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Original article

In vitro Anti-MDR Bacteria, Anti-inflammatory and Antioxidant Activities of Ceratonia siliqua L.

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Abstract
The present study demonstrated the *in vitro* antibacterial activity against multidrug-resistant (MDR) isolates, as well as the anti-inflammatory and antioxidant effects of the *Ceratonia siliqua* L. (*C. siliqua*) ethanolic extract from Al-Bayda City, located in the Green Mountain. The *C. siliqua* fruit extract was obtained using 75% ethanol. The agar-well diffusion method investigated the antibacterial activity against MDR isolates. The Albumin denaturation method was employed to demonstrate the anti-inflammatory activity, and the antioxidant activity of the extract was evaluated using DPPH scavenging activity. The extract showed promising antibacterial activity against MDR isolates, with higher inhibition zones against *S. aureus*, followed by *Ps. aeruginosa*, *K. pneumoniae*, and *E. coli*. The extract showed high inhibitory activity against albumin denaturation, with an IC_{50} of 368.5 µg/ml. Moreover, the DPPH scavenging method revealed a weaker antioxidant activity of the *C. siliqua* extract (42.86 ± 0.08 %) at a concentration of 500 µg/ml compared with the control. In summary, the fruit *C. siliqua* ethanolic extract is a promising therapeutic agent for MDR isolates and has the potential to reduce inflammation and oxidative stress.
Keywords: *C. siliqua*, Antibacterial, MDR Isolates, Anti-Inflammatory, Antioxidant.

Introduction

In the current medical field, bacterial resistance has become a pressing concern, and medicinal plants offer a viable solution [1]. Eradicating pathogens such as vancomycin-resistant enterococci (VRE) and methicillin-resistant Staphylococcus aureus (MRSA) has become increasingly complex. Approximately 2.8 million people in the US are estimated to fall ill each year due to antibiotic-resistant infections, resulting in over 35,000 deaths. Furthermore, Europe experiences approximately 33,000 fatalities annually due to antibiotic resistance [2,3]. The human body employs inflammation as a defense mechanism against pathogens; however, this response can also lead to pathological conditions and promote the development of various illnesses. High morbidity and mortality rates are often associated with gastrointestinal diseases [4]. Within the stomach, endogenous harmful agents are produced through lipid peroxidation, pepsin, HCl, and reactive oxygen species (ROS) [5]. While ROS play a role in normal physiological processes by controlling apoptosis or activating transcription factors, their unbalanced nature makes them highly reactive towards organic substrates and can cause deleterious oxidative modifications that are linked to various pathologies, including cancer, resulting from DNA mutations [6].

However, smoking, bacterial infections, excessive alcohol consumption, and regular use of nonsteroidal anti-inflammatory drugs can all lead to external influences. Nonetheless, these substances and medicinal plants have proven to be highly effective in treating a variety of conditions, such as bacterial infections, inflammatory responses, and scavenging free radicals [7].

Ceratonia siliqua L., commonly referred to as "Carob" in local circles, is a perennial tree that was originally indigenous to the Mediterranean region and other areas with comparable climates, including Libya, where it is locally named Kharob [8,9]. The carob tree is economically and environmentally significant because of its positive impact. Its fruit, known as a pod, is a vital food source and a raw material for industrial purposes. Carob pods are rich in numerous physiologically active substances such as polyphenols, sugars, cyclitols, amino acids, fibers, and minerals [10-12]. Carob extract has garnered considerable attention because of its strong antibacterial and anti-inflammatory properties [13-16]. In this study, we utilized the egg albumin precipitation method to evaluate the anti-inflammatory activity, DPPH antioxidant scavenging analysis, and the agar well-diffusion method to investigate the antibacterial activity against *S. aureus*, *E. coli*, *K. pneumoniae*, and *Ps. aeruginosa* MDR isolates. The primary objective of this study was to examine the antibacterial, anti-inflammatory, and antioxidant properties of *C. siliqua* ethanolic crude extract.

Materials and methods

Chemicals and Reagents

The chemicals used in this study were ethanol 75% and methanol (Sigma Aldrich, Germany), Muller Hinton Agar (Hi Media Laboratories Pvt. Ltd., India), egg albumin (Fisher Scientific Company), phosphate-buffered

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saline (PBS) (Sigma-Aldrich), Gallic acid (Sigma Aldrich), and 2,2-diphenyl-1-picrylhydrazyl; DPPH (write the source). Also, the standard drugs Augmentin 30µg (Hikma, Jordan), Azithromycin 15µg, Ciprofloxacin 30µg (Eugen, London), and Ibuprofen are conc. Were used (Bristol Laboratories Ltd.).

Collection of plant materials

Fresh *C. siliqua* fruits were collected from Al-Bayda, Libya, between August and September 2023. Taxonomists at the Herbarium Department of Botany, Faculty of Sciences, Omar Al-Mukhtar University, Al-Bayda, Libya, taxonomically identified and authenticated the plant. The plant was air-dried in the shade with good ventilation before being finely powdered for extract production.

Figure 1. Left: C. siliqua, the tree, and right: the powder from Green Mountain.

Preparation of crude extract

C. siliqua was pulverized and dried for 7 days in the shade before being utilized for extraction. The plant was extracted using an overnight maceration process, following Harbone [17]. A weight of 50 g was macerated in 500 ml of 75% ethanol for three days at room temperature. The supernatant was decanted after random shaking for 24 hours at room temperature. The extract was then dried and concentrated using a hood. The residue was weighed (g), and the yield is mentioned in Table 1 after being calculated in Table 1 as follows:

Yield (%) = (weight of extract/ weight of the sample) x 100

The extract was stored at 4 °C until further use.

Table 1. Characteristics and yield gram (g) and percentage (%) of C. Siliqua extract

Plant species	Families	Local name	Parts used	Weight of extracts (g)	Yield (%)	Color	Consistency
C. Siliqua	Leguminosae	Carob	Fruits	23.63	47.26	Brown	Gummy

Tested bacterial isolates

Four clinical isolates of *S. aureus*, *E. coli*, *K. pneumoniae*, and *Ps. aeruginosa* were collected from the Al-Borj Laboratory in Al-Bayda, Libya. The isolates were purified by streaking on suitable selective and differential media and identified based on Gram staining results, microscopic examination, cultural characteristics, and biochemical tests [18].

Preparation of the bacterial suspension

Each bacterial isolate's overnight growth was harvested, dissolved in 0.9% sterile normal saline, and then calibrated with MacFarland solution (0.5) to obtain a bacterial suspension of 1×10^8 CFU.

Antibacterial Assay of Crude Extract

Adjustments were made using the agar-well diffusion (cup-plate) technique [19] To evaluate the antibacterial activity of the extract. The extract (100 mg) was thoroughly mixed and dissolved in sterile DW (1 mL). Muller-Hinton agar (MHA) was then divided into 20 ml aliquots and seeded with the tested bacteria (*S. aureus*, *E. coli*, *K. pneumoniae*, and *Ps. aeruginosa*) in sterile petri dishes. After setting the agar, the plate was divided into three cups using a sterile cork borer (No. 4). Each cup received 0.1 ml of extract, and the plate was incubated at room temperature for two hours and 37 °C for 18 hours. The test was performed in triplicate. The diameters of the growth inhibition zones were measured in millimeters (mm).

Determination of the minimum inhibitory concentration (MIC)

According to Andrews [20], the agar plate dilution method was utilized to determine the tested extract's minimum inhibitory concentration (MIC). Serial dilutions of the extract were prepared at concentrations of 6.25, 12.5, 25, 50, and 100 mg/ml. The antimicrobial activities of the extract against the test bacteria were

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evaluated in a test tube containing Mueller-Hinton Broth (MHB). The bacteria were inoculated into tubes containing the diluted extract, and the plates were incubated at 37 °C for 24 h. The lowest concentration of extract that showed no turbidity was recorded as the MIC.

Albumin denaturation method

This study applied the same method as Kumari, Yasmin [21], with some modifications, such as reducing the volume by half. Ibuprofen was used as a positive control, and 0.1% extract (1.0 mg/ml) was added to each tube at 200, 400, and 600 µg/ml. Next, 1400 µL of phosphate-buffered saline (PBS) and 200 µL of egg albumin were added, followed by incubation at 37°C for 15 min and heating at 70°C for 5 min. The absorbance was then measured at 660 nm using a Jasco V-630 Spectrophotometer (Japan), and the data were processed using the Spectra Manager system. The percentage of inhibition of protein denaturation was calculated using the following formula:

$$Denaturation\ inhibition\ (\%) = (ANC - AS/ANC) \times 100\%$$

Where ANC: is the absorbance of the negative control, and **AS:** is the absorbance of the sample

DPPH scavenging activity method

The ability of the *C. siliqua* extract to neutralize free radicals was assessed using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals, following the method described by Gunduz et al. The extract was tested at concentrations of 0.062, 0.125, 0.250, and 0.5 (Results) mg/ml, while gallic acid served as a positive control at concentrations of 0.031, 0.062, 0.125, and 0.25 mg/ml. The experiment combined each extract or gallic acid concentration with 0.5 mM DPPH (in methanol) in a micro well cuvette. The absorbance was measured at 517 nm after a 30-minute incubation, and the antioxidant activity was calculated as follows:

$$Antioxidant\ Activity\ (AA) = [NCA - (SA - BA)]/NCA \times 100$$

Whereas: SA: is the absorbance of the sample, **BA:** is the blank absorbance, and **NCA** is the absorbance of the negative control.

Statistical analysis

All experiments were conducted in triplicate, and the results are presented as Mean ± Standard Deviation (M±SD) and percentages (%). All assay results were analyzed using Statistical Package for the Social Sciences (SPSS) software (version 21.0; SPSS Inc., Chicago, IL, USA). Statistical significance was set at $P<0.05$.

Results and Discussion

Antibacterial activity

C. siliqua is a widely recognized medicinal plant that has been traditionally prescribed in various parts of Africa and Asia [22, 23]. Compounds derived from this plant are effective against various microbial infections and possess anti-cancer and antioxidant properties [9, 23]. This study aimed to expand our knowledge of *C. siliqua* collected from Green Mountain by demonstrating its *in vitro* antibacterial activity against MDR isolates and its anti-inflammatory and antioxidant effects using a crude ethanolic extract.

Notably, the antibacterial properties of *C. siliqua* extend to both standard and clinical isolates. [22]. This was demonstrated in this study, which investigated the ethanolic extract of *C. siliqua* for the first time against MDR *S. aureus*, *E. coli*, *K. pneumoniae*, and *Ps. aeruginosa* isolates compared to those of Augmentin, Azithromycin, and Ciprofloxacin included in this study. The results showed that the ethanolic extract of *C. siliqua* exhibits good antibacterial activities against *S. aureus*, *E. coli*, *K. pneumoniae*, and *Ps. Aeruginosa*. Resistant isolates were found to be more effective against *S. aureus* with inhibition zones (23.3 ± 0.12 mm), followed by *Ps. aeruginosa* (20.3 ± 0.05 mm), *K. pneumoniae* (19.3 ± 0.06 mm), and *E. coli* (15.7 ± 0.03 mm) (Tables 2 and 3). These results indicate that the extract is more effective against Gram-positive and Gram-negative bacteria than the resistance controls. The effectiveness of *C. siliqua* was reported as higher than Ampicillin, Clindamycin, Gentamicin, and Amikacin against the identical clinical isolates of *S. aureus*, *E. coli*, *K. pneumoniae*, and *Ps. aeruginosa* [22]. According to the findings of this study, the extract from *C. siliqua* was effective against multidrug-resistant bacteria, including those resistant to both Augmentin and Azithromycin. The extract showed significant growth inhibition against all the tested bacteria, whereas ciprofloxacin only showed partial effectiveness against *S. aureus* and *K. pneumoniae*. Unlike synthetic antibiotics, such as ciprofloxacin, which can have side effects, the extract from *C. siliqua* could be considered an alternative therapy due to its antibacterial properties. Previous studies attributed the antibacterial activity of *C. siliqua* to the presence of diosgenin, bioactive phytosterols, and sapogenin compounds [24].

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Table 2. Antibacterial activity of C. Siliqua extract against MDR isolates

Name of Extract/Drugs	Family/ Controls	Concentrations	Bacteria used			
			S. a	E. c	Ps. a	K. p
			MZID* (mm) ± SD			
C. siliqua	Leguminosae	100 mg/ml	23.3 ± 0.12	15.7 ± 0.06	20.3 ± 0.05	19.3 ± 0.06
D. Water	Control -ve	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Augmentin	Control +ve	50 µg/ml	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Ciprofloxacin	Control +ve		0.00 ± 0.00	26.0 ± 0.00	24.0 ± 0.01	0.00 ± 0.00
Azithromycin	Control +ve		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Key: *S. a= Staphylococcus aureus, E. c= Escherichia coli, Ps. a= Pseudomonas aeruginosa and K. p= Klebsiella pneumoniae. MDIZ* (mm) = Mean diameter of growth inhibition zone in mm. Values were expressed as Mean ± standard error (SEM); n=3 in each group*

Table 3. Minimum inhibitory concentrations of C. Siliqua extract against MDR isolates

Tested bacteria	Concentration (mg/ml)				
	6.25	12.5	25	50	100
	MZID* (mm) ± SD				
Staphylococcus aureus	-	15.0 ± 0.06	18.0 ± 0.02	20.0 ± 0.03	23.3 ± 0.12
Escherichia coli	11.0 ± 0.03	11.0 ± 0.12	12.0 ± 0.01	14.0 ± 0.05	15.7 ± 0.03
Pseudomonas aeruginosa	11.0 ± 0.12	12.0 ± 0.05	15.0 ± 0.04	17.0 ± 0.02	20.3 ± 0.05
Klebsiella pneumoniae	12.0 ± 0.05	13.0 ± 0.04	16.0 ± 0.05	18.0 ± 0.12	19.3 ± 0.06

Key: MDIZ (mm) = Mean diameter of growth inhibition zone in mm. Values were expressed as Mean ± standard error (SEM); n=3 in each group. Inhibition zones were measured in mm, and cup diameter was included*

Anti-inflammatory activity

To investigate the anti-inflammatory effects of the ethanolic extract of *C. siliqua*, we evaluated its ability to inhibit albumin denaturation at 70 Â °C. The data in Table 4 illustrate the activity of *C. siliqua*. Our findings demonstrate that *C. siliqua* showed significant potential in addressing inflammatory conditions by inhibiting the denaturation of albumin induced by high temperatures in a dose-dependent manner. Moreover, the highest inhibitory activity of *C. siliqua* was observed at a concentration of 600 µg/mL, with values of 52.57%, 50.0% at 400 µg/mL, and 47.55% at 200 µg/mL, compared to ibuprofen, which exhibited a lower inhibition rate of 43.88% (Table 4). This study represents the first *in vitro* anti-inflammatory investigation of *C. siliqua* from Green Mountain, Libya, which provides a potential therapeutic option for treating inflammatory conditions. Previous research has shown that the *C. siliqua* leaf extract exhibits anti-inflammatory activity [25]. This finding is supported by the IC_{50} value of the *C. siliqua* extract (368.6 µg/ml) revealed in this study. However, previous studies reported different inhibition percentages [26, 27]. The discrepancies in previous studies may be attributed to the use of leaf and seed extracts in the previous studies. However, the precise mechanism underlying the anti-inflammatory effects of the extract remains unclear and requires further investigation.

Table 4. In-vitro anti-inflammatory activity of C. siliqua extract against albumin denaturation

Cons. (µg/ml)	Inhibition (%)	IC_{50} (µg/ml)
200	47.55	368.5
400	50.00	
600	52.57	
Control -ve	0.00	-
Ibuprofen (+ve)	43.88	ND

Key: Cons: *concentrations;* ***IC_{50}:*** *50% of inhibition concentration*

Antioxidant activity

To determine the antioxidant capabilities of the *C. siliqua* extract, its DPPH scavenging activity was assessed. The ability of the C. siliqua extracts to decrease DPPH radicals at various concentrations was examined. The results indicated that *C. siliqua* exhibited weak scavenging activity against DPPH radicals (Table 5). This finding agrees with a previous study that reported a higher IC_{50} value for *C. siliqua*, suggesting a lower

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antioxidant activity [23]. On the other hand, 500 µg/ml of *C. siliqua* in this study showed 42.86 ± 0.08 RSA% DPPH scavenging activity. Other previous studies reported an IC_{50} range of 200 to 1500 mg/ml for the DPPH scavenging activity of the *C. siliqua* plant [23, 28]. Moreover, the variations in antioxidant activities have been attributed to the differences in phenolic compound concentrations in *C. siliqua* from various geographical areas [23, 29].

Table 5. Scavenging radical activity of C. siliqua extract

Name of plant and control	Solvent	RSA* ± SD% (DPPH)	IC_{50} ± SD (µg/ml)
C. siliqua	Ethanolic	42.86 ± 0.08	ND
Gallic acid	Std.	62.01 ± 0.02	103.3 ± 0.05

Key: RSA*: *Radicals scavenging activity, (n; 3),* ***DPPH:*** *2, 2, Diphenyl -1- Picrylhydrazyl, SD: standard Division, Std: Standard.* ***IC_{50}****: value represents the sample concentration required to inhibit 50% of the DPPH free radical.*

Although this study is significant for screening and expanding the knowledge on the potential therapeutic applications of *C. siliqua* fruit extract against bacteria-resistant isolates, as well as its anti-inflammatory and antioxidant activities, it lacks phytochemical screening and the cytotoxicity of active compounds, which are considered limitations of this study. Therefore, further comprehensive research is needed on the phytochemicals and cytotoxicity of the compounds found in *C. siliqua* from the Green Mountain.

Conclusion

The research indicates that the *C. siliqua* crude ethanolic extract exhibits strong antibacterial activity against *S. aureus*, *E. coli*, *K. pneumoniae*, and *Ps. aeruginosa* antibiotic-resistant isolates. In addition, *C. siliqua* showed potent anti-inflammatory effects against albumin denaturation. This study also demonstrated that *C. siliqua* has radical scavenging activity. Further investigation is necessary to isolate and identify the pure active components and to assess the safety and potential adverse effects of the *C. siliqua* extract.

Acknowledgments

None.

Conflicts of Interest

None to declare.

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