

Optimizing Ultrasound-Assisted Extraction of *Calicotome spinosa* (L.) Link leaves using a Full-Factorial Design: linking Polyphenol Recovery to Biological Activities

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ABSTRACT

Introduction: *Calicotome spinosa* (L.) Link., is a Mediterranean medicinal plant traditionally used for the management of several health disorders. The present study aimed to optimize the extraction of total phenolic compounds from its leaves and to investigate the biological properties of the optimized extract.

Methods: Phenolic compounds were extracted by ultrasound-assisted extraction, and a three-level complete factorial design was applied to evaluate the influence of the ethanol/water ratio and sonication time on extraction efficiency. The optimized extract was characterized by determining its total flavonoid content and phenolic profile using HPLC-UV/VIS. Its antioxidant, acute oral toxicity, and anti-inflammatory activities were subsequently evaluated. **Results:** The ethanol/water ratio had the greatest effect on extraction efficiency, while both extraction parameters significantly influenced the recovery of phenolic compounds. The highest total phenolic content was obtained using 60% ethanol and 20 min of sonication. The optimized extract contained 36.94 ± 0.96 mg QE/g of flavonoids, and six phenolic compounds were identified by HPLC-UV/VIS. It also showed moderate antioxidant activity (DPPH $IC_{50} = 75.48 \pm 5.04$ μ g/mL), no signs of acute oral toxicity, and inhibited carrageenan-induced paw edema by 31.72% at 200 mg/kg. **Discussion:** The results indicate that optimizing the extraction conditions improved the recovery of phenolic compounds, which may have contributed to the observed biological activities. These findings support the value of ultrasound-assisted extraction for obtaining phenolic-rich extracts from *C. spinosa* leaves. **Conclusion:** The optimized extract of *C. spinosa* leaves constitutes a promising source of bioactive phenolic compounds with antioxidant and anti-inflammatory potential, supporting its further investigation for pharmaceutical and nutraceutical applications.

Keywords: *Calicotome*, Full-factorial design, HPLC-UV/VIS, toxicity, anti-inflammatory, antioxidant activity.

INTRODUCTION

In recent years, there has been a growing interest in natural bioactive compounds, largely driven by increasing concerns about the side effects of synthetic drugs and the alarming rise in microbial resistance to conventional treatments¹. Owing to their pharmacological potential, medicinal plants, long central to traditional healing practices, have become a major focus of contemporary scientific research. This interest is primarily attributed to their active constituents, which are capable of exerting specific physiological effects. Among these constituents, alkaloids, flavonoids, tannins, and phenolic compounds represent the most prominent classes of bioactive molecules, many of which are found in species of the *Calicotome* genus²⁻³.

Calicotome spinosa (L.) Link., a spiny shrub belonging to the Fabaceae family, is native to the Mediterranean region and grows widely throughout the Algerian Tell, from Dahra to the Aurès Mountains⁴. In Algerian traditional medicine, this species has been used to treat external conditions such as edema, swelling, and urinary retention, with the leaves being particularly employed to promote wound healing⁵. Given these traditional uses, especially in conditions associated with inflammation and tissue damage, the investigation

of the antioxidant and anti-inflammatory properties of *C. spinosa* appears particularly relevant. Previous studies have mainly focused on the phytochemical composition and biological activities of extracts obtained using conventional extraction procedures. Although several studies have reported the phytochemical composition and antioxidant activity of this species, information regarding the optimization of extraction processes and the biological validation of extracts obtained under optimized conditions remains limited.

Extraction techniques play a crucial role in determining both the efficiency and quality of bioactive compound recovery from plant materials. Among the various approaches developed in recent years, ultrasound-assisted extraction (UAE) has emerged as an efficient and environmentally friendly technique. UAE enhances the diffusion of phytochemicals from the plant matrix into the solvent by improving solvent penetration and inducing cellular disruption⁶⁻⁷. Additional advantages of this method include reduced solvent consumption, shorter extraction times, operational simplicity, and suitability for both laboratory- and industrial-scale applications^{6,8}.

Nevertheless, no single extraction technique is universally effective for the recovery of all phenolic

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compounds, as extraction efficiency is influenced by several factors, including plant matrix characteristics, the nature of the target phenolics, and the solvent system used⁹. Consequently, optimization of extraction parameters is essential to maximize both extraction yield and bioactive compound recovery.

In this regard, factorial experimental designs provide powerful tools for process optimization. A full-factorial design enables the evaluation of multiple variables and their interactions, allowing the development of predictive models to assess the effects of individual and combined factors on extraction responses^{10–11}. Because only two extraction variables were investigated in the present study (ethanol concentration and sonication time), a full-factorial design was considered appropriate to evaluate their individual and interactive effects while keeping the experimental design simple and efficient. Response surface methodology is often used to facilitate the interpretation and visualization of experimental design outcomes¹².

Despite the growing interest in *C. spinosa*, little attention has been paid to the optimization of phenolic compound extraction using ultrasound-assisted techniques. Since extraction conditions strongly influence both phytochemical recovery and biological activity, the development of optimized extraction procedures is essential for the efficient valorization of this medicinal plant. Furthermore, data concerning the anti-inflammatory activity of extracts obtained under optimized extraction conditions remain scarce. Therefore, combining extraction optimization with biological evaluation may provide a more comprehensive assessment of the therapeutic potential of *C. spinosa*.

Within this framework, the present study aims to optimize the extraction of total phenolic compounds (TPC) from *C. spinosa* leaves using ultrasound-assisted extraction. A full-factorial design was applied to investigate the effects of solvent concentration and sonication time on extraction yield and TPC content. The optimized extract was subsequently characterized by HPLC–UV/VIS analysis and evaluated for its antioxidant activity, acute oral toxicity, and anti-inflammatory potential. Unlike previous studies that investigated either the phytochemical composition or the biological activities of *C. spinosa* extracts, the present work integrates extraction optimization, HPLC characterization, and biological evaluation of the optimized extract within a single experimental approach. To the best of our knowledge, this is the first study to optimize the ultrasound-assisted extraction of phenolic compounds from *C. spinosa* leaves using a full-factorial experimental design and to evaluate the biological properties of the optimized extract in conjunction with HPLC characterization. This integrative approach provides scientific support for the traditional medicinal use of the plant and highlights its potential as a source of natural therapeutic agents.

MATERIALS AND METHODS

Chemicals and Materials

All the deuterated chemical products such as Ethanol, DPPH (1,1-diphenyl-2-picrylhydrazyl), were from Sigma-Aldrich (Germany), whereas Diclofenac pure from SAIDAL (Algeria), Folin- Ciocalteu reagent was obtained from Merck (Germany). All chemicals and solvents used in the study were of analytical grade procured.

Plant Sampling

The plant material used in this study consisted of *Calicotome spinosa* (L.) Link leaves, collected from its natural habitat in Aomar (Bouira, Algeria), located at 36°29'33"N and 3°46'16"W, at an average altitude of approximately 438 m. The plant was taxonomically authenticated at the Herbarium of the Department of Botany, National Higher School of Agronomy, Algeria. After collection, the material was dried in the dark at room temperature (Figure 1), then ground using a mechanical

grinder, and the resulting powder was stored under appropriate conditions until further use.

Animal

Male albino mice weighing 23 ± 2 g were obtained from the pharmaceutical group company SAIDAL (Algiers, Algeria) and kept under controlled conditions (20–24 °C, 12 h light/12 h dark cycle) with free access to water and standard diet food, and were allowed to acclimatize for one week prior to experimentation. The experiments on animals were conducted in accordance with the guidelines for the care and uses of laboratory animals published by the US National Institute of Health (NIH publication N° 85-23, revised 1985) and were approved by the SAIDAL ethics committee.

Optimization of extraction procedure

Selection of Solvent for enhancing extraction of total phenolic compounds: A preliminary experiment was carried out to compare the efficiency of different solvent-water mixtures (80:20, v/v) namely methanol, ethanol, and glycerol in extracting total phenolic compounds (TPC) from *C. spinosa* leaves. Each solvent was tested under identical extraction conditions, using 10 g of ground leaves were macerated for 120 min at 30°C. The resulting extracts were evaluated for their total phenolic content using the Folin-Ciocalteu method described below.

Full-factorial design: To explore and optimize the extraction parameters influencing the recovery of TPC from *C. spinosa* leaves, a full-factorial design (FFD) was applied to evaluate both the individual and interaction effects of two independent factors: the ethanol/water ratio (% v/v, X1) and the sonication time (X2). These two variables were selected because they are among the main factors affecting the extraction efficiency of phenolic compounds during ultrasound-assisted extraction. Since only two independent variables were investigated, a full-factorial design was considered the most appropriate experimental approach to evaluate their individual and interaction effects in a simple and efficient manner. Other extraction parameters, including temperature (30 °C), solid-to-solvent ratio (1:20, w/v), ultrasound power (150 W), frequency (35 kHz), and particle size, were kept constant according to previously reported extraction conditions in order to evaluate only the effects of the selected variables. The effects of the factors were assessed on two responses: extraction yield (Y1), expressed as the mass percentage of dry extract, and total phenolic content (Y2), determined using the Folin-Ciocalteu method. Each factor was studied at three levels, and the central point of the design was replicated twice, resulting in a total of ten experimental conditions, as presented in Table 1.

ANOVA and multiple linear regression were used to analyze the experimental data. The adequacy and reliability of the model were evaluated using the coefficient of determination (R^2), adjusted R^2 , F-value, p-values, and the lack-of-fit test. All statistical analyses were performed using JMP® software version 18.2.2.

The optimal extraction conditions were determined using the desirability function with the objective of simultaneously maximizing extraction yield and total phenolic content (TPC). Response surface plots were generated to illustrate the effects of the extraction variables on the studied responses, and actual versus predicted plots were used to assess the predictive performance of the model.

Table 1. Coded and real values of the extraction parameters levels used in the FFD

Factor	Unit	-1	0	+1
X1: Ethanol-water ratio	% (v/v)	60/40	80/20	100
X2: Sonication time	Min	20	60	100



Figure 1. Dried parts of *C. spinosa* leaves.

To validate the predictive ability of the optimization model, an additional extraction experiment was performed under the predicted optimal conditions (60% ethanol and 20 min of sonication). The extraction was carried out under the same experimental conditions described above, and the experimental extraction yield and total phenolic content were compared with the corresponding values predicted by the model.

Extraction procedure of TPC: Six grams of *C. spinosa* leaf powder were extracted according to the previously described conditions. The powder was mixed with an aqueous ethanol at a constant solid-to-solvent ratio of 1:20 (w/v). The extraction was performed in an ultrasonic bath (BANDELIN Sonorex Digitec, 150 W, Germany) at 30 °C with a constant frequency of 35 kHz. After filtration, the extract was concentrated using a rotary evaporator at 40°C until a dry crude extract was obtained. The resulting extracts were then stored at 4°C for subsequent analyses. Extraction yields were calculated according to the formula (1).

$$\text{Yield \%} = \frac{\text{dried extract (g)}}{\text{leaves powder (g)}} \times 100 \dots \dots \dots (1).$$

Determination of total phenolic content: The total phenolic content of *C. spinosa* extract was determined using the Folin–Ciocalteu method, as described by Siddiqui et al., 2017¹³. Aliquots of 1 mL from the extract solutions (1 mg/mL in methanol) and gallic acid standard solutions (50, 100, 150, 200, 250, and 300 µg/mL) were transferred separately into test tubes containing 450 µL of distilled water and thoroughly mixed. Subsequently, 5 mL of Folin–Ciocalteu reagent, previously diluted tenfold with distilled water (1:10, v/v), was added to each tube. After 3 min of incubation, 4.0 mL of sodium carbonate solution (7.5%, w/v) was added, and the reaction mixtures were thoroughly homogenized. The prepared solutions were then incubated in the dark for 30 min at room temperature to allow color development. Absorbance was measured at 765 nm using a UV–visible spectrophotometer, with methanol used as the blank. Results were expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g). The calibration curve was defined by the equation $y = 0.0049x - 0.023$, with a coefficient of determination (R^2) of 0.997.

Characterization and Bioactivity Assessment of TPC optimal extract

Determination of total flavonoids content: The total flavonoid content of *C. spinosa* extract was determined using quercetin as a standard, according to the method described by Ghedadba et al. 2017¹⁴. A 1 mL aliquot of sample and standard (prepared in methanol) was mixed with 1 mL of aluminum chloride solution (AlCl₃, 2% in methanol). After 10

minutes of incubation, the absorbance was measured against a reagent blank at $\lambda_{\text{max}} = 430$ nm. Flavonoid concentrations were determined using a calibration curve ($y = 0.0062x - 0.0519$, $R^2 = 0.994$), constructed with quercetin as the reference standard (6,5 µg/mL, 12,5 µg/mL, 25 µg/mL et 50 µg/mL). The results were expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g).

HPLC UV/VIS analysis of phenolic compounds: HPLC analysis was performed for qualitative identification of major phenolic compounds by comparing retention times and spectra with authentic standards. HPLC analysis was performed using an Agilent 1100 HPLC system equipped with a UV–Vis detector. A volume of 20 µL of the extract, previously dissolved in methanol (MeOH), was injected. Chromatographic separation was achieved on a reversed-phase KNAUER C18 column (250 mm × 4.6 mm, 5 µm) at room temperature. The mobile phase consisted of 1% (v/v) acetic acid in water (solvent A) and methanol (solvent B), using a gradient elution program as described in Table 2. The flow rate was maintained at 1.0 mL/min, and detection was carried out at 254 nm.

Phenolic compounds were identified by comparison of their retention times with those of authentic reference standards analyzed under the same chromatographic conditions.

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay: The DPPH radical scavenging activity of *C. spinosa* leaf extract was evaluated using the method detail by Thakral et al., 2010¹⁵. Briefly, 1 mL of the hydroalcoholic extract of *C. spinosa* (10–100 µg/mL, prepared in absolute ethanol) was mixed with 1 mL of a freshly prepared ethanolic DPPH solution (0.1 mM). The reaction mixture was incubated in the dark at room temperature for 30 min, after which the absorbance was measured at 517 nm using a spectrophotometer (Shimadzu UV-1800, Japan). Ascorbic acid was used as a positive control. For the negative control, 1 mL of ethanol was mixed with 1 mL of DPPH solution. The percentage of DPPH radical inhibition was calculated according to Equation (2).

$$\text{DPPH radical scavenging activity (\%)} = [(Ac - As)/Ac] \times 100] \dots \dots \dots (2).$$

where Ac represents the absorbance of the control, and As represents the absorbance of the sample.

Toxicity test: Acute oral toxicity of the plant extract was evaluated in accordance with the OECD guideline 423¹⁶. A group of three mice was used for the study. The animals were fasted for 12 h prior to the experiment, with free access to water. The test dose was selected based on bibliographic data indicating a low probability of toxicity. Accordingly, a single oral dose of 2000 mg/kg body weight was administered to each mouse. The plant extract was dissolved in distilled water, and each animal received a total volume of 0.5 mL by oral gavage. Animals were then observed during 14 days for signs of toxicity and mortality.

Anti-inflammatory activity: The anti-inflammatory activity of *C. spinosa* leaf extract was evaluated using the carrageenan-induced hind paw edema model, as previously described by Levy et al., 1969¹⁷. Mice were randomly divided into three experimental groups (n = 5 per group). The first group received 0.5 mL of distilled water orally and served as the negative control. The second group was treated orally

Table 2. HPLC gradient elution

Time (min)	A (%)	B (%)
0	95	5
55	5	95
57	95	5
59	95	5

with diclofenac (75 mg/kg) as the positive control, while the third group received the *C. spinosa* leaf extract at a dose of 200 mg/kg body weight (0.5 mL).

Thirty minutes after treatment, acute inflammation was induced by subplantar injection of 25 μ L of carrageenan solution (1%, w/v) into the left hind paw of each mouse. The right hind paw, which did not receive carrageenan injection, was considered as the internal control. Four hours after inflammatory induction, the mice were sacrificed by cervical dislocation. The procedure was carried out by a veterinarian trained in the handling and care of laboratory animals. Both hind paws were excised at the same anatomical level and immediately weighed using an analytical balance. Paw edema (E %) was calculated as the percentage increase in paw weight of each mouse using the following formula 3:

$$E\% = [(Left\ paw - Right\ paw) / Right\ paw] \times 100 \dots (3).$$

Anti-inflammatory activity was expressed as the percentage of edema inhibition (EI %) in treated mice relative to the control group, calculated according to the formula 4:

$$Inhibitory\ (\%) = (Control - Treated) / Control \times 100 \dots (4).$$

Data Analysis

Statistical analyses were performed using one-way ANOVA followed by Tukey's test. The results of the triple experiments are shown as mean $\pm$ SD for descriptive values. All data of anti-inflammatory assay are expressed mean $\pm$ SE for comparisons among mouse groups. A *p*-value < 0.05 was considered statistically significant.

RESULTS

Optimization study

Solvent effect on Total Phenolic Compounds: A preliminary solvent screening was performed to identify the most suitable solvent for the extraction of phenolic compounds from *C. spinosa* leaves prior to the optimization study. Three solvents with different polarities: methanol, ethanol, and glycerol were evaluated. The results showed that ethanol yielded the highest total phenolic content (TPC), with a value of 32.62 ± 2.61 mg GAE/g of dry extract, followed by methanol (16.84 ± 2.91 mg GAE/g) and glycerol (11.40 ± 0.65 mg GAE/g) (Figure 2).

Analysis of the result of full-factorial design: The full factorial design (3^2) was employed to evaluate the effects of two independent variables, the ethanol-water ratio (X_1) and sonication time (X_2), on two response variables: extraction yield (Y_1 , % dry extract) and total phenolic content (Y_2 , mg GAE/g dry extract). The primary objective was to determine the optimal extraction conditions that maximize both responses. The experimental results are presented in Table 3.

Both extraction yield and TPC varied significantly as a function of the experimental conditions. As shown in Table 3, extraction yields ranged from 10.33% to 24.90%, while TPC values varied between 20.57 and 66.90 mg GAE/g.

The highest extraction yield (24.29%) and the maximum TPC (66.90 mg GAE/g) were simultaneously obtained under moderate extraction conditions, specifically with 60% ethanol and a sonication time of 20 min (Run 8). In contrast, the lowest yields and TPC values were generally observed when using absolute ethanol (100%) combined with longer sonication times, indicating that harsher conditions negatively affected extraction efficiency.

The center-point experiments (Runs 4 and 10) showed comparable responses, suggesting good experimental reproducibility and minimal experimental error.

Effect on extraction yield (Y_1): The ANOVA results for Y_1 indicated a good model fit, with a coefficient of determination (R^2) of 0.88 and an adjusted R^2 of 0.83, as well as a non-significant lack of fit ($p = 0.3365$) (Figure 2a). Among the studied factors, the ethanol-water ratio (X_1) had a statistically significant effect on extraction yield ($p = 0.0005$), whereas sonication time (X_2) and the interaction between the two factors did not show significant effects (Table 4). The negative regression coefficient of X_1 (-5.07) indicates that increasing the ethanol proportion in the solvent significantly reduced the extraction yield (Y_1). Furthermore, the Studentized residuals were randomly and evenly distributed, suggesting the absence of systematic bias and confirming the adequacy of the model.

Effect on TPC (Y_2): The regression model for TPC exhibited strong predictive performance, with a coefficient of determination (R^2) of 0.92 and an adjusted R^2 of 0.88 (Figure 2b), indicating a good correlation between the independent variables and the response. According to the ANOVA results (Table 4), both the ethanol-water ratio (X_1 , $p =$

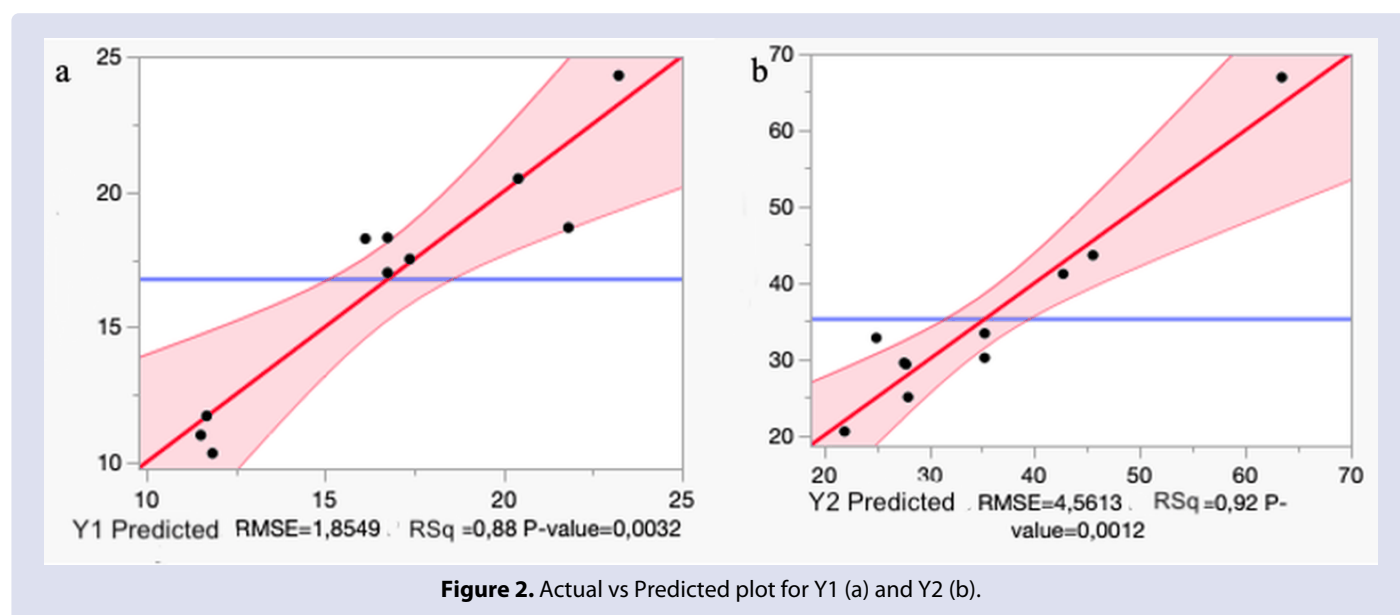


Figure 2. Actual vs Predicted plot for Y_1 (a) and Y_2 (b).

Table 3. Experimental Variables and Measured Responses of the 3² Full-factorial design.

Run	Factors		Responses	
	X1	X2	Y1 (%)	Y2 (mg EAG/g)
1	60	100	20.48	29.55
2	100	60	11.71	32.81
3	100	100	10.33	25.06
4*	80	60	17	33.4
5	100	20	11	20.57
6	80	100	18.26	29.34
7	80	20	17.51	41.18
8	60	20	24.29	66.9
9	60	60	18.67	43.63
10*	80	60	18.3	30.2

*duplicate conditions at the center point of the experimental design.

Table 4. Analysis of the effects of factors on extraction yield (Y1) and TPC (Y2).

Source	Y1					Y2				
	Df	Sum of Sq	Mean of Sq	F value	P value	Df	Sum of Sq	Mean of Sq	F value	P value
Model	3	158.810	52.936	15.386	0.0032	3	1403.909	467.970	22.493	0.0012**
X1	1	154.026	154.026	44.767	0.0005**	1	633.248	633.248	30.436	0.0015**
X2	1	2.318	2.318	0.674	0.4431 ^{ns}	1	333.015	333.015	16.005	0.0071**
X1X2	1	2.464	2.464	0.716	0.4298 ^{ns}	1	437.646	437.646	21.034	0.0037**
Lac of fit	5	19.798	3.959	4.686	0.3365	5	119.715	23.943	4.676	0.3368
Pure error	1	0.845	0.845			1	5.120	5.120		
Cor. Total	9	179.453				9	1528.74			

ns : Non significant ($p > 0.05$) *: significant ($p < 0.05$) **: Highly significant ($p \leq 0.01$). Df: Degrees of freedom. Sq: squares. Cor.: corrected.

0.0015) and sonication time (X2, $p = 0.0071$) had statistically significant effects on TPC. In addition, the interaction between the two factors (X1X2) was also significant ($p = 0.0037$), highlighting the combined influence of solvent composition and sonication duration on phenolic extraction.

The negative regression coefficients associated with X1 (−10.27) and X2 (−7.45) indicate that increasing either factor individually led to a decrease in TPC. Conversely, the positive coefficient of the interaction term (+10.46) suggests a synergistic effect under specific combinations of ethanol concentration and sonication time, which enhanced phenolic recovery. The non-significant lack of fit ($p = 0.3368$) further confirms the adequacy and reliability of the proposed model.

Optimization and predicted responses: Optimization was carried out using a multi-response desirability function with the objective of simultaneously maximizing extraction yield and TPC. The response surface plots presented in Figure 3a and 3b illustrate the combined effects of sonication time and ethanol concentration on both responses. The curvature observed in both response surfaces indicates that these parameters exert a significant influence on the extraction process.

As shown in Figure 3a, extraction yield decreased with increasing ethanol concentration, particularly when combined with longer sonication times. The highest yield was obtained under moderate extraction conditions (60% ethanol and 20 min of sonication), suggesting that excessive ethanol concentration or prolonged sonication may adversely affect extraction efficiency.

Similarly, the TPC response surface (Figure 3b) confirmed the strong influence of both factors. Maximum phenolic content was achieved at 60% ethanol and a sonication time of 20 min, corresponding to the conditions that also maximized extraction yield. A pronounced decline in TPC was observed with increasing ethanol concentration, and this decrease was more marked than that observed for extraction yield.

The desirability function identified the optimal extraction conditions as 60% ethanol and 20 min of sonication, with an overall desirability

of 98.6%. Under these conditions, the model predicted an extraction yield of 23.23% and a TPC of 63.44 mg GAE/g (Table 5). To verify the predictive ability of the model, an additional extraction was performed under the predicted optimal conditions. The experimental results showed good agreement with the predicted values (Table 5), confirming the reliability of the optimization model.

Study of phenolic extract

Total flavonoids Content: The total flavonoid content of the optimized extract was determined using the aluminum chloride (AlCl₃) colorimetric method, with quercetin as the reference standard. The results showed that the extract contained 36.95 ± 0.96 mg QE/g.

HPLC UV/VIS analysis: HPLC analysis of *C. spinosa* leaf extract revealed the presence of seven distinct compounds. The corresponding chromatogram is shown in Figure 4. Identification of the constituents was achieved by comparing the retention times of peaks detected in the extract with those of reference standards. Seven compounds were successfully identified among the detected peaks, including quercetin (Rt = 32.34 min), apigenin (Rt = 35.87 min), 2,3,4-trihydroxyflavone (Rt = 29.28 min), robinin (Rt = 27.14 min), kaempferol (Rt = 41.12 min), catechin (Rt = 34.01 min), and ascorbic acid (Rt = 2.87 min).

DPPH scavenging activity of total phenolic extract: The antioxidant activity of *C. spinosa* leaf extract was evaluated using the DPPH radical scavenging assay. The results showed that the leaf extract exhibited a moderate antioxidant activity, with an IC₅₀ value of 75.48 ± 5.04 µg/mL, compared to ascorbic acid, which showed a significantly stronger activity with an IC₅₀ of 26.87 ± 7.03 µg/mL. The percentage of DPPH radical inhibition increased in a concentration-dependent manner for both the total polyphenolic extract and ascorbic acid. At a concentration of 100 µg/mL, the total polyphenolic extract achieved 65.76 ± 0.28 % inhibition, whereas ascorbic acid reached 86.11 ± 0.01 % inhibition (Figure 5).

Toxicity test of leaves extract: The objective of this study was to evaluate the acute oral toxicity of *C. spinosa* leaves extract. Following an oral

Table 5. Predicted responses obtained from the optimization profiler

Response	Predicted Value*	Experimental value	Unit
Yield (Y1)	23.23	21.88±1.38	%
Total Phenolic Content (Y2)	63.44	65±2.68	mg GAE/g

*Under these conditions: 60% ethanol and 20 min of sonication.

Table 6. Anti-inflammatory effect of *C. spinosa* extract in carrageenan-induced paw edema in mice.

Solutions	Dose (mg/Kg)	%E ^a	%EI
Control – (water)	-	33.79 ±3.02	-
Leaf extract	200	23.07± 2.56*	31.72
Diclofenac (pure)	75	15.85±1.03*	53.09

^aValues are Mean ± SE; (n = 5); *p<0.05, compared to control. E: edema percentage. EI : edema inhibition.

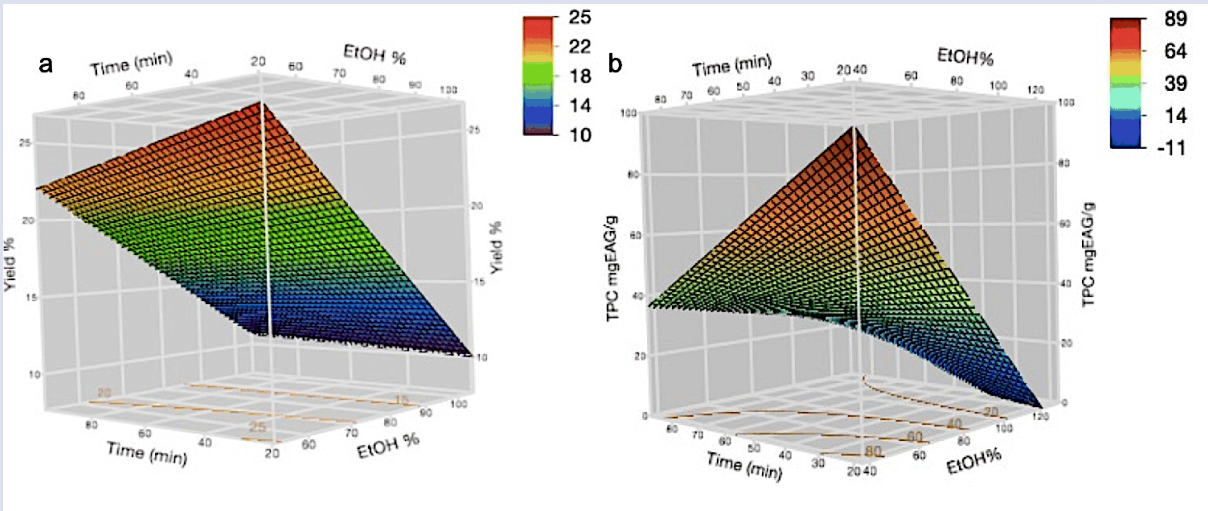


Figure 3. Response surfaces of extraction yield (a) and TPC (b).

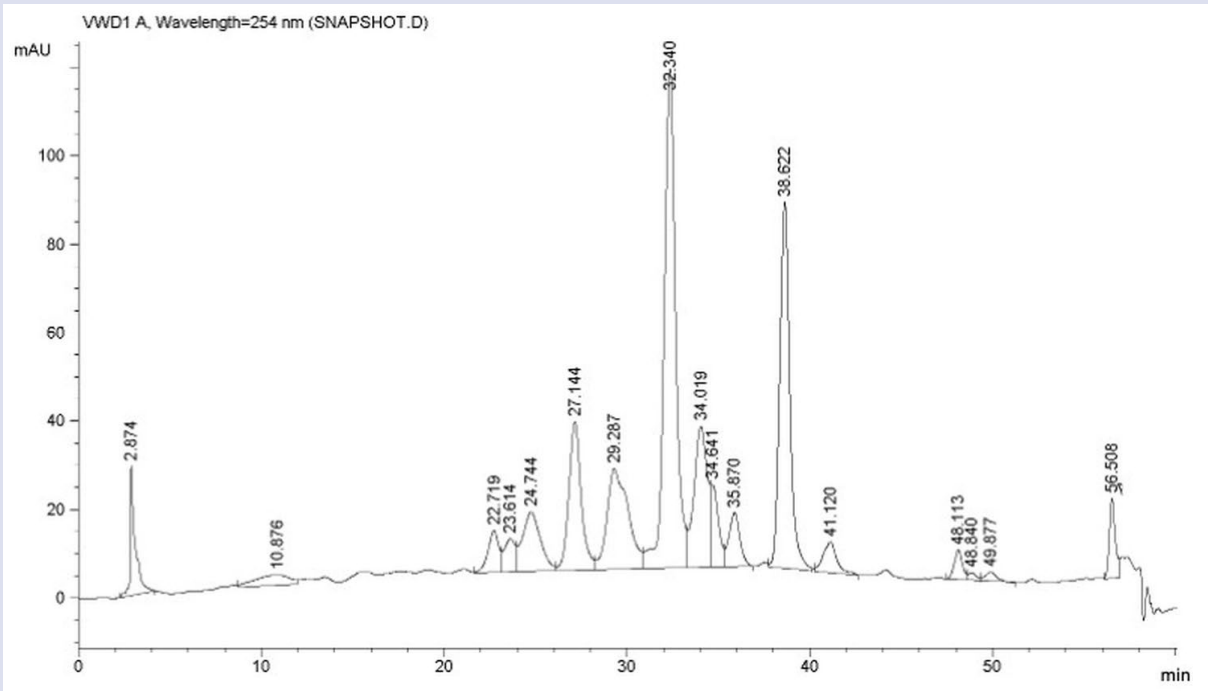


Figure 4. HPLC chromatogram at 254 nm of leaf extract of *C. spinosa*.

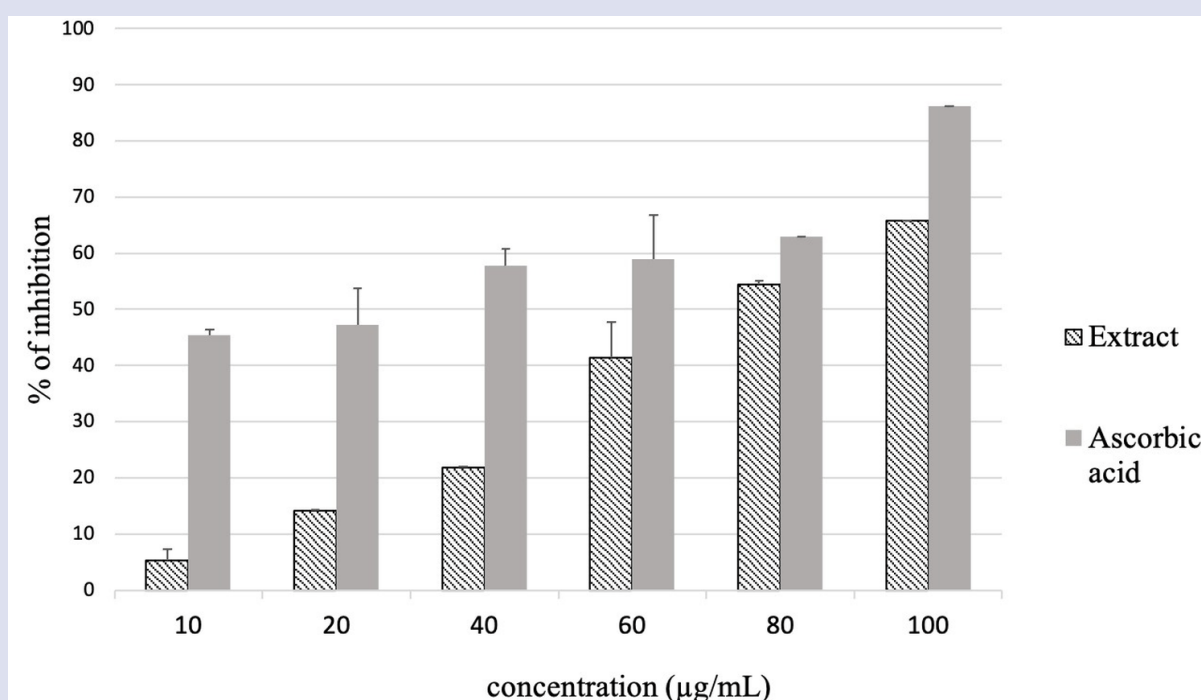


Figure 5. DPPH radical scavenging activities of various concentrations of *C. spinosa* leaf ethanolic extract and positive control (Ascorbic acid).

administration at a dose of 2000 mg/kg, none of the treated animals showed a sign of toxicity. Throughout the 14 days observation period, all animals still alive, with normal behavior and no visible adverse effects. In comparison to the control group, the mice's morphological parameters of were normal.

Anti-inflammatory effect of the compound: The anti-inflammatory activity of the phenolic extract from *C. spinosa* leaves in the carrageenan-induced paw edema model in mice is summarized in table 6. The results were recorded four hours after carrageenan injection. A significant reduction in paw edema was observed following administration of the extract at a dose of 200 mg/kg, resulting in an inhibition rate of 31.72% ($p=0.001$).

DISCUSSION

Extraction is a critical step in phenolic compound recovery, and its efficiency depends on several parameters, including solvent type, extraction time, temperature, particle size, and solvent polarity⁶. Solvent selection is particularly important, as phenolic solubility is strongly influenced by polarity²⁰. In this study, we used methanol, a solvent widely employed for TPC extraction but associated with potential health hazards upon exposure, alongside two green solvents: ethanol and glycerol, both of which are approved for use in pharmaceutical formulations²¹⁻²⁴. Recent studies have shown that differences in solvent composition, extraction techniques, harvesting season, and environmental factors can significantly affect phenolic yields. Ethanol is increasingly preferred due to its GRAS status and suitability for food and pharmaceutical applications^{22,25-28}. Based on our results, ethanol was selected as the extraction solvent for the subsequent optimization process using a full factorial design.

Full-factorial design (FFD) experiments demonstrated that higher ethanol concentrations (80–100%) resulted in lower extraction yield and TPC. This observation aligns with previous studies showing that highly concentrated ethanol exhibits reduced polarity, limiting the solubility of hydrophilic phenolics²⁹⁻³¹. In contrast, aqueous ethanol mixtures

(50–70%) offer intermediate polarity, facilitating the extraction of both polar and moderately non-polar phenolic compounds while improving solvent penetration into plant matrices^{22,31-32}. These results are consistent with recent reports identifying 60% ethanol as an optimal solvent for phenolic recovery in various plant species, including *Oryza sativa*, *Bougainvillea × buttiana*, and rosemary residues^{30-31,33}. Ultrasound-assisted extraction (UAE) significantly enhanced phenolic recovery by increasing cell wall permeability through acoustic cavitation^{6,29-30}. A sonication time of 20 min yielded the highest extraction efficiency (Yield = 24.29%, TPC = 66.9 mg GAE/g), corroborating previous findings that short UAE durations maximize extraction while minimizing compound degradation^{30,33-35}. Prolonged sonication did not further improve yields, likely due to oxidative or thermal degradation of phenolics once equilibrium was reached³⁵.

The optimal extract had flavonoid content of 36.94 ± 0.96 mg QE/g, surpassing the values reported for *C. spinosa* extracts obtained using conventional methods¹⁸⁻¹⁹. Such differences may result from variations in extraction methods, solvent polarity, plant origin, and environmental or physiological factors affecting secondary metabolite biosynthesis³⁶⁻³⁸.

High-performance liquid chromatography (HPLC) analysis of the extract allowed the identification of seven key phytochemicals: quercetin, apigenin, 2,3,4-trihydroxyflavone, robinin, ascorbic acid, catechin, and kaempferol. While these target molecules were unambiguously confirmed using authentic reference standards, high-resolution LC-MS/MS profiling remains a valuable perspective for future non-targeted metabolomic exploration. Similar phenolic profiles have been reported across *Fabaceae* species, where such compounds are strongly associated with antioxidant, anti-inflammatory, and antimicrobial activities³⁹⁻⁴².

The DPPH antioxidant assay showed moderate but significant radical-scavenging capacity, with an IC_{50} of 75.48 ± 5.04 µg/mL. Although less potent than pure ascorbic acid ($IC_{50} = 26.87 \pm 7.03$ µg/mL), the extract achieved 65.73% inhibition at 100 µg/mL, suggesting synergistic interactions among its phenolic constituents. Similar correlations

between phenolic compounds and antioxidant activity have been widely reported in numerous plant species. These compounds, including flavonoids and tannins, possess redox properties that enable them to scavenge free radicals, reduce oxidative stress, and contribute to the overall antioxidant activity of plant extracts^{42–44}. Among the compounds identified in the present study, quercetin, catechin, and kaempferol are known for their strong antioxidant properties. They can neutralize free radicals, chelate metal ions, and reduce the formation of reactive oxygen species, which may contribute to the antioxidant activity observed in the optimized extract^{45–46}. The antioxidant efficiency of phenolic compounds largely depends on their molecular structure, particularly the number and arrangement of hydroxyl groups, which influence their hydrogen- or electron-donating ability⁴⁷.

The absence of toxicity in acute tests supports the potential use of the extract in nutraceutical or phytopharmaceutical applications. According to the OECD Guideline 423, these findings indicate that the median lethal dose (LD₅₀) of extract is greater than 2000 mg/kg, suggesting a low level of acute oral toxicity¹⁶. Overall, the results support the safety of oral administration of *C. spinosa* leaves extracts.

In vivo anti-inflammatory evaluation demonstrated that the extract reduced carrageenan-induced paw edema by 31.72% at 200 mg/kg, compared with 53.09% for diclofenac. Apigenin, which was identified in the *C. spinosa* extract, is well known for its potent anti-inflammatory effects through inhibition of prostaglandin E₂ synthesis and the downregulation of pro-inflammatory cytokines such as interleukin-1 β and tumor necrosis factor- α . Similarly, catechin, another bioactive constituent detected in the extract, has been reported to suppress pro-inflammatory signaling pathways including NF- κ B and MAPK^{48–49}. In addition, Li et al. (2019) suggested that catechin and quercetin may act synergistically to enhance anti-inflammatory activity⁴⁹. Although direct quantification of serum cytokines was beyond the scope of this baseline *in vivo* evaluation, these physiological findings lay a solid foundation for future molecular mechanism studies. While the effect of the crude extract was expectedly less pronounced than that of pure diclofenac, an inhibition exceeding 30% remains highly relevant pharmacologically and provides strong scientific support for the traditional use of *C. spinosa* in managing inflammatory conditions."

CONCLUSIONS

This study demonstrates that *Calicotome spinosa* leaves constitute a promising source of bioactive phenolic compounds. Ultrasound-assisted extraction optimized at 60% ethanol for 20 min yielded an extract with high phenolic content alongside noteworthy antioxidant and *in vivo* anti-inflammatory activities. Furthermore, the acute oral toxicity evaluation showed no signs of toxicity, indicating a favorable preliminary safety profile. Nevertheless, further investigations including the isolation and precise quantification of major bioactive markers, elucidation of the underlying molecular pathways, and long-term toxicity evaluations remain essential to fully confirm the therapeutic potential and safety of this extract."

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