



Phytochemical Screening, Physicochemical Profiling, and Bioactivity Assessment of *Ceratonia Siliqua* Seed Aqueous Extract: Antioxidant and Antimicrobial Properties

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Abstract

The growing interest in plant-derived bioactive compounds has intensified the search for novel antioxidants and antimicrobials. This study investigates the phytochemical profile, antioxidant potential, and antibacterial efficacy of the aqueous extract of *Ceratonia siliqua* seeds (AECS), employing a multi-technique analytical approach for the first time. AECS was analyzed for total phenolic (TPC), flavonoid (TFC), and condensed tannin content. Key compounds were identified using HPLC–ESI–MS/MS, while FTIR and XRD characterized functional groups and crystalline structures. SEM–EDX provided morphological and elemental insights. Antioxidant activity was assessed via DPPH, ABTS, and FRAP assays, and antibacterial effects were evaluated against Gram-negative and Gram-positive bacteria using MIC and MBC tests. AECS exhibited high phenolic and flavonoid content, with HPLC–MS/MS confirming the presence of gallic acid, catechin, and quercetin. FTIR and XRD provided structural insights, while SEM–EDX revealed a porous morphology with bioactive elements. Phytochemical screening showed a total phenolic content of 30.14 ± 0.16 mg GAE/g DM, a flavonoid content of 8.60 ± 0.80 mg CAE/g DM, and a condensed tannin content of 1.49 ± 0.08 mg CAE/g DM. AECS demonstrated strong antioxidant activity (DPPH $IC_{50} = 0.02 \pm 0.06$ mg/mL; FRAP $IC_{50} = 6.54 \pm 0.01$ mg/mL) and exhibited potent antibacterial effects against all four bacterial strains, with the strongest activity observed against *E. faecium* (MIC = 0.78 ± 0.00 µg/mL). While AECS shows activity against multiple bacterial strains, its mechanism of action appears to be predominantly bacteriostatic, rather than bactericidal. This study provides the first comprehensive chemical characterization of AECS using combined spectroscopic and microscopic techniques, unveiling its structural and bioactive properties. These findings position *C. siliqua* seed extract as a promising natural antioxidant and antimicrobial agent for pharmaceutical and nutraceutical applications.

Keywords *Ceratonia siliqua* · Antioxidant activity · Bioactive compounds · *C. siliqua* extract · Phytochemicals · Antimicrobial activity

Introduction

The search for natural therapeutic agents has gained significant attention in recent years, with plants serving as a rich source of bioactive compounds. *Ceratonia siliqua* L. (*C. siliqua*) is one such plant, valued for its historical use in traditional medicine and growing scientific interest due to its diverse phytochemical composition and potential therapeutic applications.

Oxidative stress is closely associated with the development of numerous chronic diseases, including cardiovascular

disorders, neurodegenerative conditions, and certain types of cancer (Arunachalam et al. 2022). Antioxidants help counteract oxidative damage by scavenging free radicals and interrupting chain reactions that contribute to disease progression. Recent research suggests that nutraceuticals rich in antioxidants can also exert pro-oxidative effects on cancer cells, promoting apoptosis. Among these, phenolic and aromatic ring-containing antioxidants have shown high efficacy by stabilizing radical intermediates and donating hydrogen radicals (H●). Consequently, plant-based supplements with antioxidant properties are increasingly recognized for their role in mitigating oxidative stress and inflammation (Arunachalam et al. 2022). Extensive research has identified over 8000 polyphenolic compounds and more than

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6000 flavonoids in various plants (Baslam et al. 2023a, b; Jahan et al. 2022; Baslam et al. 2023a).

In addition to their antioxidant potential, natural products offer a promising alternative to combat multidrug-resistant pathogens. The rising threat of antibiotic resistance has intensified the search for plant-based antimicrobial agents, which may enhance enzymatic and non-enzymatic antioxidant defenses. Traditional medicinal plants have long been used to treat infectious diseases, and scientific exploration of their antimicrobial properties continues to yield promising results.

C. siliqua (*Ceratonia siliqua* L.) is a perennial, ever-green leguminous tree native to the Mediterranean basin (Boutasknit et al. 2020; Obeidat et al. 2012). Well adapted to arid environments, it demonstrates remarkable resistance to salinity and drought (Youssef et al. 2013). The *C. siliqua* fruit, or pod, is rich in polyphenols, flavonoids, and tannins, which contribute to its numerous health benefits (Youssef et al. 2013). The fruit of the *C. siliqua* tree, commonly known as *C. siliqua* pods, contains a plethora of bioactive compounds, including polyphenols, flavonoids, tannins, which have been associated with a wide range of health benefits (Youssef et al. 2013). Importantly, *C. siliqua* is recognized as a valuable source of gallic acid, catechol, pyrogallol, and chlorogenic acid (Youssef et al. 2013; Papagiannopoulos et al. 2004). These bioactive compounds exhibit strong antioxidant, antimicrobial, and anti-inflammatory properties. For instance, gallic acid is known for its potent antioxidant and anticancer activities, catechin has been widely studied for its role in cardiovascular health and neuroprotection, and quercetin has demonstrated significant anti-inflammatory and immunomodulatory effects (Albuquerque et al. 2025; Shirazi et al. 2019; Sun & Shahrajabian 2023). *C. siliqua* has gained recognition as a natural food additive, often used as a thickener and flavoring agent (Arribas et al. 2019). Its high dietary fiber content makes it a favorable choice for gluten-free functional foods (Arribas et al. 2019; Aguilar et al. 2015). Furthermore, *C. siliqua* has the potential to serve as an important alternative for wheat and rice fortification, providing numerous phytochemicals and nutritional benefits (Arribas et al. 2019). In the food industry, *C. siliqua*-derived products, including locust bean gum, are employed as stabilizers and thickeners (Dakia et al. 2007). While *C. siliqua* seeds are the most commercially valuable part of the fruit, the pulp and seed peel are now recognized for their potential valorization. *C. siliqua* fruit consists primarily of sugars (sucrose, glucose, and fructose) and dietary fibers (cellulose, hemicellulose, and lignin), with *C. siliqua* seed gum (galactomannan) comprising approximately 85% of the seed's composition (Albanell et al. 1996). Additionally, *C. siliqua* is rich in essential minerals such as potassium and calcium. Research suggests that the

physiological benefits of *C. siliqua* and its by-products are largely attributed to its bioactive compounds, which may support human health and prevent chronic diseases (Goulas et al. 2016). Previous studies have largely concentrated on specific parts of *C. siliqua*, such as the seeds and pods, while the aqueous extract of *C. siliqua* (AECS) remains largely unexamined. The aqueous extract was selected due to its traditional use in ethnomedicine, its safety for food and pharmaceutical applications, and the limited research on its phytochemical composition and bioactivity (Anbessa et al. 2024). Unlike organic solvents that may leave toxic residues, aqueous extraction ensures a safer and more practical approach (Mungwari et al. 2025), allowing us to explore the therapeutic potential of *C. siliqua* in a form suitable for dietary and medicinal use.

Despite the existing body of research on *C. siliqua*, there remains a significant gap in understanding its comprehensive phytochemical profile and the application of advanced analytical techniques to characterize its bioactive constituents. Previous studies have primarily focused on specific bioactive components or crude extracts without a detailed chemical profiling approach. This study aims to bridge this gap by systematically evaluating the phytochemical composition of AECS using advanced analytical techniques, assessing its antioxidant potential, and investigating its antimicrobial properties.

Advanced analytical techniques are essential for identifying and characterizing bioactive compounds in plant extracts. HPLC quantifies phytochemicals, FTIR reveals functional groups, SEM and XRD analyze microstructure and crystallinity, while EDX provides elemental composition. This integrated approach ensures a comprehensive assessment of AECS, enhancing understanding of its therapeutic potential.

To date, no comprehensive study has combined a systematic phytochemical screening, detailed chemical profiling, and bioactivity evaluation of AECS. By evaluating the antimicrobial properties of AECS, its potential as a natural alternative to traditional antibiotics can be assessed. Thus, this study contributes to the growing field of natural product research by providing an integrated analysis of the phytochemical and biological properties of *C. siliqua* aqueous extract.

In this study, we aimed to perform an extensive phytochemical screening of AECS and characterize the extract using techniques such as high-performance liquid chromatography (HPLC) analysis, Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), X-ray diffraction (XRD), and energy dispersive X-ray spectroscopy (EDX). Further, we investigated the antioxidant activity of AECS using established assays and examined its potential as an antimicrobial agent against a range of clinically relevant pathogens.

Materials and Methods

Plant Samples and Extraction

Ceratonia siliqua seeds were collected from Essaouira region, Morocco (31° 30'42" latitude N/9° 45'43 " longitude W). The plant was authenticated by botanist Professor A. Ouhammou and pharmacologist Professor A. Chait from the Faculty of Sciences Semlalia, Cadi Ayyad University. The voucher specimen MARK-AC927 was deposited in the herbarium of the Department of Biology, Faculty of Sciences Semlalia, Cadi Ayyad University, Marrakech, Morocco. The seeds were initially washed and dried. Subsequently, the dried seeds were crushed to obtain a fine powder. The powder was then subjected to extraction using distilled water (1 g of seed powder per 10 mL of water) with continuous agitation for 24 h at room temperature (cold maceration). Following the extraction process, the aqueous extract was centrifuged at 1200 rpm, filtered and lyophilized using a Christ instrument. A comprehensive microbial analysis of the aqueous extract was conducted using standard pharmacopoeial methods to ensure its purity. This evaluation aimed to confirm the absence of microbial contaminants. The resulting lyophilized dry powder was sealed in amber bottles and kept at 4 °C until its subsequent use.

Phytochemical Study

HPLC–MS/MS Analysis

High-performance liquid chromatography with photodiode array detection tandem mass spectrometer (HPLC-PDA-MS–MS). In this study, a Thermo Finnigan instrument (Thermo Electron Corporation, Waltham, MA, USA) was utilized for liquid chromatography (LC), featuring a reversed-phase column (Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 3.5 µm, Agilent, Santa Clara, CA, USA). The LC system was connected to a mass spectrometer (LCQ-Duo ion trap) equipped with an electrospray ionization source. The mobile phase consisted of water and acetonitrile (can), both infused with 0.1% formic acid. A gradient flow of the mobile phase was initiated, starting with 5% ACN and gradually increasing to 30% over a span of 60 min. In the subsequent 30 min, can concentration was elevated to 90%. The flow rate was set at 1 mL/min with a split ratio of 1:1. Spectra were scanned within the range of m/z 50–2000 in the negative mode using the full scan acquisition method. Data evaluation was carried out using the X-calibur software (X-calibur™ 2.0.7, Thermo Fisher Scientific, Waltham, MA, USA). Peak identification

was performed by comparing retention times with available standards, inbuilt MS/MS libraries, and literature references.

Phytochemical Screening

Determination of Total Phenolic Content (TPC) The total phenolic content (TPC) was determined using a modified Folin–Ciocalteu method (Singleton et al. 1999). An aliquot (0.4 mL) of the diluted extract was mixed with 1.5 mL of Folin–Ciocalteu reagent. After 5 min, 1.6 mL of sodium carbonate solution (7.5%) was added. The resulting solution was incubated in the dark at room temperature for 2 h, and absorbance was read at 765 nm. Gallic acid was used as a standard, and the results were expressed as milligrams of equivalent gallic acid per gram of dry weight (mg EAG/g DW).

Determination of Total Flavonoid Content (TFC) The total flavonoid content (TFC) was determined following the method described by Zhishen et al. (1999). A 200 µL extract was diluted with 1 mL of distilled water, followed by the addition of 60 µL of 5% NaNO₂ and 60 µL of 10% AlCl₃. After 5 min, 400 µL of 1 M NaOH was added to the mixture. The absorbance was read at 510 nm, and the TFC was expressed as milligrams of catechin equivalent per gram of dry matter (mg CAE/g DM).

Quantification of Condensed Tannins The total condensed tannin content was determined following the method described by Heimler et al. (2005). Briefly, diluted samples (400 µL) were mixed with 3 mL of methanol vanillin solution (4%) and 1.5 mL of concentrated hydrochloric acid (HCL). The mixture was incubated for 15 min, and absorbance was measured at 500 nm. The quantity of total condensed tannins was expressed as milligrams of catechin equivalent per gram of dry matter (mg catechin/g DM).

Physicochemical Profiling

Fourier Transform Infrared (FTIR) To investigate molecular vibrations within the sample particles, the synthesized silver nanoparticle solution was combined with potassium bromide in a 1:100 ratio. The resulting mixture was analyzed using an infrared spectrometer to investigate molecular vibrations within the sample particles. Infrared spectra were collected in the range of 400–4000 cm^{−1}, allowing for characterization of chemical bonds and functional groups present in the nanoparticles.

X-ray Diffraction (XRD) X-ray diffraction (XRD) patterns were obtained using a PAN analytical X'pert PRO X-ray

diffractometer from JEOL, Tokyo, Japan. Measurements were conducted within the 2θ range of 20–80°, employing a fixed current of 40 mA and a voltage of 45 kV. Analysis involved depositing a suspension of myogenic AgNPs onto a glass substrate. XRD data enabled scrutiny of the crystal structure and phase composition of the silver nanoparticles. The average crystal size was determined using Scherrer's equation, which relates size (D) to wavelength (λ), the full width at half maximum of the diffraction peak (β), Scherrer's constant (K), and Bragg's angle (θ).

Scanning Electron Microscope (SEM) Morphology, size, and shape of synthesized silver nanoparticles were analyzed using a SEM across magnification levels ranging from $\times 1,000$ to $\times 1,000,000$, following the methodology described by Baslam et al. (2024). SEM provided high-resolution imaging of nanoparticles, facilitating insights into their surface attributes. Particle size determination was accomplished by processing acquired SEM images using the ImageJ program.

Energy-Dispersive X-ray Spectroscopy (EDX) Elemental composition of samples was determined through EDX spectrometry, which provided valuable insights into the elemental makeup and distribution within the samples. This analysis is crucial in plant extracts, as it helps identify essential bioactive elements, such as minerals and trace metals, that contribute to the extract's pharmacological properties. Additionally, the particle structure and size distribution were evaluated using a transmission electron microscope (TEM) model JEM-2100 from JEOL, Tokyo, Japan. TEM allowed high-resolution imaging of particles, affording detailed insights into their morphology, structure, and size distribution.

Antioxidant Activity

Determination of DPPH Radical Scavenging Activity

The antioxidant activity of the extract was assessed using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay, as described by Mansouri et al. (2005) with modifications by Benmeddour et al. (2013). Methanolic solution (1.5 mL) of DPPH (6×10^{-5} M) was mixed with 60 µL of the extract at various concentrations (1, 2, 4, 6, and 8 mg/mL) of AECS. The mixture was then kept away from light at room temperature for 30 min. Subsequently, the absorbance was measured at 515 nm against a negative control consisting of 1.5 mL of the DPPH solution and 60 µL of methanol. Positive controls, butylated hydroxytoluene (BHT) and quercetin were included. The percentage of inhibition was calculated using the following formula:

$$\% \text{ Inhibition (mg/mL)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

where A_{control} is the absorbance of the control and A_{sample} is the absorbance of the test compound. The sample concentration that induces 50% inhibition (IC_{50}) was determined by plotting the percentages of inhibition against the sample concentrations (Baslam et al. 2024).

Determination of FRAP Activity

The ferric reducing ability power (FRAP) test was conducted following the method described by Oyaizu (1986). The assay involves the prevention of the formation of Fe(II)-ferrazine complexes when samples are incubated with ferrous iron. In the procedure, a mixture of distilled water (1 mL), phosphate buffer (2.5 mL; 0.2 M; pH=6.6), and potassium ferricyanide ($K_3[Fe(CN)_6]$) (2.5 mL; 1%) was combined with 0.5 mL of solutions of varying concentrations. After incubating for 30 min, the mixture was supplemented with 2.5 mL of distilled water, 2.5 mL of 10% trichloroacetic acid, and 0.5 mL of $FeCl_3$. The absorbance was read at 700 nm. Quercetin and BHT were used as positive controls.

Antibacterial Activity

Microbial Strains and Culture Conditions

The antibacterial activity of *C. siliqua* extract was evaluated against a panel of Gram-positive (*Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecium* (ATCC 4602)) and Gram-negative (*Pseudomonas aeruginosa* (ATCC 27653) and *Escherichia coli* (ATCC 25922)) bacteria. The bacterial cultures were cultured in Mueller–Hinton Broth (MHB) for 24 h, and the final inoculum sizes were adjusted to 1×10^8 CFU/mL (0.5 McFarland standard).

To assess the antibacterial activity, three crucial *in vitro* assays were employed: (1) the agar well diffusion method was employed to measure the inhibition zones (ZOI) caused by the plant extract, (2) the microdilution assay was performed to determine the minimal inhibitory concentration (MIC), which represents the lowest extract concentration inhibiting visible bacterial growth, and (3) the minimum bactericidal concentration (MBC) was determined as the lowest extract concentration that killed the bacteria. All tests were conducted in triplicate for each bacterial strain.

Agar Well Diffusion Inhibition Zones Test

Petri dishes containing 25 mL of Mueller–Hinton agar (MHA) were prepared and labeled accordingly. The final bacterial inocula were spread evenly on the agar surface using sterile cotton swabs, rotating the plate 90° after each application to ensure uniform distribution. Wells with a

diameter of 4 mm were aseptically created in each plate using a sterile cork-borer. Next, aliquots (100 μ L) of the plant extract, prepared at a concentration of 100 mg/mL, were loaded into each well. Gentamicin (10 μ g/disc) and DMSO were used as positive and negative controls, respectively, to confirm that any observed antibacterial activity was not due to the solvent.

The plates were allowed to stand at room temperature for 30 min to ensure proper diffusion of the plant extract before being incubated at 37 °C. After incubation, the zones of bacterial inhibition around each well were measured in millimeters, and the mean value was calculated to determine the extent of antibacterial activity (Alayed et al. 2022).

MIC and MBC/MFC Determination

The minimum inhibitory concentration and minimum bactericidal concentration of *C. siliqua* extract were determined using a broth dilution micro method in 96-well micro-titer plates, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) with some modifications (Ma W, 2006). *C. siliqua* extract and its polyphenolic fractions were dissolved in distilled water to prepare stock solutions with a concentration of 100 mg/mL. From these stock solutions, serial twofold dilutions were prepared in MHB, resulting in final concentrations ranging from 50 to 0.19 mg/mL. Microbial cultures were inoculated into the wells to achieve a final concentration of 5×10^5 CFU. Each determination was performed in duplicate, and control wells containing only MHB and MHB⁺ distilled water were included. Gentamicin was used as a positive reference standard. After incubation (24 h), the MIC was determined as the lowest concentration of the extract that completely inhibited visible bacterial growth. To determine the MBC, 20 μ L from all clear MIC wells were seeded onto agar plates. The MBC was defined as the lowest concentration of the plant extracts that killed 99.9% of the inoculum. Each determination was performed in three independent experiments, and the results were calculated.

Reagents and Chemicals

All chemicals and reagents used in the experiment were of analytical grade and were mainly obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) with a purity of $\geq 99.9\%$ was employed as the organic solvent for HPLC, while HPLC-grade deionized water was used throughout. Formic acid (purity $\geq 98\%$), prepared as a 0.1% (v/v) solution, was added to both the water and ACN to facilitate ionization in the mass spectrometer. For the phytochemical assays, the Folin–Ciocalteu reagent (purity $\geq 98\%$) and sodium carbonate (7.5% w/v, purity $\geq 98\%$) were used in the determination of total phenolic content with gallic

acid (purity $\geq 98\%$) as the standard. The total flavonoid content assay involved sodium nitrite (5% w/v, purity $\geq 98\%$), aluminum chloride (10% w/v, purity $\geq 98\%$), and a 1 M sodium hydroxide solution (purity $\geq 98\%$). Analytical grade methanol (purity $\geq 99.9\%$) was used for various solution preparations, while a 4% (w/v) methanolic vanillin solution (purity $\geq 98\%$) combined with concentrated hydrochloric acid (ACS reagent grade, $\sim 37\%$ HCl) was utilized for the quantification of condensed tannins. In the FRAP assay, a 1% (w/v) potassium ferricyanide solution (purity $\geq 98\%$), 10% (w/v) trichloroacetic acid, and ferric chloride (ACS reagent grade) were used. Additionally, gentamicin (10 μ g/disc) served as the positive control in antibacterial tests, with DMSO (analytical grade) as the negative control, and butylated hydroxytoluene (BHT) along with quercetin (both with purity $\geq 98\%$) were employed as antioxidant standards.

Statistical Analysis

The data were subjected to statistical analysis using Graph-Pad Prism Software version 9.00 (San Diego, California, USA) and were expressed as a mean $\pm$ standard error of the mean (SEM).

Results and Discussion

Phytochemical Screening

Determination of Total Phenolic Content, Total Flavonoid Content, and Quantification of Condensed Tannin

Our findings unveiled substantial and intriguing quantities of total phenol (30.1 ± 0.16 mg GAE/g), flavonoids (8.6 ± 0.8 mg CAE/g), and condensed tannins (1.5 ± 0.08 mg CE/g) within the AECS (Table 1).

Chemical Profiling

HPLC–MS/MS Analysis

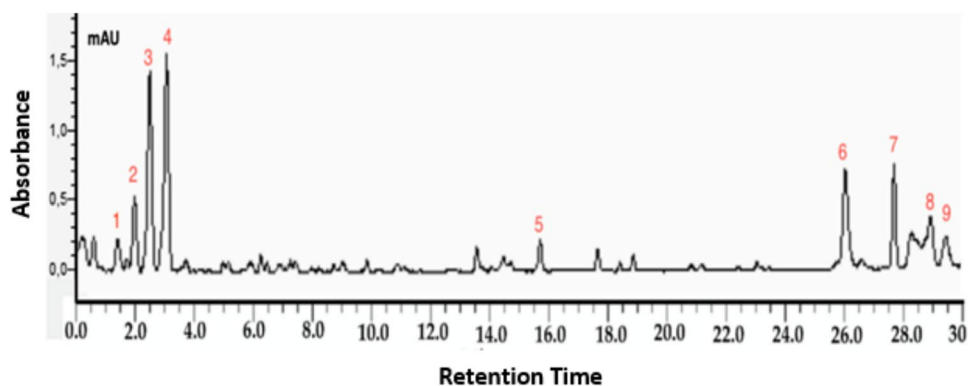
To provide valuable insights into the diverse phytochemical composition, we carried out HPLC–PDA–MS/MS analysis of AECS, as illustrated in Fig. 1. In this analysis,

Table 1 Phytochemical screening of the AECS

	Total phenolic content (mg GAE/g DM)	Total flavonoid content (mg CAE/g DM)	Tannins (mg CAE/g DM)
AECS	30.14 ± 0.16	8.60 ± 0.80	1.49 ± 0.08

GAE gallic acid equivalent, CAE catechin equivalent. Results are presented as mean $\pm$ SEM

Fig. 1 HPLC–MS/MS chromatogram of the AECS. (1) ascorbic acid, (2) resorcinol, (3) gallic acid, (4) catechin, (5) caffeic acid, (6) rutin, (7) quercetin, (8) epicatechin, (9) luteolin



phytoconstituents were identified based on their MS/MS fragments and retention times, resulting in the identification of a total of nine secondary metabolites. The chromatogram obtained from AECS sample revealed prominent peaks observed at 280 nm, each corresponding to different secondary metabolites with distinct retention times (Rt) and molecular weights (MW). The compounds, listed in order of abundance, include: Ascorbic acid (Rt: 1.69 min, MW: 176.12 g/mol), resorcinol (Rt: 2 min, MW: 110.11 g/mol), gallic acid (Rt: 3 min, MW: 170 g/mol), catechin (Rt: 3.69 min, MW: 170 g/mol), caffeic acid (Rt: 15.6 min, MW: 180 g/mol), rutin (Rt: 26 min, MW: 610.52 g/mol), quercetin (Rt: 27.8 min, MW: 302 g/mol), epicatechin (Rt: 28.5 min, MW: 290 g/mol), and luteolin (Rt: 29 min, MW: 286 g/mol).

Fourier Transform Infrared (FTIR)

The FTIR analysis of AECS is depicted in Fig. 2, revealing a prominent band observed between 3350 and 3460 cm^{-1} , signifying the stretching of the $-\text{NH}_2$ and $-\text{OH}$ group bonds. Furthermore, a peak at 1611.23 cm^{-1} was identified, corresponding to the stretching and vibration of the carbonyl group ($\text{C}=\text{O}$) present in the extract.

Moreover, the absorption peak at 1410.41 cm^{-1} can be associated with $\text{C}-\text{O}$ symmetric stretching in carboxylates ($-\text{COO}^-$) or $\text{C}-\text{H}$ bending vibrations in aromatic rings, indicating the presence of polyphenolic compounds or flavonoids. The peak at 1038.58 cm^{-1} represents the stretching vibration of the $\text{C}-\text{O}-\text{C}$ bridge of the ring, suggesting the

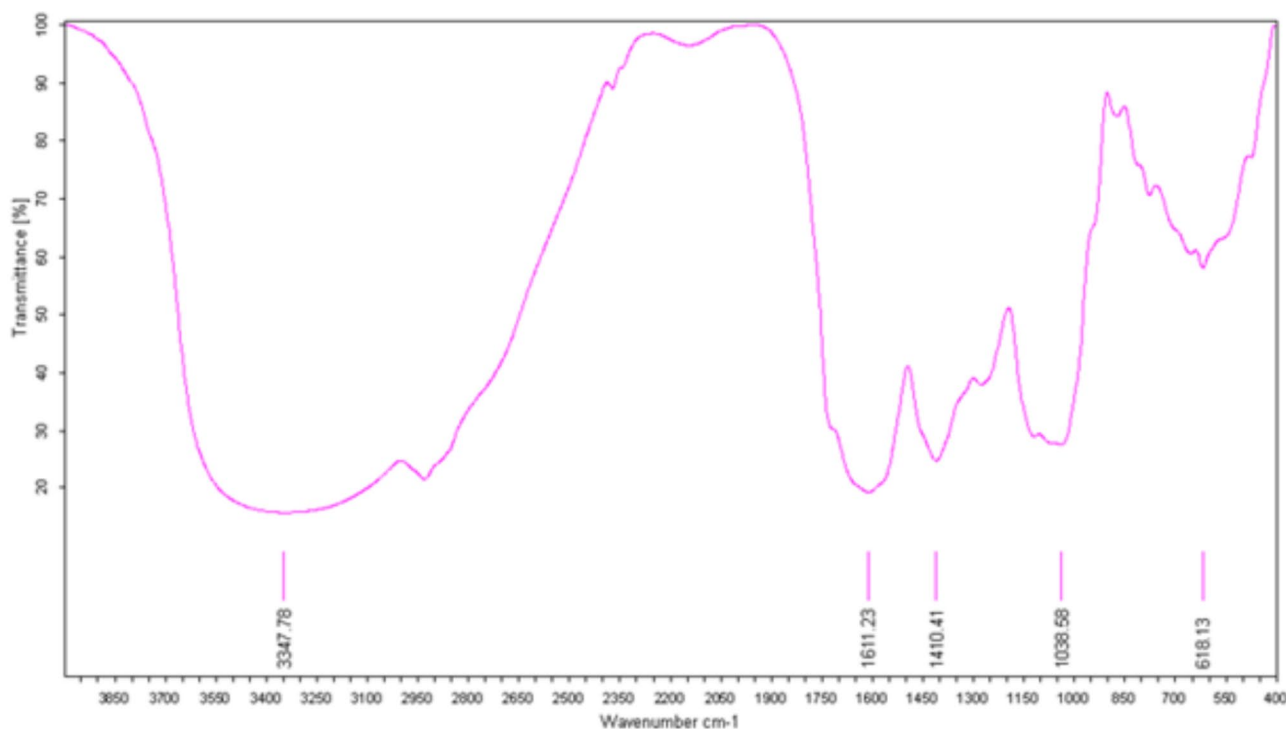


Fig. 2 FTIR of the aqueous extract of *Ceratonia siliqua* (AECS)

presence of cyclic molecules within the extract. Finally, the band observed at 618.13 cm^{-1} corresponds to out-of-plane bending vibrations of aromatic C–H bonds, further supporting the presence of aromatic compounds, possibly phenolic derivatives.

The diffractogram of AECS was obtained and is presented in Fig. 3. In this diffractogram, a spectrum of peak is observed at 2θ , spanning the range between 5 and 45° . Notably, these peaks align with crystal reflections, specifically the (020), (1–10), (110) and (111). (002) (200) and (112) reflections, respectively. These reflections are indicative of a diverse range of molecules present in the extract, including potential active ingredients.

Fig. 3 X-ray diffraction (XRD) of aqueous extract of *C. siliqua* (AECS)

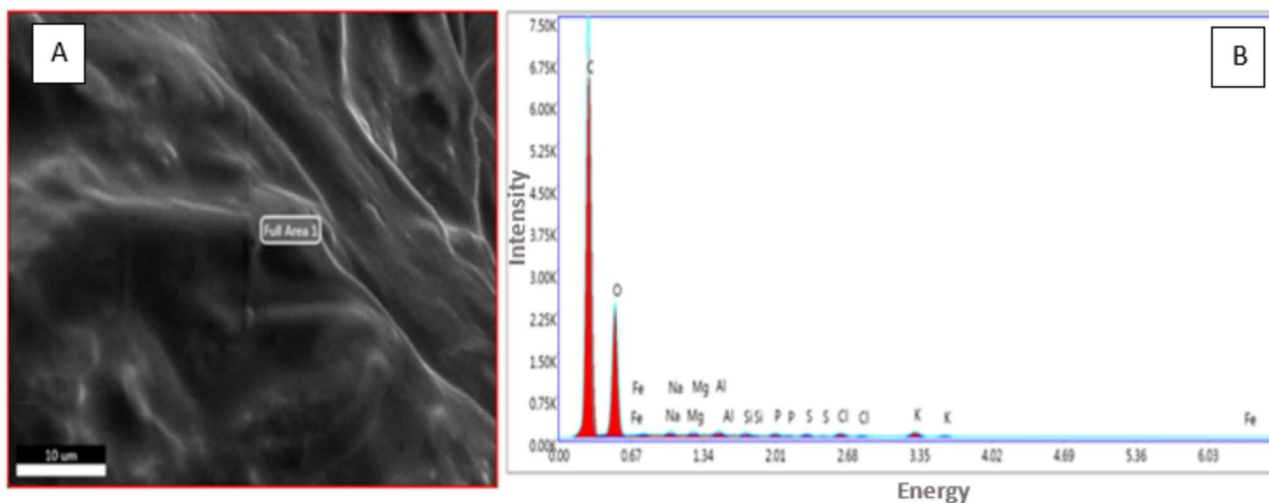
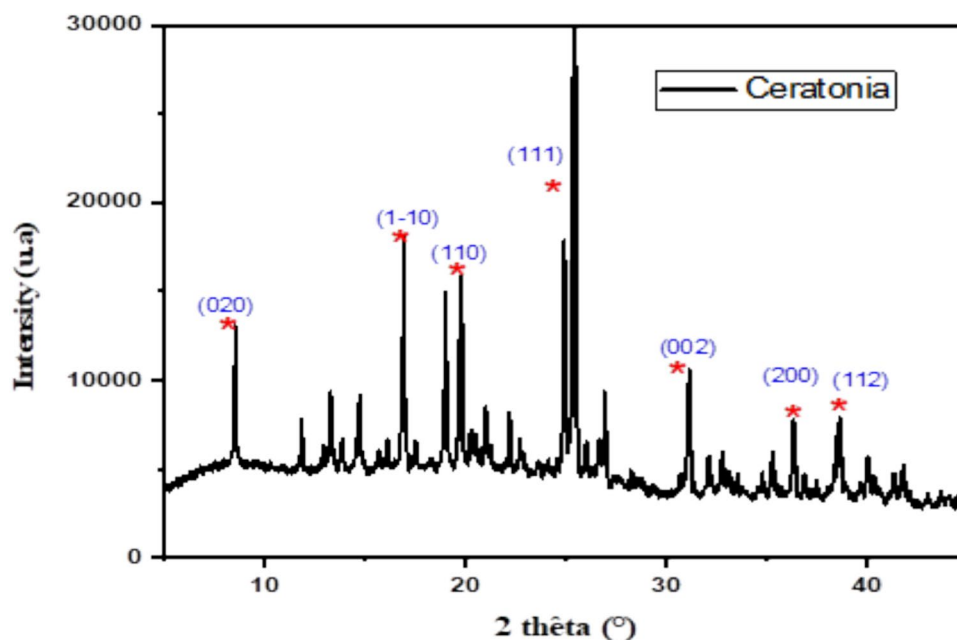


Fig. 4 Scanning electron microscope (SEM) and energy-dispersive X-ray (EDX) spectroscopy of AECS

Scanning Electron Microscope (SEM) and Energy-Dispersive X-ray Spectroscopy (EDX)

The combination of energy-dispersive EDX with SEM facilitated microanalysis of AECS, providing valuable insights into its chemical composition in terms of atomic and weight percentages. The SEM image and EDX analysis, as depicted in Fig. 4a, illustrated the amorphous arrangement of molecules within the extract. This structural depiction showcased globular fibers stabilized by specific mineral elements, as revealed by the EDX analysis. Furthermore, the elemental analysis results (Fig. 4b) of the extract underscored a significant mineralogical composition.

Notably, elements such as K and Cl were predominant in both weight and atomic percentages, as elaborated in Table 2. Additionally, other elements were detected at lower levels, including P, S, Si, Al, Mg, Na, and Fe. These findings indicate that AECS can be considered as a valuable source of mineral elements, which could potentially augment its efficacy in various biological applications.

Antioxidant Activity

Regarding standard antioxidant assays, AECS demonstrated a noteworthy antioxidant effect through both the DPPH free radical scavenging and reducing power assays. The superior antioxidant activity was evidenced by the lower IC_{50} values. Specifically, AECS exhibited significant antioxidant activity, with the lowest IC_{50} value observed in the DPPH assay ($IC_{50} = 0.24 \pm 0.06$ mg/mL), followed by reducing power assay ($IC_{50} = 6.54 \pm 0.008$ mg/mL). When comparing these results to those of reference antioxidants, it is evident that AECS exhibited superior efficacy compared to butylated hydroxytoluene (BHT) and quercetin for DPPH assay and slightly less efficacy than quercetin and BHT for reducing power assay (Table 3).

Antibacterial Activity

The antibacterial potential of AECS was evident through the inhibition zone diameter against the tested bacteria. At a concentration of 100 mg/mL, AECS displayed a significant mean inhibition zone of $19.0 \text{ mm} \pm 2.16$ when assessed against *S. aureus* (ATCC 25923). In parallel, when comparing AECS to Gentamicin against *E. faecium* (ATCC 4602), both AECS and Gentamicin exhibited similar inhibition zones of $11.3 \text{ mm} \pm 0.47$ and $12.22 \text{ mm} \pm 0.05$, respectively. However, in the case of gram-negative bacteria, the antibacterial activity of AECS appeared to be relatively subdued. The inhibition zone diameter for *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27653) were observed to be $16.00 \text{ mm} \pm 0.82$ and $12.67 \text{ mm} \pm 1.89$, respectively. In contrast, the positive control Gentamicin displayed larger inhibition zones, measuring $23.6 \text{ mm} \pm 0.08$ and $22.1 \text{ mm} \pm 0.03$ mm against *E. coli* and *P. aeruginosa*, respectively (Table 4, and Fig. S1).

To determine the effective concentration of AECS, we conducted broth microdilution tests to establish both the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). As depicted in Table 5, AECS exhibited a bactericidal effect against the tested bacteria, with MIC values as follows: *E. faecium* (6.25 mg/mL) *S. aureus* (0.78 mg/mL), *E. coli* (6.25 mg/mL), and *P. aeruginosa* (25 mg/mL). These MIC values signify the lowest extract concentration at which visible bacterial growth is inhibited, underscoring its inhibitory potential. However, it

Table 2 Elemental analysis of the AECS

	K	Cl	P	S	Si	Al	Mg	Na	Fe
Weight percentage(%)	2.31 ± 0.02	1.03 ± 0.01	0.62 ± 0.01	0.56 ± 0.01	0.50 ± 0.01	0.60 ± 0.01	0.43 ± 0.03	0.61 ± 0.01	0.03 ± 0.01
Atomic percentage(%)	0.80 ± 0.01	0.40 ± 0.02	0.24 ± 0.01	0.27 ± 0.01	0.24 ± 0.01	0.30 ± 0.04	0.24 ± 0.02	0.36 ± 0.01	0.01 ± 0.01

Results are presented as mean $\pm$ SEM.

Table 3 The antioxidant activity of the AECS by DPPH and FRAP

Antioxidant assay	Plant extract (IC ₅₀ mg/mL)	Standard antioxidant (IC ₅₀ mg/mL)	
		Quercetin	BHT
DPPH	0.24 ± 0.06	1.77 ± 0.05	1.85 ± 0.04
FRAP	6.54 ± 0.01	2.61 ± 0.02	1.04 ± 0.01

Results are presented as mean ± SEM.

is noteworthy that the MBC was found to be greater than 50 mg/mL, indicating that while AECS effectively inhibits bacterial growth, it may not necessarily result in complete bacterial eradication.

Discussion

In this study, we identified several bioactive compounds in *C. siliqua*, including gallic acid, catechin, and quercetin, which are known for their antioxidant properties. These compounds have the potential to mitigate oxidative stress in plants facing abiotic stressors such as drought and salinity. Beyond their antioxidant effects, these bioactive compounds offer a wide range of potential applications. Firstly, they can be integrated into breeding programs to develop stress-tolerant crop varieties, contributing to improved agricultural sustainability (Villalobos-López et al. 2022). Secondly, these compounds may serve as key ingredients in the formulation of biostimulants, promoting healthy plant growth, especially in challenging environments (Campobenedetto et al. 2021; Aremu et al. 2015). Thirdly, their potential as environmentally friendly biopesticides can aid in sustainable

pest management practices (Luzi et al. 2019; Iacopini et al. 2008). Moreover, the pharmaceutical and nutraceutical industries may benefit from their health-promoting properties, and their use in environmental remediation further showcases their versatility (Yeshe et al. 2022).

Analytical techniques, including HPLC–MS/MS allowed us to identify specific compounds presents, including gallic acid, catechin, and quercetin (Fig. 1). Corsi et al. (2002), detected the presence of gallic acid, (–) epigallocatechin-3-gallate, and (–) epicatechin-3-gallate in *C. siliqua* extracts pods and leaves. This finding aligns with the results of Goulas and Georgiou (2020), which also confirmed the predominance of gallic acid as primary phenolic compound in water extract. These compounds contribute to the rich bioactive composition of the extract. Notably, Abidar et al. (2020), reported that the flavonoid content in *C. siliqua* leaves is approximately 3 × higher than that of phenolic acids and 2 × as much as catechin. These substantial quantities underscore the value of *C. siliqua* leaves as a significant natural source of bioactive compounds. Gallic acid is renowned for its robust antioxidant properties, offering protection against oxidative stress (Mahindrakar et al. 2020; Badhani et al. 2015). In the same line, catechin, a prominent member of the flavonoid family, plays a pivotal role in mitigating the impact of abiotic stress, including salinity, drought, extreme temperatures, and oxidative stress, which disrupt cellular homeostasis and hinder plant growth and development (Sheng et al. 2023; Wang et al. 2022). Catechin, a well-documented antioxidant property, functions as a key defender against these stressors by acting as a potent scavenger of ROS, which are generated in excess under abiotic stress conditions (Wang et al. 2022; Bernatoniene and Kopustinskiene 2018), causing thereby oxidative damage to

Table 4 Inhibition zones (in mm) of the AECS (100 mg/mL) against microbial strains

	Bacterial strains	Reference	Diameter inhibition zone (mm)	
			AECS	Gentamicin
Gram-positive bacteria	<i>S. aureus</i>	ATCC 25923	19.0 ± 2.16	23.4 ± 0.02
	<i>E. faecium</i>	ATCC 4602	11.3 ± 0.47	12.2 ± 0.05
Gram-negative bacteria	<i>E. coli</i>	ATCC25922	16.0 ± 0.82	23.6 ± 0.08
	<i>P. aeruginosa</i>	ATCC 27653	12.7 ± 1.89	22.1 ± 0.03

Results are presented as mean ± SEM.

Table 5 Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the aqueous extract of the AECS (50 mg/mL) against microbial strains

	Bacterial strains	Reference	MIC (mg/mL)	MBC (mg/mL)
Gram-positive bacteria	<i>S. aureus</i>	ATCC 25923	6.25	> 50
	<i>E. faecium</i>	ATCC 4602	0.78	> 50
Gram-negative bacteria	<i>E. coli</i>	ATCC25922	6.25	> 50
	<i>P. aeruginosa</i>	ATCC 27653	25.0	> 50

Results are presented as mean ± SEM.

cellular components such as proteins, lipids. Additionally, catechin modulates signaling pathways involved in stress responses by activating the synthesis of stress-related proteins and trigger the activation of antioxidant enzymes (Fan et al. 2017). As such, understanding the role of catechin in abiotic stress responses is crucial for harnessing its potential in enhancing woody plant tolerance to adverse environmental conditions.

The diverse phytochemical composition of AECS suggests potential synergistic interactions among its bioactive compounds, which could enhance its overall biological activity. Synergy occurs when multiple compounds work together to produce a greater effect than the sum of their individual activities Tallarida 2011). For instance, flavonoids such as catechin, epicatechin, quercetin, and luteolin are known to exhibit strong antioxidant properties by scavenging free radicals and modulating oxidative stress pathways (Zahra et al. 2024). When combined with phenolic acids like gallic acid and caffeic acid, their antioxidant efficacy may be amplified through complementary mechanisms, such as metal ion chelation and inhibition of pro-oxidant enzymes (Tungmunthum et al. 2018). Additionally, rutin, a glycosylated flavonoid, can enhance the bioavailability and stability of quercetin, further strengthening its pharmacological effects (Carrillo-Martinez et al. 2024). These interactions highlight the importance of considering the whole extract rather than isolated compounds, as the natural matrix may optimize bioactivity through synergistic effects.

The characterization techniques (FTIR, SEM with EDX, and XRD) shed light on the extract chemical composition and physical structure (Figs. 2, 3, and 4, respectively), highlighting the presence of key functional groups and diverse molecules. The detection of mineral elements (i.e., K and Cl) suggests potential interaction with bioactive compounds. The presence of key functional groups ($-NH_2$ and $-OH$) indicates the existence of bioactive compounds like gallic acid, catechin, and quercetin, which possess antioxidant properties that can neutralize harmful free radicals, thereby safeguarding cells against oxidative damage (Hsouna et al. 2011). Furthermore, the diverse molecular composition unveiled by the diffractogram analysis suggests the presence of various compounds within the extract. The crystal reflections observed in the diffractogram may suggest the crystalline nature of gallic acid, catechin, and quercetin, contributing to their stability and potential biological activity. Additionally, the detection of mineral elements implies that these elements may interact with the bioactive compounds, enhancing their bioavailability and potential physiological effects (Sharifi-Rad et al. 2020; More et al. 2012). The study by Karmous et al. (2022), highlighted the promising role of *C. siliqua* in the synthesis of zinc oxide nanoparticles (ZnONPs) with antibacterial properties. The functional groups derived from *C. siliqua*, identified through FTIR analysis, act as capping

ligands, stabilizing the NPs and influencing their bioactivity (Dimkpa et al. 2022). These capping ligands consist of various phytochemicals, including flavones, alcohols, phenols, amines, carboxylic acids, sugars, and ketones, which interact with the zinc surface and aid in the stabilization of the NPs (Iqbal et al. 2021). The reduction of metal salts into NPs is primarily facilitated by the presence of sugars, while carboxylic and phenolic acids serve as effective capping agents, preventing agglomeration and governing the biosynthesis process. The screening of polyphenols and phenolic acids in *C. siliqua* further underscores the substantial contribution of flavonoids and other phenolic compounds to the capping and stabilization of NPs (Singh et al. 2011; Beattie and Haverkamp 2011). These findings demonstrate the potential of using *C. siliqua* as a natural source for environmentally friendly and cost-effective synthesis of stable and bioactive nanoparticles. The use of plant extracts as reducing and stabilizing agents in nanoparticle synthesis offers a sustainable alternative to conventional physical and chemical methods.

It is worth noting that the antioxidant activity detected in the *C. siliqua* extract may represent the plant's adaptive response to environmental stressors (Table 3). The complex interplay between antimicrobial activity, antioxidant potential, and stress tolerance reflects the multifaceted nature of plant responses to environmental challenges. Plants synthesize antioxidants as a defense mechanism against oxidative stress, which can be induced by both biotic and abiotic stressors. This oxidative stress, characterized by an overproduction of ROS, can compromise the plant's immune system, thereby increasing its susceptibility to pathogenic infections (Saijo et al. 2020). Antioxidants serve to neutralize these ROS, effectively protecting plant cells from oxidative damage.

Our study also showcased broad-spectrum antibacterial activity of *C. siliqua* extract, which is a highly encouraging finding (Tables 4 and 5). This potent antibacterial effect can be attributed to the abundance of total phenols and flavonoids, known for their antibacterial properties (Meziani et al. 2015). These bioactive compounds have the potential to disrupt microbial cell membranes and metabolic processes, inhibiting bacterial growth (Meziani et al. 2015; Tian et al. 2018). The antibacterial activities of the *C. siliqua* extract were evaluated against a panel of relevant pathogens, revealing significant inhibitory effects on both Gram-positive and Gram-negative bacteria. This broad-spectrum antibacterial activity is promising and can be attributed to the abundance of total phenols and flavonoids with strong anti-radical activity. These bioactive compounds disrupt microbial cell membranes, interfere with essential metabolic processes, and hinder bacterial growth and proliferation. The observed antibacterial activity of *C. siliqua* extract may be attributed to its ability to disrupt key microbial processes, including

attachment and invasion, motility, biofilm formation, and quorum sensing. By interfering with these processes, *C. siliqua* extract has the potential to compromise the integrity of bacterial cells, increase cell permeability, and ultimately result in the leakage of cytoplasmic contents (Abd El-Hack et al. 2022, 2020; Abdel-Moneim et al. 2021; Abou-Kassem et al. 2021). Our findings align with previous studies, demonstrating the antibacterial potential of woody plant extract (Ojala et al. 2000; Shafiee et al. 2016; Raffa et al. 2017; Khatun et al. 2017).

The robust antimicrobial defenses observed in our study can enhance a plant's resilience against challenges posed by biotic and abiotic stresses (Tables 4 and 5) (Ali et al. 2018). Of these defenses, antimicrobial peptides are among the most common and prominent chemical barriers that plants have developed to resist biotic stresses (Rehman et al. 2021). Yet, the direct link between antimicrobial properties and abiotic stress tolerance in plants is not well-studied. This dynamic interplay between antimicrobial activity and stress tolerance underscores the intricate nature of plant responses to environmental pressures (Li et al. 2021).

These compounds likely exert their antibacterial effects through multiple mechanisms. First, the phenolic compounds, such as gallic acid and caffeic acid, are known to disrupt microbial cell membranes (Oulahal and Degraeve 2022). This membrane destabilization leads to increased permeability, resulting in leakage of cytoplasmic contents and eventually cell death (Wong et al. 2021). In addition, flavonoids like quercetin and rutin can interfere with bacterial metabolic processes by inhibiting key enzymes involved in energy production and cell division, thereby hindering bacterial growth and proliferation (Ullah et al. 2020).

Moreover, these bioactive compounds may interfere with microbial processes critical for infection and virulence, including biofilm formation, quorum sensing, motility, and bacterial adhesion (Samrot et al. 2021). By inhibiting biofilm formation, the extract reduces the bacteria's ability to adhere to surfaces and form protective layers, making them more susceptible to antimicrobial agents (Sharma et al. 2023).

Altogether, our study on *C. siliqua* contributes to the broader understanding of plant tolerance in woody crops. The wealth of bioactive compounds, potent antioxidant activity, and broad-spectrum antibacterial properties make this plant a promising candidate for enhancing woody plant resilience against adverse environmental conditions. Our research contributes valuable insights to the field, offering innovative strategies to address the challenges encountered by woody crops. Further research is warranted to elucidate the underlying mechanisms under abiotic stress conditions.

Conclusions

In conclusion, this study highlights the phytochemical composition, antioxidant capacity, and antimicrobial activity of *Ceratonia siliqua* aqueous extract. Key bioactive compounds, including gallic acid, catechin, and quercetin, were identified, contributing to its strong antioxidant and antimicrobial properties. Characterization techniques such as HPLC–MS/MS, FTIR, and XRD provided critical insights into the extract's chemical profile, with HPLC–MS/MS proving particularly effective in identifying and quantifying phenolic compounds. These findings reinforce the therapeutic potential of *C. siliqua* and its application in natural product-based treatments.

Further research should explore the mechanisms of action underlying its bioactivity, assess its pharmacokinetics and toxicity *in vivo*, and evaluate its efficacy against multidrug-resistant pathogens. Comparative studies on different extraction and analysis methods could also enhance the optimization of bioactive compound isolation. These investigations will support the development of *C. siliqua*-derived formulations for pharmaceutical and nutraceutical applications.

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Declarations

Competing Interests The authors declare no competing interests.

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