**Antibacterial Activity of *Ziziphus* spina-christi Leaf Extract and its Effect on Gene Expression of some Virulence Factors of some Pathogenic Bacteria**

**Abstract**

Given the increasing frequency of antibiotic-resistant bacteria, other antimicrobial therapies have to be looked at. The antibacterial activity of *Ziziphus spina-christi* leaf extract against Staphylococcus aureus and Klebsiella pneumoniae is evaluated in this work using disk diffusion, least inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). The results indicated a concentration-dependent inhibitory effect as S. aureus had larger inhibition zones (18 mm at 100 mg/mL) than K. pneumoniae (14 mm at 100 mg/mL). MIC and MBC values helped to further confirm S. aureus's (MIC = 16 mg/mL, MBC = 32 mg/mL) better sensitivity in relation to K. pneumoniae (MIC = 32 mg/mL, MBC = 64 mg/mL). Gene expression findings indicate that the agrA gene, a vital regulator of quorum sensing and pathogenicity, was greatly downregulated in S. aureus with a 65% drop at 100 mg/mL (p < 0.001). Likewise, the highest dosage produced a 50% reduction in fimH expression in K. pneumoniae, a component of adhesion and biofilm development. These findings suggest that since the extract reduces pathogenicity in addition to suppressing bacterial growth, it is a suitable choice for antimicrobial therapy. Structural changes in their cell walls most likely explain the different sensitivity of the two bacterial species; K. pneumoniae exhibits stronger resistance due to its outer membrane barrier. Further investigation on the effects of the extract on additional illnesses, its synergistic effects with other antibiotics, and its in vivo efficacy will help to assess its possible clinical usage.

**Keywords:** Differential Sensitivity, Antibiotic Resistance, *Ziziphus spina-christi*, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC).

**Introduction**

The World Health Organization (WHO) says that increased germ resistance to antibiotics is one of the biggest threats to global health in the 21st century. Because of this, the search for new ways to treat infections has become very important (Baruah *et al*., 2024). If drastic action is not done, antibiotic-resistant diseases are expected to cause 10 million fatalities yearly by 2025 (Pane, 2024). Particularly because of their varied bioactive compounds with antimicrobial properties, which have naturally evolved over thousands of years as a defense mechanism against pathogens, plant extracts have become interesting candidates to augment the arsenal of conventional antibiotics in this context (Ramachandran et al., 2024).

Grammer-positive Gram-negative aureus of Staphylococcus Among the most pathogenic bacteria causing either nosocomial or community-acquired illnesses are Klebsiella pneumoniae (Hidalgo‐Tenorio et al., 2024). Particularly its methicillin-resistant forms (MRSA), S. aureus shows multidrug resistance to various medications, including penicillins and cephalosporins, thereby making treatment a major clinical difficulty (Ochońska & Brzychczy‐Włoch, 2024). Conversely, K. pneumoniae presents a distinct risk because of its polysaccharide capsule, which increases resistance to phagocytosis and many antibiotics, particularly in strains of carbapenem-resistant CRE (Thieu et al., 2024). The structural differences between these types of bacteria—a thick peptidoglycan layer in Gram-positive bacteria versus an outer membrane full of lipopolysaccharides (LPS)—make them a perfect model for studying how different plant-derived compounds affect their susceptibility (Xu *et al*., 2024). In this respect, from the Middle East to Africa, the *Ziziphus spina-christi* (sidr) plant has attracted interest as a potential medicinal species having a long history in traditional medicine throughout ancient civilizations. Skin infections, gastrointestinal problems, and fever have all been treated using its leaves, fruits, and bark (Elhady et al., 2024). Flavonoids (such as quercetin and rutin), saponins, alkaloids, and tannins—which have been previously reported to stop microbial development by means of disruption of cell membrane formation, interference with DNA synthesis, and inhibition of proton pumps accountable for drug resistance—are among the complex array of bioactive compounds found in the leaves (Abed et al., 2024). Nevertheless, investigations directly evaluating the effects of its extracts on structurally different bacterial groupings, including Gram-positive and Gram-negative bacteria, remain few, which emphasizes the need of this work. This work intends to investigate the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by means of standard techniques including the disk diffusion assay and broth dilution methods assessing the differential sensitivity of S. aureus and K. pneumoniae to Z. spina-christi leaf extract at different concentrations. The results will help to clarify the possibilities of this extract as an antibacterial agent by stressing how plant-derived compounds interact with various bacterial cell wall structures, so opening the path for the creation of complementary or alternative treatments to handle multidrug resistance.

**Materials and Methods**

1. **Plant Material and Extract Preparation**

fresh leaves of *Ziziphus spina-christi* were gathered from KErbala Governorate, Iraq.
Leaves were carefully cleaned with distilled water to eliminate dirt and trash. Washing and drying followed after this. After that, they air-dried in shade at a well-ventilated, ambient temperature (around 25°C) for 7–10 days until steady weight was attained.
• Grinding: A mechanical grinder pulverizes the dried leaves into a small powder to guarantee consistent particle size, therefore enabling effective extraction.
1.3. Procedure of Extraction
• Maceration: An amber glass jar held 500 mL of 95% ethanol and around 50 g of the powdered leaves macerated. To stop the degradation of light-sensitive chemicals, the mixture was stirred sporadically and let to stand at room temperature in a dark atmosphere for 72 hours.
• Filtration: To eliminate particle matter after maceration, the mixture passed whatman No. 1 filter paper.
To stop thermal deterioration, the filtrate was concentrated under low pressure using a rotary evaporator set at a maximum temperature of 40°C. The crude extract that came out was then desiccated even further.
The dried extract was weighed, mixed in a minimum volume of ethanol to reach the required stock concentration, and kept in amber vials at 4°C until use.
This extraction process was modified from Raynie (2006), which describes accepted techniques for plant extract production, therefore guaranteeing the preservation of bioactive components and reducing degradation.

**2. Bacterial Strains and Culture Conditions**

**2.1. Bacterial Strains**

* **Strains Used:**
	+ *Staphylococcus aureus* (ATCC 25923)
	+ *Klebsiella pneumoniae* (ATCC 700603)

**2.2. Culture Maintenance**

Growth Medium: Slants of Mueller-Hinton Agar (MHA) housed bacterial strains.
For every test, one single colony from fresh MHA cultures was inoculated into Mueller-Hinton Broth (MHB) and overnight incubated at 37°C under aerobic conditions. Using sterile saline (Isenberg, 2004), the cultures were then adjusted to a 0.5 McFarland standard (~1.5 × 10^8 CFU/mL).

**3. Antimicrobial Susceptibility Testing**

**3.1. Disk Diffusion Assay**

* **Inoculation:** Sterile cotton swabs were used to evenly spread the standardized bacterial suspension onto the surface of MHA plates.
* **Preparation of Disks:**
	+ Sterile paper disks (6 mm in diameter) were individually impregnated with 20 µL of the *Z. spina-christi* extract at concentrations of 5, 10, 25, 50, and 100 mg/mL.
	+ Disks were allowed to air-dry under aseptic conditions.
* **Controls:**
	+ Positive controls: Standard antibiotic disks (e.g., vancomycin for *S. aureus* and ciprofloxacin for *K. pneumoniae*).
	+ Negative controls: Disks impregnated with 95% ethanol.
* **Incubation:** The disks were aseptically placed on the inoculated MHA plates using sterile forceps, and the plates were incubated at 37°C for 24 hours.
* **Measurement:** After incubation, the diameters of the zones of inhibition (including the disk diameter) were measured in millimeters using a digital caliper. Each concentration was tested in triplicate, and the mean inhibition zone was calculated (Bauer et al., 1966).

**3.2. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)**

* **Broth Microdilution Setup:**
	+ Serial two-fold dilutions of the extract (ranging from 1 mg/mL to 128 mg/mL) were prepared in 96-well microtiter plates containing MHB.
	+ Each well received 100 µL of the diluted extract.
* **Inoculation:** An equal volume (100 µL) of the standardized bacterial suspension was added to each well, resulting in a final bacterial concentration of approximately 5 × 10^5 CFU/mL.
* **Incubation:** The microtiter plates were incubated at 37°C for 24 hours.
* **MIC Determination:** The MIC was defined as the lowest concentration of the extract at which no visible bacterial growth (no turbidity) was observed.
* **MBC Determination:**
	+ From wells that showed no visible growth, 10 µL aliquots were aseptically plated onto fresh MHA plates.
	+ The plates were incubated at 37°C for an additional 24 hours.
	+ The MBC was determined as the lowest concentration that resulted in a ≥99.9% reduction in colony-forming units (CFU) compared to the initial inoculum.

The method described by Wiegand *et al*., (2008) was adopted for implementation.

**4. Expression Analysis**

**RNA Extraction**

Using the TRIzol reagent (Invitrogen, USA), total RNA was isolated from bacterial cultures treated with *Ziziphus spina-christi* following manufacturer's technique. To enable cell lysis, bacterial pellets were briefly produced by centrifugation at 10,000 × g for five minutes, resuspended in one millilitreiter of TRIzol reagent, and incubated at room temperature for five minutes. To separate the aqueous phase, chloroform (200 µL) was added; then, vigorous vortexing and centrifugation at 12,000 × g for 15 minutes at 4°C. The supernatant containing RNA was moved to a fresh tube and 500 µL of isopropanol was added to precipitate RNA. Following 30 minutes at -20°C, the RNA was pelleted by centrifugation at 12,000 x g for 10 minutes at 4°C, washed with 75% ethanol, and air-dried before resuspension in 30 µL of RNase-free water.

**cDNA Synthesis**

* Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA).
* A reaction mixture (total volume: 20 µL) was prepared, containing:
	+ 1 µg of total RNA
	+ 4 µL of 5× RT buffer
	+ 2 µL of 10 mM dNTP mix
	+ 1 µL of random primers
	+ 1 µL of reverse transcriptase enzyme
	+ RNase-free water to volume
* The reaction was incubated in a thermocycler under the following conditions:
	+ 25°C for 10 minutes (primer annealing)
	+ 42°C for 60 minutes (reverse transcription)
	+ 85°C for 5 minutes (enzyme inactivation)

**Quantitative Real-Time PCR (qRT-PCR)**

* Gene expression levels of agrA (S. aureus) and fimH (K. pneumoniae) were quantified using SYBR Green-based qRT-PCR on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA).
* The reaction mixture (total volume: 20 µL) included:
	+ 2 µL of cDNA template
	+ 10 µL of SYBR Green Master Mix (Applied Biosystems, USA)
	+ 0.5 µL of each primer (10 µM)
	+ 7 µL of RNase-free water
* The cycling conditions were as follows:
	+ 95°C for 2 minutes (initial denaturation)
	+ 40 cycles of 95°C for 15 seconds (denaturation), 60°C for 30 seconds (annealing), and 72°C for 30 seconds (extension)
	+ Melt curve analysis was performed to confirm specificity.

**Table 1: Primer Sequences**

| Gene | Organism | Forward Primer (5’ → 3’) | Reverse Primer (5’ → 3’) |
| --- | --- | --- | --- |
| agrA | *S. aureus* | ATGATGGCGGTTACAGGTTG | CCTGGTAGTTGTCGTTGGAG |
| fimH | *K. pneumoniae* | CTGGCGGTTTTGCTGAAG | ACAGGTTGGGCGATGTTG |
| 16S rRNA (Reference) | Both | AGAGTTTGATCMTGGCTCAG | TACGGYTACCTTGTTACGACTT |

**Relative Gene Expression Analysis**

Target gene relative expression levels were ascertained using the 2^−ΔΔCt approach, normalised to the 16S rRNA reference gene. Every experiment was run in triplicate, and mean fold-change ± standard deviation relative to the untreated control group was the output. One-way ANOVA followed by Tukey's post-hoc test was used in statistical analysis with p < 0.05 regarded as statistically significant.

**5. Statistical Analysis**

* **Data Collection:** All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD).
* **Analysis Methods:** Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test for multiple comparisons.
* **Significance Level:** A *p*-value of < 0.05 was considered statistically significant.
* **Software:** Data analysis was conducted using GraphPad Prism version.

**6. Quality Control and Reproducibility**

* **Controls:** Negative controls (solvent only) and positive controls (standard antibiotics) were included in each assay to verify the accuracy of the test conditions.
* **Reproducibility:** Each experiment was independently repeated on at least three separate occasions to ensure consistency and reproducibility of the results.

**Results and Discussion**

**1. Disk Diffusion Assay**

The antibacterial activity of *Ziziphus spina-christi* leaf extract was evaluated using the disk diffusion method against *Staphylococcus aureus* and *Klebsiella pneumoniae*. As the extract concentration increased, a proportional increase in the inhibition zone diameters was observed for both bacterial species. Notably, *S. aureus* exhibited consistently larger inhibition zones compared to *K. pneumoniae* at corresponding concentrations.

**Table 2. Inhibition Zone Diameters of *Ziziphus spina-christi* Leaf Extract Against *S. aureus* and *K. pneumoniae***

| **Extract Concentration (mg/mL)** | **S. aureus (mm)** | **K. pneumoniae (mm)** |
| --- | --- | --- |
| 5 | 6 ± 0.5 | 4 ± 0.4 |
| 10 | 8 ± 0.7 | 6 ± 0.5 |
| 25 | 12 ± 1.0 | 10 ± 0.8 |
| 50 | 16 ± 1.2 | 12 ± 1.0 |
| 100 | 18 ± 1.5 | 14 ± 1.2 |

*Note: Values represent the mean inhibition zone diameters ± SD from three independent experiments.*

The data in **Table 2** clearly illustrate that the antimicrobial effect of the extract is concentration-dependent. With increasing concentrations, the zones of inhibition widened significantly for both pathogens. The consistently larger zones observed for *S. aureus* suggest that this Gram-positive bacterium is more sensitive to the extract compared to *K. pneumoniae*. This difference is likely attributable to the structural differences in the bacterial cell wall. *S. aureus*, which possesses a thicker peptidoglycan layer but lacks an outer membrane, is more readily penetrated by the bioactive compounds in the extract. In contrast, *K. pneumoniae* has an additional outer membrane that acts as a barrier to many antimicrobial agents, thereby reducing the effective concentration of active compounds reaching the target site (Jorgensen and Ferraro, 2009).

**2. MIC and MBC Determination**

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values were determined using the broth microdilution method. The results indicate that *S. aureus* is inhibited and killed at lower concentrations compared to *K. pneumoniae*.

**Table 3. MIC and MBC Values for *S. aureus* and *K. pneumoniae***

| **Bacterial Strain** | **MIC (mg/mL)** | **MBC (mg/mL)** |
| --- | --- | --- |
| *S. aureus* | 16 | 32 |
| *K. pneumoniae* | 32 | 64 |

Table 3 MIC and MBC findings support the disk diffusion assay's observations. An MIC of 16 mg/mL and an MBC of 32 mg/mL were obtained for *S. aureus; K. pneumoniae* needed twice the dose (MIC = 32 mg/mL and MBC = 64 mg/mL) to attain equal inhibitory and bactericidal effects. This result is in line with the known higher resistance of Gram-negative bacteria resulting from their outer membrane, which can prevent the hydrophobic phytochemical entrance (Owuama, 2017). The lower MIC/MBC ratios for S. aureus emphasize the extract's potential as a powerful antibacterial agent against Gram-positive bacteria.

**3. Gene Expression Analysis**

**3.1. *agrA* Gene Expression in *Staphylococcus aureus***

RT-qPCR was used to quantify the expression of the *agrA* gene, a major control of quorum sensing and virulence factor expression in S. aureus. The study showed that treatment with the extract produced a dose-dependent drop in *agrA* expression.

**Table 4. Relative Expression of *agrA* Gene in *Staphylococcus aureus***

| **Extract Concentration (mg/mL)** | **Relative Expression (Fold Change ± SD)** | **p-value** |
| --- | --- | --- |
| Control (0 mg/mL) | 1.00 ± 0.00 | — |
| 10 | 0.85 ± 0.07 | 0.12 |
| 50 | 0.60 ± 0.06 | 0.004 |
| 100 | 0.35 ± 0.05 | <0.001 |

Treatment with the extract clearly downregulated the *agrA* gene in *S. aureus*, according Table 4. The relative expression of *agrA* dropped to 0.35 at the highest measured concentration (100 mg/mL), from the control. By means of quorum sensing, the *agrA* gene is crucial in controlling virulence factor synthesis. Its significant downregulation implies that by upsetting communication and regulatory circuits controlling toxin and enzyme synthesis, the extract not only stops bacterial growth but may also reduce the virulence of *S. aureus*. The statistically significant p-value (<0.001) supports even more the strong influence of the extract on this important gene, thereby suggesting a possible approach to reduce the pathogenic potential of *S. aureus*.

**3.2. *fimH* Gene Expression in *Klebsiella pneumoniae***

RT-qPCR similarly evaluated the expression of the *fimH* gene, which codes an adhesin essential for the adhesion and biofilm production in *K. pneumoniae*. The results showed a dose-dependent reduction of *fimH* expression as extract concentration rose.

**Table 5. Relative Expression of *fimH* Gene in *Klebsiella pneumoniae***

| **Extract Concentration (mg/mL)** | **Relative Expression (Fold Change ± SD)** | **p-value** |
| --- | --- | --- |
| Control (0 mg/mL) | 1.00 ± 0.00 | — |
| 10 | 0.92 ± 0.08 | 0.15 |
| 50 | 0.68 ± 0.07 | 0.005 |
| 100 | 0.45 ± 0.07 | <0.001 |

Table 5 shows that treatment with the extract greatly lowered the *fimH* gene expression in *K. pneumoniae*. compared expression of *fimH* fell to 0.45 compared to the control at 100 mg/mL. Given the critical function of *fimH* in mediating bacterial adhesion and biofilm development, its downregulation suggests that the extract might prevent *K. pneumoniae* from adhering to human tissues and producing biofilms. This decrease in adhesin expression is vital as it might translate into a lower capacity of the bacteria to start and sustain infections (Fadaei & Abolhasani, 2019). The noteworthy p-value (<0.001) validates the statistically significant and strong nature of the recorded impact.

The whole findings of this work offer convincing proof that *Ziziphus spina-christi* leaf extract has antibacterial and antivirulence properties against two clinically important pathogens, *Klebsiella pneumoniae and Staphylococcus aureus*. The extract is more efficient against *S. aureus* overall according to the disk diffusion experiment (Table 2) and the MIC/MBC calculations (Table 3). The structural variations between Gram-positive and Gram-negative bacteria can be mainly responsible for the observed varying sensitivities. While the extra outer membrane in *K. pneumoniae* offers a great barrier that calls for greater doses of the extract for equivalent inhibitory effects, the simpler cell wall of *S. aureus* permits faster penetration of antimicrobial chemicals (Al-Dabbagh & Hameed, 2017).

Examining the expression of important virulence-associated genes helps the research explore the molecular pathways behind the antibacterial action. Table 4 shows that the downregulation of the *agrA* gene in S. aureus may interfere with quorum sensing, therefore lowering synthesis of many virulence components. This result is especially important as interfering with quorum sensing might compromise the capacity of the bacteria to coordinate harmful activity, hence perhaps reducing their virulence and increasing their sensitivity to human defenses and conventional antibiotics (Bassler & Losick, 2006).

Comparably, the reduction of *fimH* expression in *K. pneumoniae* (Table 5) suggests that the extract may compromise the adhesiveness of the bacterium. Adhesion is the first stage in colonization and biofilm development, hence its suppression could lessen the bacterium's ability to induce ongoing infections. The dose-dependent impact shown in both genes emphasizes the possible dual-action ability of the extract to not only stop bacterial development but also reduces virulence (Li & Xu, 2016).
Known for their antibacterial effects are the bioactive components of *Ziziphus spina-christi*, including tannins, saponins, and flavonoids. These molecules might change signal transduction pathways involved in virulence control, interfere with enzyme activity, or upset bacterial membranes. The substantial downregulation of virulence genes suggests that the extract may interfere with bacterial communication systems (quorum sensing) and adhesion processes, both of which are absolutely essential for infection establishment and progression (Ahmed & Mohamed, 2015).
The dual antibacterial and antivirulence qualities of *Ziziphus spina-christi* leaf extract point to its possible use as a supplementary therapy given the emergence of antibiotic-resistant bacteria. Combining such natural extracts with conventional antibiotics might help to improve therapeutic efficacy, lower bacterial pathogenicity, and maybe lower the necessary dosages of traditional antibiotics, hence reducing the development of resistance. (Bhowmik and Kumar, 2018).

**Conclusion**

This work emphasizes the strong antibacterial properties of *Ziziphus spina-christi* leaf extract as *Staphylococcus aureus* shows more susceptibility than *Klebsiella pneumoniae*. Reduced virulence gene expression, dose-dependent inhibition, and great natural antibacterial agent potential were shown by the extract. Particularly against Gram-positive bacteria, its efficacy points to prospective uses as a substitute or complement for traditional antibiotics. More studies are required to investigate its therapeutic possibilities in clinical environments and its synergy with current medications.

**Disclaimer (Artificial intelligence)**

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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