anti-inflammatory activity of two types of extracts from *Glochidion littorale* leaves

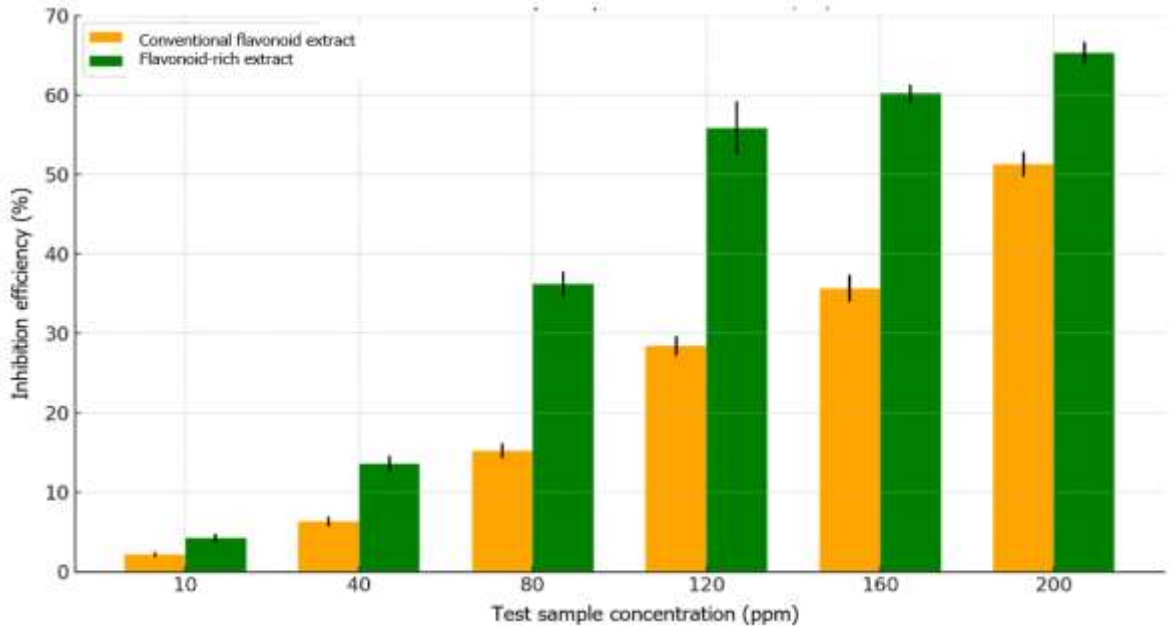

Figure 6: Cytotoxic activity of two extracts on SK-LU-1 lung cancer cell line

demonstrated markedly more potent inhibition, reducing NO production to 31.39 ± 0.95 µM—closely approximating the inhibitory effect of the positive control L-NAME, which recorded 45.59 ± 1.87 µM. These findings confirm that both extracts possess anti-inflammatory activity, with the flavonoid-rich extract exhibiting superior efficacy. This effect is likely attributable to the inhibition of inducible nitric oxide synthase (iNOS), the enzyme responsible for NO production during inflammation.[16] The enhanced anti-inflammatory activity observed in the flavonoid-rich extract supports the conclusion that flavonoids play a key bioactive role in the therapeutic potential of *Glochidion littorale* leaf extracts. Statistically, the difference between the flavonoid-rich and conventional extracts was significant ($p < 0.05$) at concentrations above 80 µg/mL, highlighting the impact of optimization. Recent studies on flavonoids from similar plants corroborate these results, showing inhibition of NF-κB and cytokine pathways as key mechanisms.[17, 18, 19] The flavonoid-rich extract cytotoxicity against selected cancer cell lines was also evaluated against SK-LU-1 lung cancer cells. The results demonstrated that both flavonoid-containing extracts from *Glochidion littorale* leaves exhibited dose-dependent inhibitory effects on the proliferation of SK-LU-1 lung cancer cells. As the concentration increased, the inhibitory efficiency also increased accordingly, with the highest value recorded for the flavonoid-rich extract at 65.32 ± 1.40% at 200 µg/mL. At this concentration, the flavonoid-rich extract achieved

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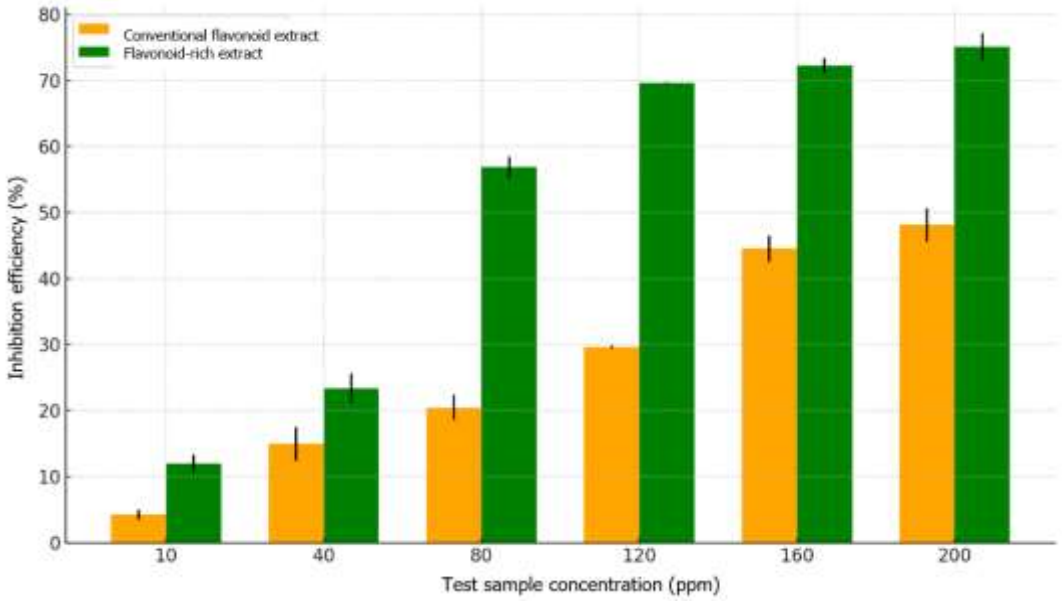

Figure 7: Cytotoxic activity of two extracts on the HepG2 liver cancer cell line

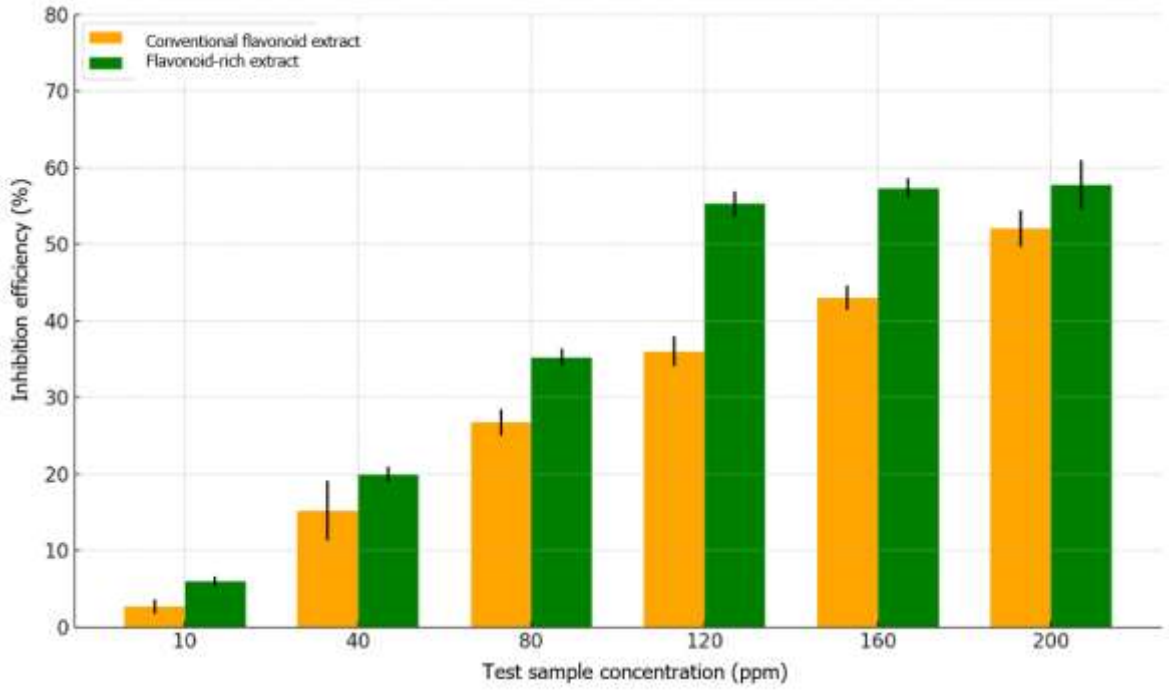

Figure 8: Cytotoxic activity of two extracts on the MKN7 gastric cancer cell line

approximately 65% inhibition, while the conventional extract achieved only about 50%, clearly indicating the superior anticancer effect of the flavonoid-enriched formulation. This difference became particularly pronounced at concentrations of 80 µg/mL and above, suggesting that higher flavonoid content plays a decisive role in the suppression of cancer cell growth. With inhibition exceeding 60%, the flavonoid-rich extract from *G. littorale* leaves demonstrates promising potential as a candidate for supporting in vitro treatment of lung cancer. At the IC_{50}, that is, the concentration required to inhibit 50% of cell viability, both extracts exhibited cytotoxic activity, although not at a high level (IC_{50} > 100 µg/mL). The flavonoid-rich extract demonstrated notably greater potency, reducing the IC_{50} value by approximately 1.5-fold compared to the conventional extract. However, the cytotoxicity of both extracts remained significantly lower than that of ellipticine, a potent positive control compound. These findings align with recent studies showing that flavonoids induce apoptosis in lung cancer cells via autophagic and metastatic pathway modulation.[20, 21]

Additionally, in the inhibition of HepG2 liver cancer cells assay, both types of *G. littorale* extracts demonstrated increasing inhibitory effects on HepG2 liver cancer cells in a concentration-dependent manner. As the dosage increased from 10 to 200 µg/mL, the inhibition of cell proliferation became more evident. The flavonoid-rich extract showed markedly superior activity, achieving a maximum inhibition rate of 75.10 ± 2.06% at 200 µg/mL, compared to only 48.11 ± 2.54% for the conventional extract. Even at 80 µg/mL, the flavonoid-rich extract achieved 56.87 ± 1.65% inhibition, whereas the conventional extract remained around 20%. The IC_{50} value of the flavonoid-rich extract was nearly half that of the conventional extract, indicating a

significant improvement in efficacy. Moreover, the extract was more potent against HepG2 cells than SK-LU-1 cells, reinforcing the hypothesis that flavonoids are the primary active constituents responsible for the observed cytotoxic effects. This is consistent with the literature on flavonoids targeting liver cancer through apoptosis induction and synergy with chemotherapeutics.[22, 23]

In the inhibition of MKN7 gastric cancer cells, both extracts displayed moderate dose-dependent cytotoxic effects. At 200 µg/mL, the flavonoid-rich extract achieved an inhibition rate of 57.79 ± 3.24%, slightly higher than that of the conventional extract (52.05 ± 2.41%).

The difference in inhibition became more apparent at concentrations of 80 µg/mL and above. Although the inhibitory effect at lower concentrations (10–40 µg/mL) was limited, the flavonoid-rich extract consistently outperformed the conventional one across the concentration range. Although the extracts exhibited cytotoxic effects against MKN7 cells, the activity remained relatively weak (IC_{50} > 100 µg/mL), even for the flavonoid-rich extract. The improvement observed with the optimized extract was less pronounced compared to its effects on HepG2 and SK-LU-1 cells. This suggests that MKN7 cells may be more resistant to the bioactive constituents of *G. littorale* leaf extracts. Recent research on flavonoids in gastric cancer supports this, noting variable efficacy across cell lines due to differential pathway targeting.[24, 25]

Overall, the cytotoxic activities were statistically comparable across cell lines, with HepG2 showing the most significant response ($p < 0.01$ compared to other lines). These results validate the findings using additional citations and emphasize the need for further mechanistic studies.

Conclusion

Through systematic investigation and experimental validation, the study identifies three primary factors (ethanol concentration, extraction temperature, and ultrasonic extraction duration) that have the most significant influence on the flavonoid yield from *Glochidion littorale* leaf extract. These variables were subsequently incorporated into a Box–Behnken Design (BBD), which helped to identify optimal extraction conditions as ethanol concentration of 68%, temperature of 60 °C, and ultrasonic time of 25 minutes. These conditions gave a maximum flavonoid content yield comparable to the predicted model value. The anti-inflammatory activity results revealed a dose-dependent trend, with increased flavonoid concentrations correlating with enhanced biological efficacy. These findings suggest that flavonoids are the principal bioactive components responsible for the anti-inflammatory effects of *G. littorale* leaf extracts. Also, the flavonoid-rich extract exerted pronounced cytotoxic activity against three human cancer cell lines: SK-LU-1 (lung), HepG2 (liver), and MKN7 (gastric) in a concentration-dependent manner. Overall, the study highlights the potential of *Glochidion littorale* as a promising natural source of bioactive flavonoids with anti-inflammatory and anticancer properties. Further research is warranted to isolate specific active compounds and elucidate their underlying mechanisms of action.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

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

Acknowledgments

We are grateful to Dr. Duong Thieu Van (Faculty of Pharmaceutical and Nursing Sciences, Tay Do University for her identification of the material. This study was partly funded by Nam Can Tho University, Vietnam (code 001).

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ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

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