In vitro characterization of *Ceratonia siliqua* in terms of antimicrobial activity, antioxidant capacity, and blood compatibility

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Abstract

This study investigated the biological properties of the ethanolic extract of *Ceratonia siliqua* fruits, focusing on antimicrobial activity, antioxidant capacity, and hemocompatibility. Traditional antimicrobial activity was evaluated with the agar diffusion method, and uses Candida, Staphylococcus, and Pseudomonas species, and other bacteria, and uses varied concentrations of 50, 100, 150, 200, and 250 mg/ml. The extract showed a concentration-dependent inhibitory effect, where 250 mg/ml had the highest activity and produced inhibition zones of 20 mm for Candida, 14 mm for Staphylococcus, and 15 mm for Pseudomonas. The DPPH radical scavenging assay was used to evaluate antioxidant activity and showed excellent free radical inhibition of 83.95% at 100 mg/ml. The dose-response relationship was confirmed by the gradual decline of activity at lower concentrations. Hemocompatibility by hemolysis assay has been checked on a human erythrocytes, and the extract presented zero percent of hemolysis at every concentration that was tested (12.5 to 100 mg/ml). This shows its blood compatibility is an excellent compared to what is observed with positive control. Overall, this research shows the plant extract has shown important antimicrobial ability and antioxidant benefits while the initial safety also seems good which is why it could offer a possibilities as natural origin for the medicine and science uses in coming times.

Keywords: *Ceratonia siliqua*, plant extraction, Staphylococcus, DPPH, Hemolysis,

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Introduction

Microbial resistance to antibiotics is increasing and escalating globally, leading to higher rates of morbidity and mortality associated with bacterial infections. This has prompted researchers to explore alternative natural sources, particularly medicinal plants, due to their wide range of biologically active compounds that have shown promising potential as natural antimicrobials (1). Beyond antimicrobial activity, oxidative stress is increasingly recognized as a major factor contributing to the development of many chronic diseases. Reactive oxygen species (ROS) can damage essential cellular components such as proteins, lipids, and nucleic acids, leading to cellular dysfunction and disease progression (2). Natural plant extracts rich in phenolic compounds and flavonoids are widely investigated due to their ability to neutralize free radicals and reduce oxidative damage. Therefore, evaluating the antioxidant potential of medicinal plants has become an important step in identifying biologically active natural compounds. One of the most widely used methods for assessing antioxidant capacity is the DPPH radical scavenging assay, which measures the ability of compounds to donate hydrogen atoms or electrons to stabilize free radicals (4,5). The hemolysis assay is a recognized indicator for assessing biosafety and determining the compatibility of a tested substance with hematopoiesis and the integrity of the red blood cell membrane (6). The absence of hemolysis is a preliminary indicator of biocompatibility and potential for future therapeutic use of any biological extract (7). *Ceratonia siliqua* is gaining popularity in both traditional medicine and modern science for its rich and diverse array of bioactive compounds. Recent reviews show the plant's extracts exhibit some antioxidant and antimicrobial activity, calling for rigorous controlled in vitro study evaluations (8). *Ceratonia siliqua* (carob) is known to contain a wide range of bioactive phytochemicals, including polyphenols, flavonoids, tannins, gallic acid derivatives, and dietary fibers (9). Several studies have demonstrated that these compounds contribute to their antioxidant, antimicrobial, anti-inflammatory, and metabolic regulatory activities (10). In particular, the high phenolic content of carob fruits has been strongly associated with free radical scavenging capacity and inhibition of microbial growth (11). Thus, Although several studies have reported the phytochemical composition of *Ceratonia siliqua*, limited studies have simultaneously evaluated its antimicrobial activity, antioxidant capacity, and hemocompatibility in a single experimental framework. Therefore, the present study aims to provide an integrated biological evaluation of the ethanolic extract of *Ceratonia siliqua* fruits under in vitro conditions.

Materials and Methods

1. Preparation of the Plant Extract

The fruits of *Ceratonia siliqua* were collected from local markets. They were then cleaned and dried at room temperature, away from direct sunlight,

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and ground into a fine powder. Extraction was carried out in the Pharmacognosy Laboratory at the Faculty of Pharmacy, Jabir Ibn Hayyan University of Medical and Pharmaceutical Sciences. Extraction was carried out using the maceration method with 70% ethanol as the extraction solvent. The procedure was performed according to standard phytochemical extraction protocols with slight modifications (9). The plant powder was macerated in the solvent for 72 hours with periodic stirring. The extract was then filtered using filter paper and concentrated using a rotary evaporator under reduced pressure to obtain the crude extract, which was stored at 4°C until use.

2. Antibacterial test

The agar well diffusion method was performed following standardized antimicrobial testing procedures as described by (11) and CLSI guidelines. Some changes were made that are not a major to the standard protocols when testing. Standard strains of microbes such as Candida spp., Staphylococcus spp. as well as the Pseudomonas spp. were handled and grown on the Mueller-Hinton agar for bacterial types and a Sabouraud dextrose agar for fungal ones before testing could begin. The microbial mixture was made using sterile saline and density was matched to 0.5 McFarland standard, close to an 1 x 10^8 CFU/ml for bacteria. After getting agar plates ready and adding microbial suspension to them, wells having 6 mm diameter were made. Then, a 100 microliter of the plant extract with strengths like 50, 100, 150 and also 250 mg/ml was poured inside. Plates were kept still for one hour so extract could move around after which incubation took place at 37°C for 24 hours for bacteria; fungi needed 48 hours instead. The inhibition zone sizes were calculated in a millimeter units and a clearly visible zone was taken as proof for antimicrobial action. Every experiment used three separate repeats while findings are shown as an average with the standard deviation.This technique works by observing how substances move through solid medium and if it stops microbes from growing; the principle is described by (10 and 11).

3. Antioxidant Activity Assessment

Antioxidant activity evaluation used an offline (DPPH) assay. The DPPH (1,l-diphenyl-2-picrylhydrazyl) radical cation technique was adapted to assess the ability of one hundred pure chemical compounds to scavenge free radicals. The DPPH reagent was DPPH 200 ul as a control in the first well of microplate. In a 96-well microplate, 100 uL DPPH reagent was mixed with 100 uL of 100 µL of DPPH solution was mixed with 100 µL of *Ceratonia siliqua* extract at different concentrations and incubated in dark at the room temperature for 30 mintues to measure scavenging activity (13). The absorbance was determined at 514 nm using an ELISA reader (TECAN, Grading, Austria). After incubation, 100 per cent methanol was used as a blank (11).The following formula was used to calculate the DPPH scavenging effect: Radical scavenging (%)= [control – sample/ control] x 100

4. Hemolysis Assay

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On the blood of one healthy donor, hemolysis experiments were performed. The hemolysis was identified using Triton X-100 as a positive control indicator. A sterilized phosphate buffer saline solution was employed as a negative control, allowing_ the stock solution to be stored at room temperature shaking plate for 2-4 hours on the plate. After the on an incubation period, the sample was centrifuged at 10,000 rpm. In a 96-well plate, Supernatant was read using a microplate scanning spectrophotometer at 550 nm. None of the extract concentrations used (125, 250, 500, and 1000) micrograms/ml caused hemolysis in all the blood samples examined (6).

5. Statistical Analysis

All experiments were conducted with three independent replicates, and results were presented as mean ± standard deviation (Mean ± SD). Statistical methods that are a proper were applied for checking significant differences, where value for the significance was at less than p of 0.05 according to (13).

Results and discussion

1. Antimicrobial activity

Microbial resistance to conventional antibiotics has become a major global health concern, leading to increased morbidity and mortality associated with infectious diseases (2). As a result, there is growing interest in exploring natural products, particularly plant-derived compounds, as alternative antimicrobial agents. Many medicinal plants contain bioactive phytochemicals such as phenolic compounds, flavonoids, and alkaloids that possess both antimicrobial and antioxidant properties. These compounds can interact with microbial cell membranes, interfere with enzyme systems, and induce oxidative imbalance in microbial cells, thereby inhibiting microbial growth. The data in Table 1 and Figure 1 indicate that concentration is the primary factor determining the antimicrobial activity of the extract. The extract's effect against Candida (12 mm) began to appear at a concentration of 150 mg/ml, and this effect became more pronounced at the higher concentration of 250 mg/ml, reaching 20 mm. The relatively high concentrations used in this study are consistent with preliminary screenings of crude plant extracts. Crude extracts usually require higher concentrations compared with purified compounds because they contain complex mixtures of active and inactive phytochemicals. Similar concentration ranges have been reported in antimicrobial screening studies of plant extracts. On the other hand, the absence of zones of inhibition is recorded at 50 mg/ml and 100 mg/ml. In relation to Staphylococcus and Pseudomonas, sensitivity was recorded at only the highest concentration, which had 14 and 15 mm inhibition diameters, respectively. The results indicate a clear concentration-dependent antimicrobial effect of the *Ceratonia siliqua* extract, as the inhibition zones increased with increasing extract concentration (Figure 1). Such dose–response behavior is commonly observed in plant extracts due to the gradual increase in the availability of bioactive phytochemicals at higher concentrations. The observed antimicrobial activity may be attributed to the presence

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of phenolic compounds, flavonoids, and other secondary metabolites known to interfere with microbial cell membrane integrity, disrupt metabolic pathways, and inhibit essential enzymatic activities in microorganisms. Phenolic compounds can also cause protein denaturation and increased membrane permeability, ultimately leading to microbial cell damage. Similar antimicrobial effects of plant-derived phenolic compounds have been reported in previous studies investigating medicinal plant extracts (12). The variation in sensitivity among the tested microorganisms may also be related to differences in their cell wall structure, particularly between Gram-positive and Gram-negative bacteria, which can influence the penetration of bioactive compounds into microbial cells.

Table 1. Antibacterial activity of the plant extract against selected microorganisms

Inhibition zone diameter (mm)				**Microorganism**	**No.**
250	**150**	**100**	**50**		
20 ± 1.1	12 ± 0.8	0 ± 0	0 ± 0	candida	1
14 ± 0.9	0 ± 0	0 ± 0	0 ± 0	Staph	2
15 ± 0.7	0 ± 0	0 ± 0	0 ± 0	Pseudomonas	3

Values are expressed as mean ± SD (n = 3); p < 0.05 was considered statistically significant

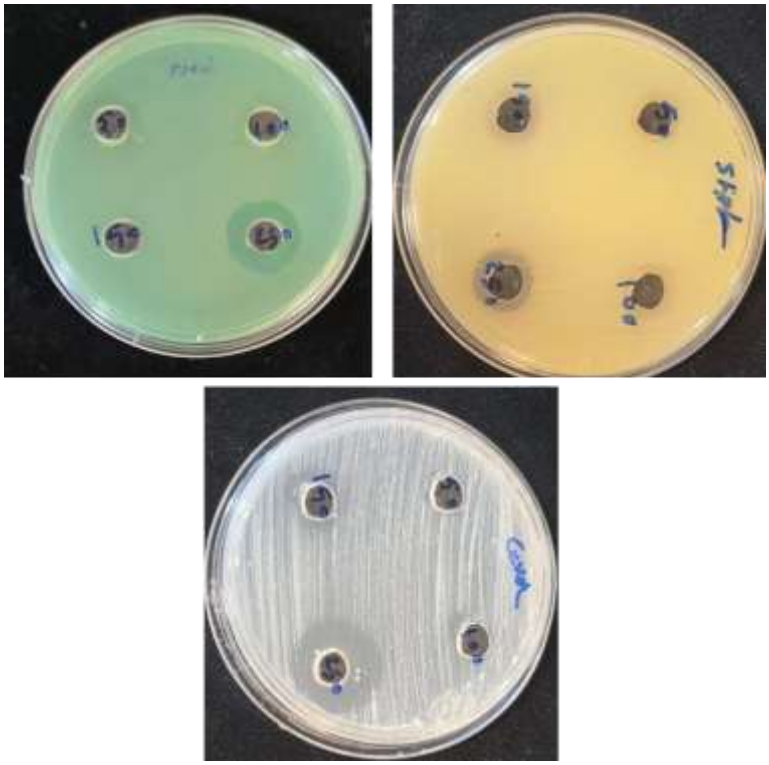

Figure 1. Antibacterial activity of the plant extract at different concentrations against tested microorganisms

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2. Antioxidant activity using DPPH assay

Table (2) and figure (2) from the DPPH assay show that *Ceratonia siliqua* extract gives a clear indication of having antioxidant activity and that this activity depends on the concentration of the extract. The greatest percent of free radical inhibition is at a concentration of 100 mg/ml (83.95%), and there is a gradual decrease in radical scavenging activity with a decrease in concentration of the extract at 50 mg/ml (inhibition of 75.82%), 25 mg/ml (inhibition of 65.87%), and 12.5 mg/ml (inhibition of 39.52%). This decreases scavenging activity corresponds to increased absorbance value (signals lower DPPH radical consumption at lower concentration). This extract has more hydrogen-donating and/or electron donating compounds (like phenolic compounds) and/or flavonoids that are responsible for antioxidant activity based on this concentration. Compared with the control sample, which exhibited high absorbance, the marked decrease in absorbance at all tested concentrations indicates the strong free radical scavenging ability of the extract. This reduction reflects the presence of hydrogen- or electron-donating compounds capable of neutralizing DPPH radicals. Such antioxidant activity is commonly associated with phenolic compounds and flavonoids present in plant extracts. Previous studies have demonstrated that the antioxidant activity measured by the DPPH assay is strongly correlated with the phenolic content of plant extracts (4,5,14). Similar findings have been reported in several medicinal plants where higher phenolic content resulted in stronger radical scavenging activity.

Table 2. Antioxidant activity of *Ceratonia siliqua* fruit extract determined by the DPPH radical scavenging assay

Scavenging %	**Absorbance**	**Concentration (mg/ml)**	**Sample number**
83.95 ± 1.10	0.1748 ± 0.01	100	**1**
75.82 ± 1.35	0.2633 ± 0.02	50	**2**
65.87 ± 1.40	0.3717 ± 0.02	25	**3**
39.52 ± 1.75	0.6587 ± 0.03	12.5	**4**
	1.0891 ± 0.04	control	

Values are expressed as mean ± SD (n = 3); $p < 0.05$ was considered statistically significant.

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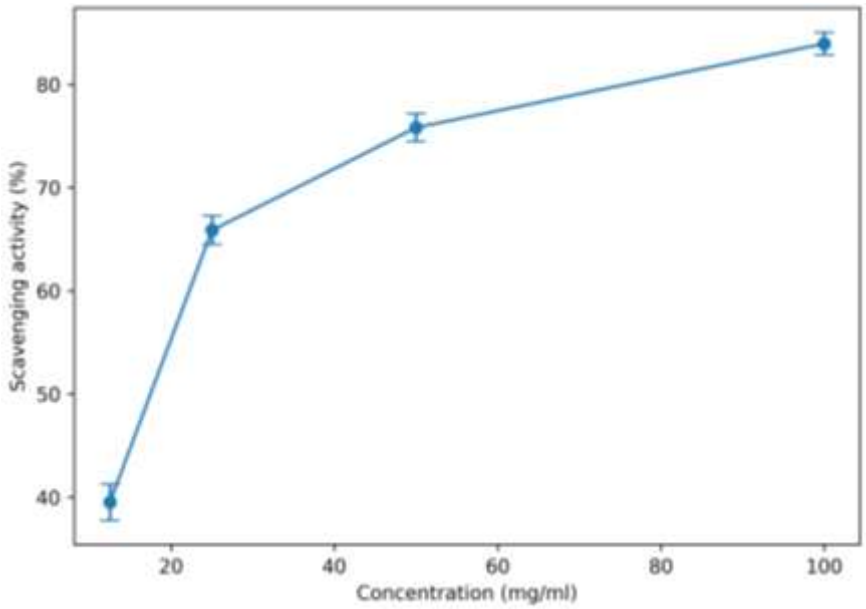

Figure 2. Concentration-dependent DPPH radical scavenging activity of *Ceratonia siliqua* extract. Values are expressed as mean ± SD (n = 3).

3. Hematological safety assessment of plant extract

The hemolysis test results in Table 3 and Figure 3 indicate that the extract (*Ceratonia siliqua*) exhibits a high degree of safety against hemolysis at all tested concentrations (12.5–100 mg/ml), showing no hemolysis (0%) compared to the positive control sample, which showed 100% hemolysis. The relatively low absorption values compared to the positive control sample indicate no damage to the red blood cell membrane, reflecting the extract's good biocompatibility. The positive control sample exhibited total hemolysis (100%), supporting the effectiveness and validity of the experimental system, while the negative control sample exhibited no hemolysis. These results indicate that the plant extract does not induce hemolytic damage to erythrocyte membranes at the tested concentrations, suggesting good hemocompatibility. Similar observations have been reported in hemocompatibility studies of bioactive compounds and biomaterials, where the absence of hemolysis indicates good biological safety and compatibility with blood components (8).

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Table (3): Effect of *Ceratonia siliqua* extract on red blood cell breakdown

Hemolysis %	Test result	Concentration (mg/ml)
0	0.9291 ± 0.03	**100**
0	0.9079 ± 0.02	**50**
0	0.7434 ± 0.03	**25**
0	0.5901 ± 0.02	**12.5**
100	1.5437 ± 0.04	**POSITIVE**
0	0.2526 ± 0.01	**NEGATIVE**

Values are expressed as mean ± SD (n = 3); $p < 0.05$ was considered statistically significant

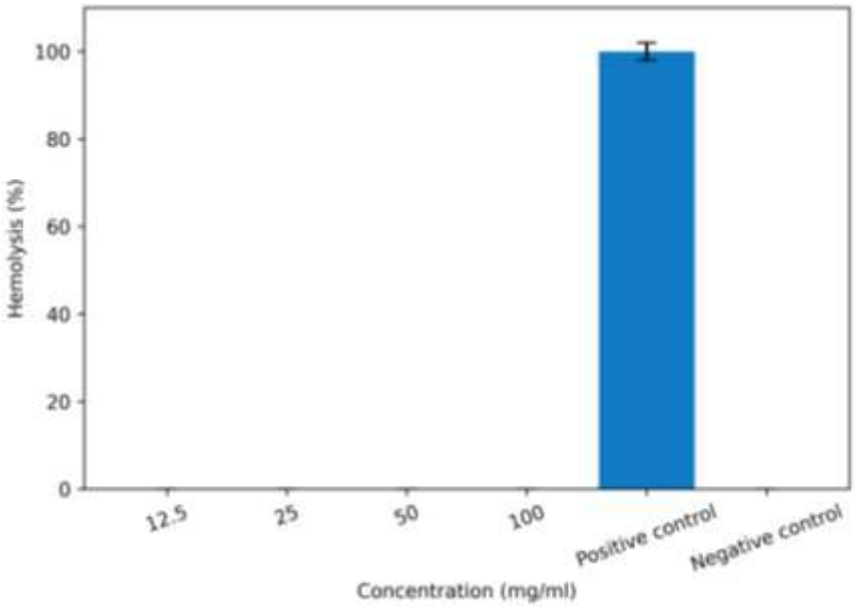

Figure 3. Hemolytic activity of *Ceratonia siliqua* extract on human red blood cells at different concentrations. Values are expressed as mean ± SD (n = 3).

Conclusion

The results of this study indicate that that the carob (Ceratonia siliqua) fruit extract ethanol was active at a concentration-dependent manner exhibiting greater antimicrobial ability against pathogenic microorganisms. Additionally, this carob ethanol extract also neutralized free radicals at DPPH concentration supporting antioxidant properties to the extent likely conferred primarily through phenols and flavonoids present within the carob. Additionally, the hemolysis assay demonstrated an excellent biocompatibility and feasible for early studies of safety with all concentrations tested were not hemolytically active, suggesting that the

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extract of Ceratonia siliqua would be a viable natural source of pharmaceutical and biological bioactive compounds; however, further analysis will be required to isolate and evaluate their efficacy in various biological systems.

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