

Antibacterial and Antivirulence Activities of *Capparis spinosa* L. against *Acinetobacter baumannii* and *Streptococcus mutans*

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Abstract

The urgent hunt for new antimicrobial medicines has been spurred on by the increasing threat of multidrug-resistant bacterial diseases. Against two therapeutically important pathogens, *Acinetobacter baumannii* and *Streptococcus mutans*, this work assessed the antibacterial and antivirulence effects of *Capparis spinosa* L. leaf and flower extract. Assessed were antibacterial effects using minimum bactericidal concentration (MBC), minimum inhibitory concentration (MIC), and disk diffusion tests. Furthermore, quantitative real-time PCR (qRT-PCR) examined the expression levels of the virulence-associated genes *ompA* and *gtfB*. Concerning concentration-dependent antibacterial activity, *S. mutans* shown more sensitivity than *A. baumannii*. For *S. mutans*, MIC values were 16 mg/mL and for *A. baumannii* they were 32 mg/mL. Significantly downregulation of *ompA* and *gtfB* at sub-inhibitory extract concentrations ($p < 0.001$) indicated by gene expression analysis suggested compromised adhesion and biofilm development capacity. These results show *C. spinosa* extract as a potential natural agent that not only prevents bacterial development but also disturbs important pathogenic processes. It is advised to investigate its active components and in vivo efficiency further.

Keywords: Antibacterial activity, Antivirulence strategy, *Capparis spinosa* L., Multidrug-resistant bacteria.

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Introduction

Public health systems all over are seriously challenged by the global rise of antimicrobial resistance (AMR). Usually resulting in longer hospital admissions, more medical expenses, and greater fatality rates, infections brought on by multidrug-resistant (MDR) bacteria are becoming more difficult to cure (World Health Organisation, 2022). Consequently, research of alternative treatment approaches—especially those using natural compounds with demonstrated bioactivity—is much needed. Among the great variety of therapeutic plants, *Capparis spinosa* L., also known as the caper plant, has shown promise but little study has been done on it.

Native to the Mediterranean region and certain areas of Asia, perennial plant *Capparis spinosa* has long been prized for both food and medicinal qualities. Treatment for rheumatism, liver illnesses, and other inflammatory diseases (Sekeroglu et al., 2020) is among traditional applications. Rich in bioactive chemicals including flavonoids (particularly rutin and quercetin), alkaloids, glucosinolates, and many terpenoids, recent phytochemical studies on *C. spinosa* leaves, flowers, and fruits have discovered *C. spinosa* is a rather appealing option for the creation of new antibacterial agents as these molecules are well known for their antimicrobial, antioxidant, and anti-inflammatory properties. Though *C. spinosa* has a strong

phytochemical profile, few research have methodically investigated the molecular pathways by which it produces antibacterial effects, especially with regard to virulence gene expression in pathogenic bacteria. It is important to close this gap as contemporary antimicrobial treatments concentrate not only on stopping bacterial growth but also on reducing bacterial pathogenicity, hence making pathogens less likely to cause disease (Clatworthy et al., 2007). We concentrate in this work on two clinically important pathogens: *Streptococcus mutans* and *Acinetobacter baumannii*. Often resistant to several kinds of antibiotics, *A. baumannii* is well known for producing serious hospital-acquired infections, especially in critical care units (Howard et al., 2023). Strongly linked to the outer membrane protein A (OmpA), a fundamental component in adherence to host cells, invasion, and biofilm formation (Nie et al., 2020) is its pathogenicity. Conversely, *S. mutans* is a main agent in dental caries as it depends mostly on its capacity to generate extracellular polysaccharides by means of glucosyltransferase B (GtfB), therefore enabling strong biofilm development on tooth surfaces (Bowen et al., 2018). Targeting these virulence elements—OmpA and GtfB—offers a creative therapy strategy. Even without total bacterial eradication, the pathogenic potential of these organisms can be greatly lowered by compromising the adhesion, invasion, or formation of protective biofilms by the bacterium. As a potential complement to conventional antibiotics, this antivirulence approach has attracted growing interest (Rasko & Sperandio, 2010).

This work aims primarily to explore the antibacterial activity of *C. spinosa* leaf and flower extract against *A. baumannii* and *S. mutans* and to evaluate its effects on the expression of the *ompA* and *gtfB* genes, respectively. We use quantitative real-time PCR (qRT-PCR) to track changes in gene expression levels; disk diffusion assays to evaluate preliminary antibacterial activity; broth microdilution techniques to ascertain minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC). This work aims to give fresh understanding of the antibacterial and antivirulence capabilities of *Capparis spinosa* by combining conventional botanical knowledge with modern molecular technologies. The results might support not just the continuous worldwide quest for alternate approaches to reduce the growing challenge of antibiotic resistance but also the scientific confirmation of conventional medical treatments.

Materials and Methods

Plant Material Collection and Extract Preparation

Fresh leaves and flowers of *Capparis spinosa* L. were collected during the peak flowering season (April–May 2024) from wild populations located in the semi-arid region of Al-Najaf, Iraq. Botanical identification was performed by a plant taxonomist at the Department of Botany, University of Al-Najaf, and a voucher specimen (No. CPS-2024-05) was deposited in the departmental herbarium for future reference.

The plant materials were thoroughly washed with sterile distilled water to remove dirt and debris, then air-dried in a shaded, well-ventilated environment at ambient temperature ($25\pm2^{\circ}\text{C}$) for two weeks until complete dryness was achieved, minimizing potential degradation of phytochemicals (Harborne, 1998). The dried samples were ground into a fine powder using a sterile mechanical grinder and stored in airtight glass containers at 4°C until extraction.

Extraction was performed using the maceration method. Briefly, 100 g of powdered material was immersed in 1 L of 70% ethanol (v/v) and left to macerate for 72 hours at room temperature with occasional stirring. The mixture was then filtered through Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure using a rotary evaporator (Heidolph, Germany) set at 40°C to obtain a semi-solid crude extract, which was further dried in a desiccator. The dried extract was weighed and reconstituted in dimethyl sulfoxide (DMSO) to prepare stock solutions at concentrations suitable for subsequent assays (Sasidharan et al., 2011).



Bacterial Strains and Culture Conditions

Two clinical strains were used in this study:

- *Acinetobacter baumannii* ATCC 19606
- *Streptococcus mutans* ATCC 25175

Bacterial strains were obtained from the American Type Culture Collection (ATCC) and maintained on Mueller-Hinton agar (MHA) slants at 4°C. Prior to experiments, bacteria were sub-cultured twice onto fresh MHA plates to ensure active growth. Bacterial suspensions were prepared in sterile saline (0.85% NaCl) and adjusted to the turbidity equivalent of a 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL) (Isenberg, 2004).

Antibacterial Susceptibility Testing

Disk Diffusion Assay

The preliminary antibacterial activity of *C. spinosa* extract was evaluated using the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2023).

- Mueller-Hinton agar plates were uniformly inoculated with bacterial suspensions using sterile cotton swabs.
- Sterile 6 mm filter paper disks were impregnated with 20 μ L of extract solutions at concentrations of 5, 10, 25, 50, and 100 mg/mL, then placed on the inoculated plates.
- Positive control disks contained ciprofloxacin (5 μ g) for *A. baumannii* and chlorhexidine (0.12%) for *S. mutans*.
- Disks impregnated with 1% DMSO served as negative controls.
- Plates were incubated at 37°C for 24 hours under aerobic conditions (for *A. baumannii*) and 5% CO₂ conditions (for *S. mutans*).

After incubation, inhibition zone diameters were measured in millimeters (including the disk diameter) using a digital caliper. All tests were performed in triplicate.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC were determined by the broth microdilution method following CLSI protocols (CLSI, 2023).

- Two-fold serial dilutions of the extract (ranging from 1 to 128 mg/mL) were prepared in Mueller-Hinton broth (MHB) within 96-well microtiter plates.
- Each well was inoculated with 100 μ L of bacterial suspension, achieving a final concentration of 5×10^5 CFU/mL.
- Plates were incubated for 24 hours at 37°C under the appropriate atmospheric conditions.

MIC was defined as the lowest concentration at which no visible turbidity was observed.

To determine MBC, aliquots from wells showing no visible growth were sub-cultured onto fresh MHA plates and incubated as described. MBC was defined as the lowest concentration resulting in a $\geq 99.9\%$ reduction in CFU compared to the initial inoculum (Wiegand et al., 2008; Salim, et al., 2025).

Time-Kill Assay

To assess the bactericidal kinetics of the extract, time-kill assays were performed (Pankey & Sabath, 2004).



- Bacterial suspensions were treated with extract concentrations corresponding to 1× MIC and 2× MIC.
- Aliquots were taken at 0, 2, 4, 6, 8, and 24 hours, serially diluted, and plated on MHA.
- Colony counts (CFU/mL) were determined after 24-hour incubation.
- Bactericidal activity was defined as a $\geq 3 \log_{10}$ reduction in CFU/mL relative to the initial inoculum.

RNA Extraction and cDNA Synthesis

Following extract treatment at sub-inhibitory concentrations ($\frac{1}{2}$ MIC), total RNA was extracted from bacterial cells using TRIzol reagent (Invitrogen, USA) following manufacturer instructions.

- Bacterial pellets were lysed in 1 mL TRIzol, and RNA was separated using chloroform, precipitated with isopropanol, washed with ethanol, and resuspended in RNase-free water.
- RNA purity and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).
- cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) with random primers according to the manufacturer's protocol.

Quantitative Real-Time PCR (qRT-PCR)

The expression levels of the *ompA* gene in *A. baumannii* and *gtfB* gene in *S. mutans* were evaluated using SYBR Green-based qRT-PCR (Applied Biosystems StepOnePlus system).

Each 20 μ L PCR reaction mixture contained:

- 2 μ L cDNA template
- 10 μ L SYBR Green Master Mix
- 0.5 μ L of each specific primer (10 μ M)
- 7 μ L RNase-free water

Thermal cycling conditions:

- 95°C for 2 minutes (initial denaturation)
- 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.
- A melting curve analysis was conducted to confirm amplification specificity.

The 16S rRNA gene was used as the internal reference for normalization. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001).

Statistical Analysis

All experiments were conducted in triplicate and the results are expressed as the mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used to assess statistical significance, followed by a Tukey's post hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant. All analyses were performed using GraphPad Prism version 9.0.



Results and Discussion

Disk Diffusion Assay

The initial evaluation of antibacterial activity through the disk diffusion assay revealed a clear, concentration-dependent inhibitory effect of *Capparis spinosa* L. extract against both *Acinetobacter baumannii* and *Streptococcus mutans*. The relationship between extract concentration and inhibition zone diameter was linear, indicating a direct proportionality between the amount of active phytochemicals and the antibacterial effect exerted.

As presented in Table 1, *S. mutans* exhibited significantly larger zones of inhibition compared to *A. baumannii* at all concentrations tested. At 100 mg/mL, the inhibition zone for *S. mutans* reached an impressive 20.5 ± 1.2 mm, whereas *A. baumannii* demonstrated a relatively modest zone of 15.3 ± 1.0 mm. These findings suggest that *S. mutans*, a Gram-positive bacterium, is inherently more susceptible to plant-based extracts. This is consistent with previous reports indicating that the thick peptidoglycan layer of Gram-positive bacteria facilitates better interaction and penetration of hydrophobic plant-derived molecules compared to the additional outer membrane present in Gram-negative bacteria (Silhavy et al., 2010).

Interestingly, even against *A. baumannii*, a notoriously drug-resistant Gram-negative pathogen, the extract demonstrated notable inhibition, reinforcing the potential of *C. spinosa* as a broad-spectrum antibacterial agent. Given the global concern over MDR *A. baumannii* strains (Howard et al., 2023), finding natural agents capable of even moderate inhibition is of significant clinical relevance.

Table 1. Inhibition Zone Diameters (mm) of *C. spinosa* Extract

Extract Concentration (mg/mL)	<i>A. baumannii</i> (mm)	<i>S. mutans</i> (mm)
5	7.2 ± 0.5	10.1 ± 0.6
10	9.5 ± 0.7	13.8 ± 0.8
25	11.7 ± 0.9	16.2 ± 0.9
50	13.4 ± 1.0	18.3 ± 1.1
100	15.3 ± 1.0	20.5 ± 1.2

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC values, as shown in Table 2, provided quantitative confirmation of the extract's inhibitory and bactericidal properties.

For *S. mutans*, the MIC was 16 mg/mL and the MBC was 32 mg/mL. In contrast, *A. baumannii* required double these concentrations (MIC = 32 mg/mL; MBC = 64 mg/mL), further corroborating the notion that Gram-negative bacteria exhibit greater resistance due to their robust outer membrane acting as a permeability barrier to many antimicrobial agents (Ghosh et al., 2020).

Notably, the MIC/MBC ratios (2:1) for both organisms suggest a bactericidal rather than merely bacteriostatic effect of the extract, which is highly desirable in antimicrobial development. According to Pankey and Sabath (2004), a MIC/MBC ratio ≤ 4 indicates bactericidal activity, thus supporting the promising therapeutic profile of *C. spinosa*.

Table 2. MIC and MBC Values of *C. spinosa* Extract

Bacterial Strain	MIC (mg/mL)	MBC (mg/mL)
<i>A. baumannii</i>	32	64
<i>S. mutans</i>	16	32



Time-Kill Assay

Time-kill kinetic assays provided dynamic insights into the bactericidal efficiency of the extract over time. At 1× MIC concentration:

- *S. mutans* exhibited a $>3 \log_{10}$ CFU/mL reduction within 8 hours.
- *A. baumannii* demonstrated a more delayed response, requiring up to 24 hours to achieve similar bactericidal effects.

At 2× MIC concentration:

- *S. mutans* was completely eradicated within 6 hours.
- *A. baumannii* achieved total killing within 18 hours.

These results confirm that *C. spinosa* exerts a time- and concentration-dependent killing effect. The faster clearance of *S. mutans* is in line with previous studies demonstrating that Gram-positive organisms often succumb more rapidly to plant-derived antimicrobials (Singh et al., 2019). However, the extract's ability to kill *A. baumannii*, albeit at a slower rate, remains highly significant given the organism's reputation for extreme resilience.

The bactericidal profile observed here is particularly important for clinical applications, especially in immunocompromised patients where rapid bacterial clearance is crucial to prevent systemic infections (Magill et al., 2022).

Gene Expression Analysis (qRT-PCR Results)

The expression of key virulence-associated genes, *ompA* in *A. baumannii* and *gtfB* in *S. mutans*, was significantly suppressed upon treatment with sub-inhibitory concentrations of *C. spinosa* extract.

As detailed in Table 3:

- *ompA* expression was downregulated by approximately 58%, suggesting impaired adhesion and biofilm formation potential.
- *gtfB* expression decreased by about 62%, indicating a likely disruption in the production of glucans essential for biofilm integrity.

Both reductions were statistically significant ($p < 0.001$).

These findings are consistent with the growing body of literature emphasizing that plant-derived compounds can interfere with bacterial quorum sensing, adhesion mechanisms, and biofilm matrix production, rather than solely exerting direct bactericidal effects (Kalia, 2013).

Moreover, targeting virulence factors without necessarily killing the bacteria offers a distinct therapeutic advantage: it reduces selective pressure for resistance, as the bacterial survival is not directly threatened (Rasko & Sperandio, 2010). This antivirulence strategy is increasingly being recognized as a revolutionary approach in the post-antibiotic era.

Table 3. Relative Expression of *ompA* and *gtfB* Genes

Gene	Bacterial Strain	Relative Expression (Fold Change)	p-value
<i>ompA</i>	<i>A. baumannii</i>	0.42 ± 0.05	<0.001
<i>gtfB</i>	<i>S. mutans</i>	0.38 ± 0.04	<0.001



Comparative Analysis with Previous Studies

While several plant compounds have been demonstrated to kill *S. mutans* and *A. baumannii* bacteria, few research have really examined how to suppress virulence genes. For instance, the Punica granatum (pomegranate) ethanol solution killed *S. mutans* bacteria but not tested *gtfB* gene expression (Koo et al., 2017). Water-based *Nigella sativa* extracts similarly were able to prevent *A. baumannii* from proliferating but not *ompA* expression (Al-Dabbagh & Hameed, 2017).

Therefore, the emphasis of this work on both antibacterial and antivirulence effects closes a significant void in present knowledge and reveals how *C. spinosa* has latent therapeutic value.

Conclusion

This research showed that an extract of *Capparis spinosa* L. is very good at killing *Acinetobacter baumannii* and *Streptococcus mutans* bacteria and viruses. The extract stopped the growth of bacteria and greatly reduced the production of the *ompA* and *gtfB* genes. These results show that it might be a good natural way to fight bacteria that are resistant. In the future, researchers should focus on finding active chemicals and testing how well they work in living things.

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