Scientiae Radices 5(3) 130-139 (2026)

Full Paper

Preliminary Phytochemical Characterization and Antibacterial Activity of Capparis spinosa L. Leaf Extract with LC-MS-Based Metabolite Profiling

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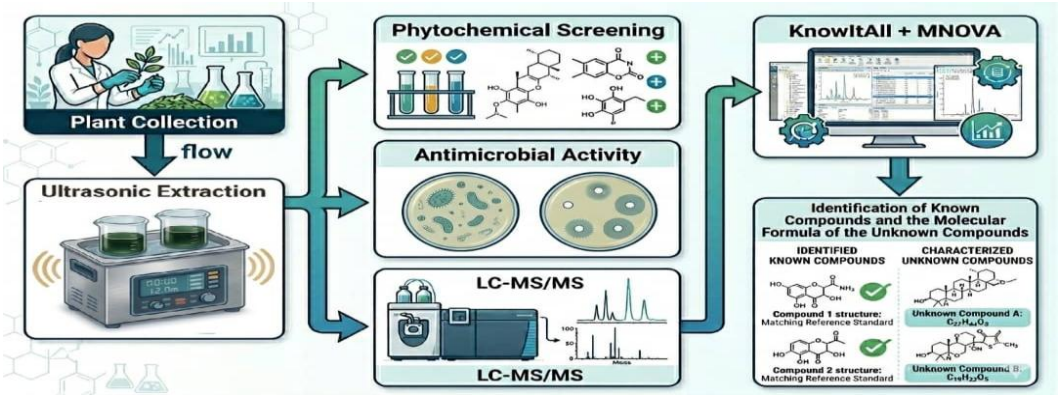
Abstract

Capparis spinosa L. plant metabolites were extracted using ultrasonic wave and prepared for metabolism analysis by liquid chromatography-mass spectrometry (LC-MS/MS). The analysis revealed many phytochemicals such as flavonoids, phenolics, glycosides, tannins, saponins and terpenoids; however, no alkaloids were demonstrated in the extract. The antimicrobial activity of the C. spinosa extract was evaluated using the disc diffusion method against four bacterial species: Escherichia coli (ATCC 8739), Pseudomonas aeruginosa (ATCC 27853), Staphylococcus aureus (ATCC 6538), and Enterococcus faecalis (ATCC 29212). Growth inhibition was observed only for E. coli. The LC-MS/MS metabolomics approach permitted the identification of 6 high-confidence metabolites and 33 possible (tentative) metabolites based on spectral matching from the C. spinosa extract. The most abundant classes of metabolites detected were organic acids, amino acids, and phenolic compounds, which agreed with the phytochemical screening. The low antimicrobial activity of the extract was most probably associated with the extraction conditions and metabolite specificity.

Keywords Capparis spinosa L., ultrasound-assisted extraction, LC–MS/MS, phytochemical screening, antibacterial activity, disc diffusion, metabolites, natural products.



Received: 2026.04.25
Accepted: 2026.08.02
Published: 2026.08.11
DOI: 10.58332/scirad2026v5i3a01

ISSN: 2956-4808
DOI: 10.58332/scirad2026v5i3a01

1. Introduction

The use of medicinal plants is an enormous resource for bioactive agents (compounds) that can have therapeutic uses, including the discovery of agents with antimicrobial properties [1]. The plant Capparis spinosa L. (also called the caper) has been investigated more than many other medicinal plants because of the high number of active phytochemicals, or phytochemicals with a long or traditional history of use in folk medicine [2]. Capers have a large variety of active phytochemicals that consist of flavonoids, phenolic acids, alkaloids and glycosides and have been shown to exert pharmacological effects due to the pharmacologically active compounds present in their phytochemicals (both flavonoid and glycoside) [3]. Phytochemical screening is a critical tool to identify bioactive constituents contained in extracts from a plant and to provide information about whether or not there are biological activities present in an extract of a given plant [4]. Many of these bioactive compounds have demonstrated capabilities to exert antioxidant, anti-inflammatory and antimicrobial functions/characteristics and are therefore excellent candidates for further research [5]; however, there can be a large range of different levels of bioactive compounds present in a plant sample depending on factors such as geographic origin, part of the plant that is used, and the method of extraction that is utilized to isolate the bioactive compounds [6]. In the last several years, there has been considerable interest in using ultrasound-assisted extraction to effectively isolate phytochemical compounds from plant materials because this method of extraction increases the yield of extracted phytochemicals compared with traditional extraction methods and allows the extracted phytochemicals to retain their chemical and physical properties [7]. Moreover, recent developments in liquid chromatography/mass spectrometry (LC-MS/MS) technology have allowed a much greater degree of separation and characterization of complex mixtures of metabolites found within a given plant (a metabolome) [8]. The integration of spectral databases and software tools such as KnowItAll and MNOVA allows for both automated and manual validation of compound identification, improving the accuracy of metabolite profiling [9]. Despite the reported medicinal value of C. spinosa, studies on its antimicrobial activity have shown inconsistent results, likely due to variations in extraction procedures and experimental conditions [10]. Therefore, a comprehensive investigation combining phytochemical screening, antimicrobial evaluation, and LC-MS-based metabolite profiling is necessary to better understand its biological potential. This study aims to characterize the phytochemical composition of C. spinosa L. leaf extract, evaluate its antibacterial activity against selected pathogenic strains, and identify its chemical constituents using LC-MS/MS analysis supported by advanced software tools.

2. Results and discussion

2.1 Preliminary phytochemical screening

Preliminary phytochemical analysis of the hydro-ethanolic extract of C. spinosa L. leaves indicated the presence of various secondary metabolites, including flavonoids, cardiac glycosides, phenols, and tannins.

Table 1. Phytochemical screening of C. spinosa L. extract.

Phytochemical Compounds	Result
Alkaloids	–
Flavonoids	+
Phenols	+
Terpenoids	+
Glycosides	+
Tannins	+
Saponins	+

Phytochemical screening of the C. spinosa L. extract revealed the presence of phenols, flavonoids, glycosides, tannins, saponins, and terpenoids (Table 1). Alkaloids were not detected. These findings partially align with those of [11], who reported the presence of polyphenols, flavonoids, and glycosides; however, in contrast to our extract, they reported the absence of alkaloids, tannins, saponins, terpenoids, and steroids in C. spinosa L. leaf extracts from Iraq. Conversely, [12] identified alkaloids, flavonoids, steroids, glycosides, carbohydrates, tannins, phenolic compounds, and saponins in methanolic fruit extracts of C. spinosa L., with terpenes absent. The discrepancies between the current study and that of [12] may be attributed to differences in plant parts (leaves vs. fruit) and extraction methods used, as well as the geographical location.

2.2 Antibacterial activity

ISSN: 2956-4808
DOI: 10.58332/scirad2026v5i3a01

The inhibition zone diameters resulting from the antibacterial assay of C. spinosa L. extract (Fig. 1) against various pathogenic bacterial strains are summarized in Table 2.

Table 2. Antibacterial activity of C. spinosa L. extract against selected pathogenic bacterial strains.

Bacterial Strains	250 mg/mL	125 mg/mL	62.5 mg/mL	31.25 mg/mL	Negative Control (DMSO)	Positive Control (CIP)
E. coli (ATCC 8739)	18 ± 0.3	18 ± 0.4	16 ± 0.2	16 ± 0.5	–	35
P. aeruginosa (ATCC 27853)	–	–	–	–	–	30
S. aureus (ATCC 6538)	–	–	–	–	–	32
E. faecalis (ATCC 29212)	–	–	–	–	–	26

E. coli = Escherichia coli; P. aeruginosa = Pseudomonas aeruginosa; S. aureus = Staphylococcus aureus; E. faecalis = Enterococcus faecalis. Data are presented as mean inhibition zone (mm) ± standard deviation (SD) of three independent experiments performed in triplicate. Ciprofloxacin (CIP) was used as the positive control, while 10% dimethyl sulfoxide (DMSO) served as the negative control. The symbol (–) indicates no detectable antibacterial activity.

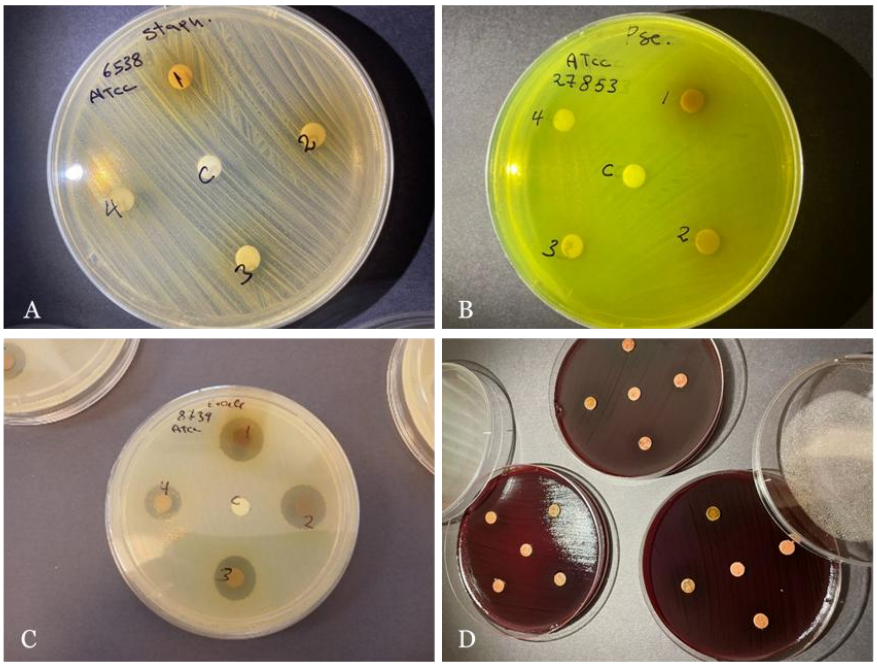


Fig 1. Inhibition zones demonstrating the antimicrobial activity of C. spinosa L. extract at various concentrations (1 = 250, 2 = 125, 3 = 62.5, and 4 = 31.25 mg/mL); 10% DMSO served as a negative control and ciprofloxacin as a positive control (C) against four bacterial strains: (A) Staphylococcus aureus, (B) Pseudomonas aeruginosa, (C) Escherichia coli, and (D) Enterobacter cloacae.

The antimicrobial activity of Capparis spinosa L. leaf extract observed in this study presents both corroborating and conflicting evidence compared to existing literature. Our findings align with [13] regarding the susceptibility of E. coli; however, they contrast with [14], who reported inactivity against this strain. These discrepancies underscore the importance of methodological considerations: our study employed a hydro-ethanolic solvent and ultrasonic extraction, whereas [14] utilized methanol and potentially different extraction methods. Such variations can alter the extracted phytochemical profile and subsequent antimicrobial activity. Furthermore, differences in bacterial strains and assay conditions can also influence the observed results.

2.3 LC-MS/MS compound identification using KnowItAll and MNOVA

In this study, a two-step analytical approach was employed to identify and characterize the compounds in the sample (Tables 3-6, Fig. 2). First, KnowItAll was used for automatic analysis and comparison of isolated compound chromatogram peaks and fragmentations against the MoNA, GNPS, and NIST databases, followed by manual analysis using MNOVA to validate and further explore the compounds. This comprehensive

ISSN: 2956-4808
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approach ensured that the identified compounds were carefully filtered and analyzed for their relevance as natural products.

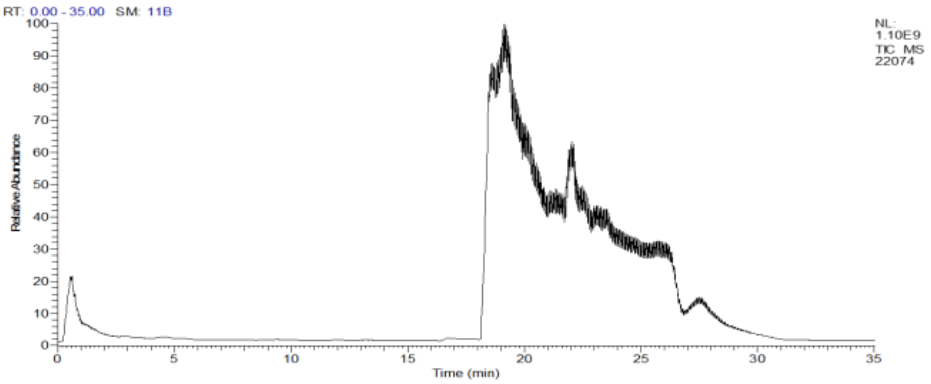

Fig 2. LC-MS/MS chromatogram of C. spinosa L. extract.

2.3.1. Automatic analysis using KnowItAll

Automated analysis was performed using KnowItAll software as the first step, based on LC-MS/MS data acquired at the Nawah Scientific multidisciplinary research center. Although the instrument was an LC-MS/MS platform, data acquisition relied primarily on full MS scans (Fig. 3-5), with only occasional, low-intensity MS2 events. Candidate compounds were manually evaluated for relevance to natural product chemistry. In total, seven candidate compounds were retained, with individual matching scores ranging from 80% to 91% in negative ionization mode. The tentative assignment of each compound was based on MS1 spectral similarity and molecular formula agreement with database entries; these compounds are therefore considered putative natural products, pending further confirmation by targeted MS/MS experiments or authentic standards.

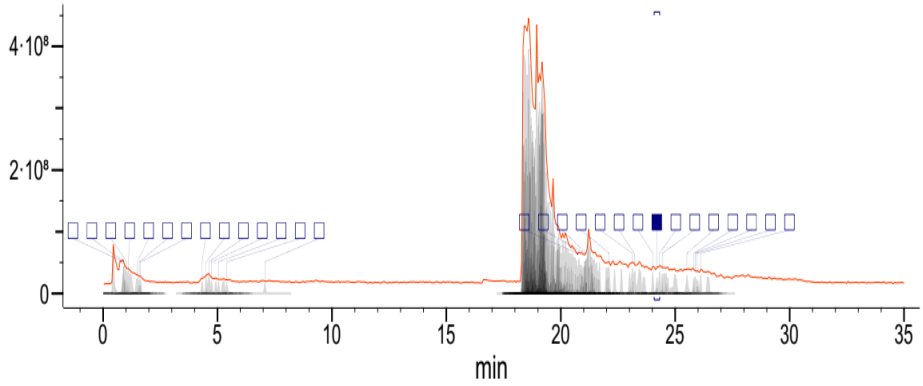

Fig 3. LC-MS/MS analysis of C. spinosa L. extract using KnowItAll software.

2.3.2. Manual validation using MNOVA

Following the automatic analysis with KnowItAll, a manual analysis was conducted using MNOVA software to cross-validate the results and further investigate the positive ionization mode data. In this manual step, a total of 61 compounds were isolated from the chromatogram, of which 33 were selected according to their fitness scores, identified based on their retention times in the chromatogram. Each compound was analyzed in detail, and their molecular weights were calculated.

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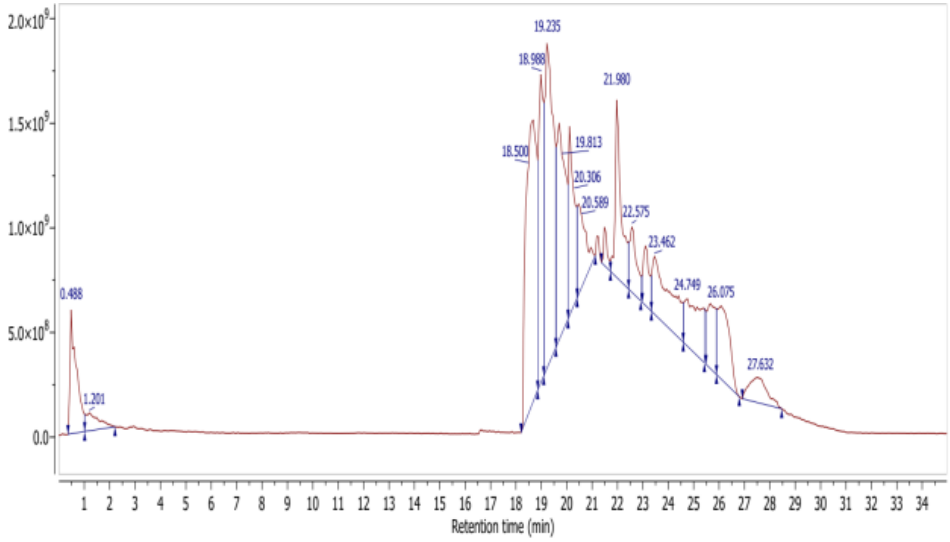

Fig 4. LC-MS/MS analysis of C. spinosa L. extract using MNOVA software.

Table 3. Compounds were detected in the hydro-ethanolic extract of Capparis spinosa L. using LC-MS/MS analysis, analyzed through KnowItAll software.

Compound Name	Symbol	Molecular formula	Molecular weight	Retention time (min)
Chorismic acid	C1	$C_{10}H_{10}O_6$	227	1.1040
Fraxetin	C2	$C_{10}H_8O_5$	209	1.4956
Fraxidin glucoside	C3	$C_{19}H_{24}O_7$	385	1.5815
1-(1-Hydroxybutyl)-1,3,4,5,6,7-hexahydro-2-benzofuran-4,5,6,7-tetrol	C4	$C_{12}H_{20}O_6$	261	1.6411
6-Phosphogluconate	C5	$C_6H_{13}O_{10}P$	277	4.3266
Dehydrovariabilin	C6	$C_{17}H_{14}O_4$	283	25.512

Table 4. Compounds with their corresponding match score and record ID in the database and PubChem CID

Symbol	Match score	Record ID	Database	PubChem CID
C1	86.83	88789	MoNA	12039
C2	88.85	154744	MoNA	5273569
C3	80.33	255380	MoNA	21633144
C4	86.80	10734	MoNA	51136324
C5	91.83	80191	MoNA	91493
C6	82.62	5630	MoNA	624785

Table 5. Overview of compound fragmentations.

Compound symbol	Molecular formula	Molecular weight	Fragmentation
C1	$C_{10}H_{10}O_6$	227	227, 210, 193, 172, 149.91, 63
C2	$C_{10}H_8O_5$	209	209, 191, 168, 132, 104, 91, 62
C3	$C_{19}H_{24}O_7$	385	385, 340, 315, 287, 252, 210, 183, 168, 132, 105, 62
C4	$C_{12}H_{20}O_6$	261	261, 218, 190, 168, 132, 91, 62
C5	$C_6H_{13}O_{10}P$	277	277, 252, 237, 208, 193, 172, 160
C6	$C_{17}H_{14}O_4$	283	283, 275, 214, 197, 160, 134, 104, 59

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DOI: 10.58332/scirad2026v5i3a01

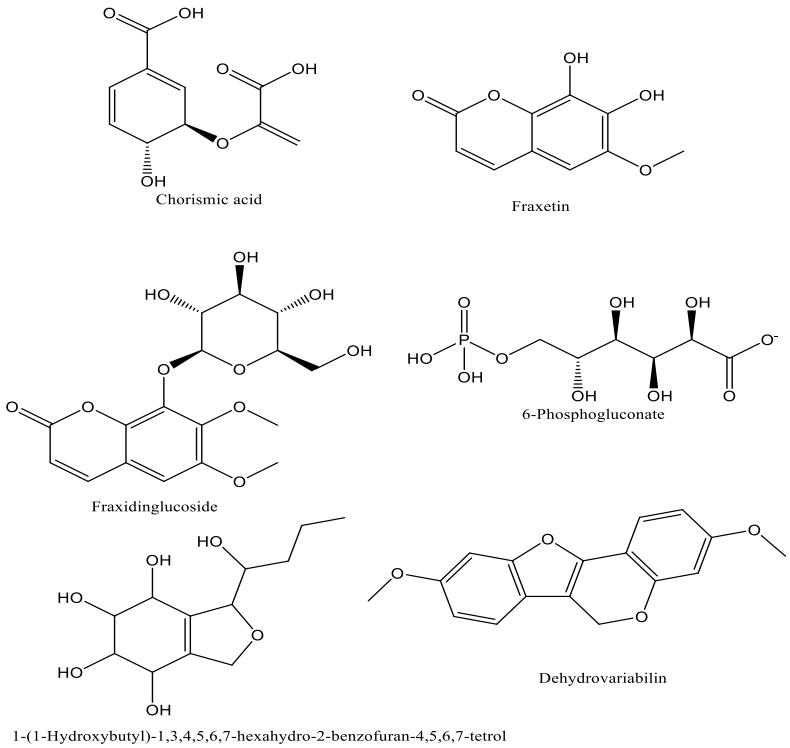

Fig 5. Structures of extract compounds from LC-MS/MS analysis of C. spinosa L. by KnowItAll software.

Table 6. Unknown compounds in the extract identified by MNOVA software.

Cmpd	Formula	Calc. Mass	DBE	Abs. Err (ppm)	Err (mDa)	Err (ppm)	Fitness	RT (min)
CP1	$C_{16}H_{24}NOS$	279.16514	5.5	0.24	-0.07	-0.24	0.925	0.36
CP2	$C_7H_{12}N$	111.10425	2.5	1.76	-0.19	-1.76	0.987	0.48
CP3	$C_{16}H_{30}O_4$	287.22169	2.0	4.78	1.37	4.78	0.938	0.54
CP4	$C_{14}H_{30}N_4S$	287.22639	2.0	1.22	-0.35	-1.22	0.938	0.60
CP5	$C_{16}H_{32}N_2OS$	301.23081	2.0	4.15	1.24	4.15	0.990	0.78
CP6	$C_6H_{19}N_{10}O_6$	328.15618	2.5	0.35	-0.11	-0.35	0.977	1.67
CP7	$C_{22}H_{21}N_3$	328.18082	14.0	0.72	0.24	0.72	0.883	1.85
CP8	$C_{10}H_{23}N_4O_6S$	328.14111	1.5	0.17	-0.05	-0.17	0.956	1.91
CP9	$C_{26}H_{48}N_9O_8S_3$	711.28607	7.5	0.09	-0.06	-0.09	0.732	18.44
CP10	$C_{23}H_{30}N_8O_8S$	579.19801	13.0	0.09	0.05	0.09	0.831	18.56
CP11	$C_{19}H_{37}NO$	296.29479	2.0	4.27	1.26	4.27	0.913	18.56
CP12	$C_{14}H_{31}NOS_2$	294.19198	0.0	0.22	0.06	0.22	0.920	19.69
CP13	$C_{14}H_{23}N_5O_6$	358.17211	6.0	0.15	-0.05	-0.15	0.977	19.69
CP14	$C_{20}H_{20}N$	275.16685	11.5	0.74	0.20	0.74	0.994	18.98
CP15	$C_{41}H_{83}N_7O_3S_2$	786.60716	4.0	0.13	-0.10	-0.13	0.957	19.57
CP16	$C_{40}H_{87}N_4O_4S_3$	784.59622	-0.5	1.05	0.83	1.05	0.826	19.69
CP17	C_8H_{10}	107.08553	4.0	4.59	0.49	4.59	0.985	19.75
CP18	$C_{44}H_{81}N_7O_5$	788.63720	8.0	0.22	-0.17	-0.22	0.995	19.93
CP19	$C_{51}H_{83}N_2O_2S$	788.62480	11.5	0.33	0.26	0.33	0.973	19.99
CP20	$C_{51}H_{83}N_2O_2S$	788.62480	11.5	0.33	0.26	0.33	0.973	19.99
CP21	$C_{46}H_{78}N_9O_2$	789.63512	12.5	0.07	-0.05	-0.07	0.900	20.05
CP22	$C_{64}H_{90}O_4S$	955.66326	20.0	2.35	-2.25	-2.35	0.811	20.11
CP23	$C_{41}H_{88}N_3O_4S_3$	783.60097	-0.5	3.77	-2.95	-3.77	0.846	20.17
CP24	$C_{52}H_{95}N_3OS_4$	906.64307	7.0	0.01	-0.01	-0.01	0.779	21.01
CP25	$C_{47}H_{86}N_9O_{10}$	937.65704	9.5	0.00	0.00	0.00	0.916	12.31
CP26	$C_{50}H_{79}N_7O_4S_2$	906.57077	15.0	0.05	-0.05	-0.05	0.886	21.54
CP27	$C_{58}H_{89}N_7O_3$	932.70997	18.0	0.22	-0.20	-0.22	0.898	21.66
CP28	$C_{61}H_{105}NOS_2$	932.77103	10.0	0.02	0.02	0.02	0.852	23.04
CP29	$C_{58}H_{109}NO_4S$	916.81501	5.0	0.06	0.06	0.06	0.901	24.59
CP30	$C_{60}H_{94}N_2OS_2$	923.68803	15.0	0.02	0.02	0.02	0.825	25.66
CP32	$C_{10}H_{31}N_8O_2$	296.26427	-0.5	4.11	-1.21	-4.11	0.960	26.98
CP33	$C_{17}H_{35}N_4$	296.29401	5.2	1.89	0.56	1.89	0.922	27.93

DBE = double bond equivalence; RT = retention time; Cmpd. = compound; Calc. Mass = calculated mass; Err = error.

ISSN: 2956-4808
DOI: 10.58332/scirad2026v5i3a01

3. Materials and methods

3.1 Plant material (sampling and identification)

Capparis spinosa L. leaves, sourced from the Al-Murassas region of eastern Libya, were identified using the botanical library at the Department of Botany, Faculty of Science, University of Benghazi, Libya, following the Flora of Libya. Post-collection, the leaves were cleaned, shade-dried, and pulverized for extraction (Fig. 6).

Fig 6. C. spinosa L.: (A) raw plant; (B) dried plant; (C) powdered plant material.

3.2 Sample preparation and ultrasound-assisted extraction

An ethanolic extract of C. spinosa L. leaves was prepared according to the method of Abdulridha & Saliem [15]. Briefly, 10 g of powdered leaves were mixed with 200 mL of 70% ethanol and sonicated for 60 minutes at 40 kHz in a dark environment at room temperature; this procedure was repeated 30 times. The extract was then filtered through Whatman No. 4 filter paper under vacuum, and the ethanol was removed using a rotary evaporator at 40°C. The resulting extract was stored at -18°C until needed (Fig. 7).

Fig 7. Schematic representation of the extraction and purification procedure for C. spinosa L.: (A) an ultrasonic process facilitates extraction; (B) solid-liquid separation is achieved via vacuum filtration; (C) the extract is concentrated using a rotary evaporator; (D) crude extract of C. spinosa L.

3.3 Preliminary phytochemical screening

Standard procedures were employed to identify secondary metabolites in C. spinosa L. leaf extract using established methods [16], [17], [18], [19], [20].

1. Alkaloid detection (Dragendorff's test). Two drops of Dragendorff's reagent were added to 1 mL of the extract. Formation of a creamy precipitate indicated the presence of alkaloids.

ISSN: 2956-4808
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2. Phenolic acids detection (ferric chloride test). Four drops of 10% alcoholic ferric chloride solution were added to the extract. A bluish-black color indicated the presence of phenolic compounds.

3. Flavonoids detection (alkaline reagent test). The extract was treated with a 10% NaOH solution. A strong yellow color indicated the presence of flavonoids.

4. Terpenoids detection (Salkowski's test). One milliliter of the extract was dissolved in 10 mL of chloroform. An equal volume of concentrated sulfuric acid (H_2SO_4) was carefully added along the test tube wall. A red color in the chloroform layer and a yellow-green fluorescence in the sulfuric acid layer indicated the presence of triterpenoids and steroids.

5. Tannin detection ($FeCl_3$ test). Four milliliters of extract were treated with 4 mL of ferric chloride solution. A bluish-black precipitate indicated the presence of hydrolyzable tannins.

6. Saponin detection (froth test). Five milliliters of extract were diluted to 20 mL with distilled water and shaken vigorously in a graduated cylinder for 15 minutes. The formation of persistent foam indicated the presence of saponins.

7. Glycoside detection (Keller–Kiliani test). Two milliliters of glacial acetic acid were added to the extract, followed by one drop of ferric chloride ($FeCl_3$) solution. A brown ring at the interface indicated the presence of glycosides.

3.4 Antimicrobial bioassay

The antibacterial activity of Capparis spinosa L. leaf crude extract was evaluated against four American Type Culture Collection (ATCC) reference bacterial strains, including two Gram-negative bacteria, Escherichia coli (ATCC 8739) and Pseudomonas aeruginosa (ATCC 27853), and two Gram-positive bacteria, Staphylococcus aureus (ATCC 6538) and Enterococcus faecalis (ATCC 29212). A stock solution of the crude extract was prepared at a concentration of 250 mg/mL by dissolving 1.250 g of the dried extract in 5 mL of 10% (v/v) dimethyl sulfoxide (DMSO). Serial two-fold dilutions of the stock solution were subsequently prepared to obtain final concentrations of 125, 62.5, and 31.25 mg/mL. The antibacterial activity was determined using the Kirby–Bauer disc diffusion method on Mueller–Hinton agar (MHA) plates, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2017). Fresh bacterial suspensions were prepared in sterile normal saline, and the turbidity of each suspension was adjusted to the 0.5 McFarland standard, corresponding to approximately 1.5×10^8 CFU/mL, to ensure a standardized bacterial inoculum. The bacterial suspensions were then evenly spread over the surface of MHA plates using sterile cotton swabs. Sterile paper discs (6 mm in diameter) were impregnated with 30 µL of each extract concentration (250, 125, 62.5, and 31.25 mg/mL) and carefully placed onto the inoculated agar plates. Following incubation at 37°C for 24 h, the antibacterial activity of the extract was assessed by measuring the diameter of the inhibition zones surrounding each disc, and the results were recorded in millimeters (mm). All experimental procedures were carried out at Al-Saleem Laboratory, Benghazi, Libya.

3.5 Characterization by liquid chromatography-mass spectrometry (LC-MS/MS) analysis

Bioactive compounds within an ethanolic extract of C. spinosa L. leaves were analyzed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) at the Nawah Scientific multidisciplinary research center, Egypt. Prior to analysis, the extract was centrifuged at 12,000 rpm for 10 minutes. The LC system comprised two pumps, an automated injector, and an Agilent Eclipse ColumnComp.CC (5 µm particle size, 15 cm length, 4.6 mm internal diameter). Two mobile phases were employed: mobile phase A – 2% (deionized water, 5 mM ammonium formate, 0.1% formic acid, and 0.1% acetic acid) and mobile phase B – 2% (methanol, 5 mM ammonium formate, and 0.1% formic acid). A constant flow rate of 0.500 mL/min was maintained. The separation protocol combined gradient and isocratic elution steps: initial conditions (0.00–1.00 min) used 2% mobile phase B; gradient elution 1 (1.00–17.50 min) involved a linear increase of mobile phase B to 95% using a curve-5 gradient profile; this was followed by isocratic elution (17.50–25.51 min) at 95% mobile phase B; gradient elution 2 (25.51–30.01 min) again saw a linear increase of mobile phase B to 95% using a curve-5 gradient profile, followed by an equilibration phase (30.01–35.00 min) with a linear decrease of mobile phase B to 2% to re-equilibrate the column for the next injection. The total run time was 35.00 minutes, with the column temperature maintained at 40°C and pressure monitored throughout the analysis. For mass spectrometry detection, a Thermo TSQ Altis mass spectrometer was employed, operating in both positive and negative ionization modes with centroid acquisition. Specific mass spectrometric parameters included a capillary voltage of 3200 V in negative ion mode and 3500 V in positive ion mode, with a nebulizing gas flow of 1.5 L/min and a dry gas flow rate of 12 L/min at a desolvation line temperature of 325°C. Full-scan mass spectra were recorded over a mass range

ISSN: 2956-4808
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of 100 to 1500 Da. KnowItAll's automated spectral matching functionalities were employed for rapid identification of compounds based on the obtained LC-MS data, while MNOVA's structure verification tools were used to manually validate molecular formulas and fragmentation patterns, ensuring accurate compound annotation. This methodological approach enabled the effective detection and characterization of bioactive compounds present in the ethanolic extract of C. spinosa L. leaves.

4. Conclusion

The present study provides a comprehensive evaluation of the phytochemical composition, antimicrobial activity, and metabolite profile of Capparis spinosa L. leaf extract. Preliminary phytochemical screening confirmed the presence of several important secondary metabolites, including flavonoids, phenols, glycosides, tannins, saponins, and terpenoids, indicating a chemically diverse extract with potential biological relevance.

Despite this rich phytochemical composition, the antimicrobial assessment revealed limited antibacterial activity, with observable inhibition restricted to Escherichia coli, while no activity was detected against Staphylococcus aureus, Pseudomonas aeruginosa, and Enterococcus faecalis. These findings suggest that the extract, under the applied conditions, does not possess broad-spectrum antibacterial efficacy, and highlight the influence of extraction method, compound concentration, and microbial susceptibility.

LC-MS/MS analysis, combined with KnowItAll and MNOVA software, enabled the identification of multiple compounds, including organic acids, coumarins, benzofuran derivatives, carbohydrate-related metabolites, and terpenoids. The detection of both known and unknown compounds, particularly those with high molecular weights and heteroatom-rich formulas, suggests the presence of complex metabolites such as peptides or glycosylated compounds. Overall, this study demonstrates that C. spinosa leaf extract is metabolically diverse, though its antimicrobial potential is limited under the tested conditions. Future studies should focus on compound isolation, alternative extraction techniques, and bioactivity-guided fractionation to better explore its pharmacological potential.

Acknowledgements

The authors sincerely thank the University of Benghazi and the Departments of Chemistry, Pharmacognosy, Pathology, and Pharmacology, as well as the Central Medical Laboratory, Garyounis Campus, and Al-Saleem Medical Laboratory, Microbiology Unit, for their valuable support and cooperation.

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ISSN: 2956-4808
DOI: 10.58332/scirad2026v5i3a01

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ISSN: 2956-4808
DOI: 10.58332/scirad2026v5i3a01