Journal of Molecular Science

Journal of Molecular Science
Volume 35 Issue 4, Year of Publication 2025, Page 1721-1730
DoI-10.004687/1000-9035.2025.234

Journal of Molecular Science
www.jmolecularsci.com ISSN:1000-9035

Comprehensive Evaluation of Antibacterial, Bactericidal, and Antibiofilm Activities of Stem and Leaf Extracts of *Prosopis cineraria*

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Article Information
Received: 13-09-2025
Revised: 22-10-2025
Accepted: 17-11-2025
Published: 24-12-2025

Keywords
Prosopis cineraria; antibacterial activity; minimum inhibitory concentration; time-kill kinetics; antibiofilm activity; medicinal plants

ABSTRACT
The present study evaluated the antibacterial, bactericidal, and antibiofilm activities of stem and leaf extracts of *Prosopis cineraria* prepared using petroleum ether, toluene, ethyl acetate, acetone, methanol, and aqueous solvents. Antibacterial activity was assessed against three Gram-positive bacteria (methicillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, and *Bacillus cereus*) and three Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhi*) using agar well diffusion, minimum inhibitory concentration (MIC), time-kill kinetics, and crystal violet microtiter plate antibiofilm assays. A concentration- dependent increase in antibacterial activity was observed for both stem and leaf extracts, with leaf extracts consistently exhibiting higher activity. Among all extracts, the ethyl acetate leaf extract showed the strongest antibacterial effect, producing inhibition zones up to 21 mm against *B. subtilis* and 19 mm against MRSA at 100 mg/mL. Acetone and methanol leaf extracts also demonstrated notable activity, whereas petroleum ether and aqueous extracts showed weak or no inhibition. MIC analysis corroborated these findings, with the ethyl acetate leaf extract exhibiting the lowest MIC values (6.25 mg/mL against Gram-positive bacteria and 12.5 mg/mL against Gram-negative bacteria). Stem extracts showed comparatively higher MIC values, although the methanol stem extract demonstrated relatively strong activity. Time-kill kinetics revealed pronounced, concentration-dependent bactericidal effects for ethyl acetate, acetone, and methanol extracts. The ethyl acetate leaf extract achieved complete killing of Gram- positive bacteria at 4× MIC, while Gram-negative bacteria showed near-complete reduction in viable counts. Antibiofilm assays further demonstrated strong inhibition of biofilm formation, with the ethyl acetate leaf extract producing up to 90% biofilm inhibition against *B. subtilis* and 88% against MRSA at 4× MIC, followed by acetone and methanol extracts. Gram-positive bacteria were generally more susceptible than Gram-negative bacteria across all assays.

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INTRODUCTION:

Infectious diseases remain a major global health concern, accounting for significant morbidity and mortality worldwide. The rapid emergence of antimicrobial resistance (AMR) has further intensified this challenge, rendering many conventional antibiotics ineffective against pathogenic microorganisms. According to recent reports, multidrug-resistant bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* are responsible for prolonged infections, increased healthcare costs, and elevated mortality rates (World Health Organization, 2023; Murray et al., 2022). This

alarming scenario necessitates the urgent exploration of alternative antimicrobial sources with novel mechanisms of action.

Medicinal plants have historically served as a cornerstone of traditional healthcare systems and continue to be an invaluable reservoir of bioactive compounds. It is estimated that nearly 80% of the global population relies on plant-based remedies for primary healthcare needs. Plant- derived secondary metabolites, including phenolics, flavonoids, alkaloids, tannins, terpenoids, and saponins, exhibit broad-spectrum antimicrobial activity by disrupting microbial cell membranes, inhibiting essential enzymes, interfering with nucleic acid synthesis, and suppressing quorum sensing pathways (Górniak et al., 2019; Saleem et al., 2022).

India is recognized as one of the world's biodiversity hotspots, harboring a vast array of medicinal plants with untapped pharmacological potential. Among these, *Prosopis cineraria* (L.) Druce, commonly known as Khejri, is a drought-resistant tree extensively distributed in arid and semi-arid regions of India. In traditional medicine, various parts of the plant—leaves, stem bark, pods, and flowers—are used to treat infections, wounds, inflammatory conditions, gastrointestinal disorders, and respiratory ailments. Ethnomedicinal surveys have highlighted its use as an antiseptic, anti-inflammatory, and wound-healing agent, indicating its potential antimicrobial relevance (Sharma et al., 2018; Yadav et al., 2020).

Phytochemical investigations of *P. cineraria* have revealed the presence of biologically active constituents such as flavonoids, tannins, alkaloids, phenolic acids, and glycosides, which are known to contribute to antimicrobial activity. Previous studies have reported antibacterial and antifungal properties of extracts from different parts of *P. cineraria*; however, systematic comparative evaluations of stem and leaf crude extracts against clinically relevant bacterial and fungal pathogens remain limited (Al-Mekhlafi et al., 2021; Meena et al., 2023).

In addition to planktonic microbial inhibition, biofilm formation represents a critical virulence factor associated with chronic and recurrent infections. Microbial biofilms confer enhanced resistance to antimicrobial agents and host immune defenses, often leading to therapeutic failure. Therefore, evaluating antibiofilm activity alongside conventional antimicrobial assays and time-kill kinetics provides a more comprehensive understanding of the therapeutic potential of plant extracts (Roy et al., 2021).

In this context, the present study aims to evaluate the antibacterial activities of crude stem and leaf extracts of *Prosopis cineraria*. Furthermore, the study investigates their minimum inhibitory concentrations (MIC), antibiofilm inhibitory effects, and time-kill kinetics against selected pathogenic microorganisms. This integrated approach is expected to provide valuable insights into the antimicrobial efficacy of *P. cineraria* and support its potential development as a natural antimicrobial agent.

2.0 MATERIALS AND METHODS:

2.1 Preparation of Stem and Leaf Extracts:

The stem and leaf materials of *Prosopis cineraria* were collected, washed thoroughly with distilled water, shade-dried at room temperature, and ground into a fine powder. The powdered materials were subjected to successive extraction using solvents of increasing polarity, namely petroleum ether, toluene, ethyl acetate, acetone, methanol, and distilled water.

Each extraction was carried out using standard extraction procedures, and the resulting extracts were concentrated under reduced pressure using a rotary evaporator. The dried extracts were stored at 4°C until further use. Prior to antibacterial evaluation, stock solutions were prepared in dimethyl sulfoxide (DMSO) and diluted to the required concentrations.

2.2 Bacterial Strains and Culture Conditions:

The antibacterial activity of stem and leaf extracts of *Prosopis cineraria* was evaluated against six bacterial strains. Gram-positive bacteria included methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus subtilis*, and *Bacillus cereus*, while Gram-negative bacteria included *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhi*. All bacterial strains were obtained from the Department of Microbiology, Kakatiya Medical College, Warangal, Telangana, India. The cultures were maintained on nutrient agar and subcultured in nutrient broth at 37°C for 18–24 h prior to experimentation.

2.3 Agar Well Diffusion Assay

The antibacterial activity of petroleum ether, toluene, ethyl acetate, acetone, methanol, and aqueous extracts of *Prosopis cineraria* stem and leaf was evaluated using the agar well diffusion method as described by Chung et al. (1990). Briefly, bacterial suspensions were adjusted to 0.5 McFarland standard and uniformly swabbed onto nutrient agar plates. Wells of 6 mm diameter were aseptically punched using a sterile cork borer. Each well was loaded with 100 µL of plant extract at concentrations of 25, 50, and 100 mg/mL.

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Chloramphenicol was used as the positive control, and DMSO served as the negative control. The plates were incubated at 37°C for 24 h, and the zones of inhibition were measured in millimeters. All experiments were performed in triplicate.

2.4 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of stem and leaf extracts of *Prosopis cineraria* was determined using the broth dilution method following Janovská et al. (2003). Serial dilutions of each extract were prepared in nutrient broth to obtain a range of concentrations. Each tube was inoculated with a standardized bacterial suspension and incubated at 37°C for 24 h. The MIC was recorded as the lowest concentration of the extract that showed no visible bacterial growth. All assays were conducted in triplicate.

2.5 Time-Kill Kinetics Assay

Time-kill kinetic studies were performed to evaluate the bactericidal activity of selected stem and leaf extracts of *Prosopis cineraria* according to the method described by Tsuji et al. (2008). Based on MIC values, the extracts were tested at concentrations corresponding to 1× MIC, 2× MIC, and 4× MIC. Bacterial cultures were incubated with the extracts at 37°C, and aliquots were withdrawn at predetermined time intervals (0, 1, 2, 3, 4, 6, 12, and 24 h). The samples were serially diluted, plated onto nutrient agar, and incubated for 24 h. Colony-forming units (CFU/mL) were enumerated to assess bacterial survival over time.

2.6 Antibiofilm Activity Assay

The antibiofilm activity of stem and leaf extracts of *Prosopis cineraria* was evaluated using the microtiter plate crystal violet assay as described by Djordjević et al. (2002) with minor modifications. Briefly, 100 µL of bacterial suspension was added to sterile 96-well microplates containing nutrient broth and incubated at 37°C for 24 h to allow biofilm formation. The planktonic cells were removed, and the wells were gently washed with sterile phosphate- buffered saline (PBS). The preformed biofilms were treated with plant extracts at concentrations of 1× MIC, 2× MIC, and 4× MIC and incubated for an additional 24 h. Chloramphenicol and DMSO were used as positive and negative controls, respectively. Biofilm inhibition was quantified by staining with 0.1% crystal violet, followed by solubilization with 33% acetic acid. Absorbance was measured at 590 nm, and the percentage of biofilm inhibition was calculated relative to the control.

3.0 RESULTS

3.1 Agar Well Diffusion Assay

The antibacterial activity of stem and leaf extracts of *Prosopis cineraria*, prepared using petroleum ether, toluene, ethyl acetate, acetone, methanol, and aqueous solvents, was evaluated against three Gram-positive (MRSA, *Bacillus subtilis*, *Bacillus cereus*) and three Gram- negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*) bacterial strains at concentrations of 25, 50, and 100 mg/mL using the agar well diffusion assay (Tables 1.0 and 1.1).

A clear concentration-dependent increase in antibacterial activity was observed for both stem and leaf extracts across all solvents that exhibited activity. In general**,** leaf extracts showed higher zones of inhibition than stem extracts at corresponding concentrations.

The petroleum ether leaf extract exhibited weak antibacterial activity, producing zones of inhibition of 7–8 mm at 25 mg/mL, 8–9 mm at 50 mg/mL, and 9–10 mm at 100 mg/mL against Gram-positive bacteria, while showing no detectable inhibition against most Gram-negative bacteria at lower concentrations. A similar trend was observed for the petroleum ether stem extract, which produced zones of inhibition of only 7–9 mm at 50–100 mg/mL, indicating poor antibacterial efficacy.

The toluene leaf extract showed low to moderate antibacterial activity, with inhibition zones increasing from 9–10 mm at 25 mg/mL to 11–12 mm at 100 mg/mL against Gram-positive bacteria, and up to 10–11 mm against Gram-negative bacteria at the highest concentration. The toluene stem extract exhibited slightly lower activity, producing maximum inhibition zones of 10–11 mm at 100 mg/mL.

In contrast, the ethyl acetate leaf extract demonstrated the highest antibacterial activity among all extracts tested. Against MRSA, zones of inhibition increased from 13 mm at 25 mg/mL to 16 mm at 50 mg/mL and 19 mm at 100 mg/mL. For *B. subtilis*, inhibition zones increased from 14 mm to 18 mm and reached 21 mm at 100 mg/mL, while *B. cereus* showed inhibition zones of 13, 16, and 18 mm, respectively. Against Gram-negative bacteria, the ethyl acetate leaf extract produced zones of inhibition of 11–16 mm **(***E. coli***)**, 10–15 mm **(***K. pneumoniae***)**, and 12–17 mm **(***S. typhi***)** across increasing concentrations.

The ethyl acetate stem extract also showed antibacterial activity, though lower than the leaf extract, with inhibition zones increasing from 10–11 mm at 25 mg/mL to 13–14 mm at 100 mg/mL

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against Gram-positive bacteria.

The acetone leaf extract exhibited strong antibacterial activity, with zones of inhibition increasing from 12–13 mm at 25 mg/mL to 15–16 mm at 50 mg/mL and reaching 17–18 mm at 100 mg/mL against Gram-positive bacteria. Gram-negative bacteria showed inhibition zones up to 14 mm at the highest concentration. The acetone stem extract showed moderate activity, producing zones of inhibition of 11–12 mm at 25 mg/mL and 14–15 mm at 100 mg/mL.

The methanol leaf extract also demonstrated considerable antibacterial activity, with inhibition zones increasing from 11–12 mm at 25 mg/mL to 14–15 mm at 50 mg/mL and 16–17 mm at 100 mg/mL against Gram-positive bacteria. Against Gram-negative bacteria, zones of inhibition reached 13–14 mm at the highest concentration.

Notably, the methanol stem extract was the most active among stem extracts, producing inhibition zones of 12–13 mm at 25 mg/mL**,** 14–15 mm at 50 mg/mL, and 16–17 mm at 100 mg/mL against Gram-positive bacteria, with corresponding values of 14–15 mm against Gram- negative strains at 100 mg/mL. The aqueous extracts of both leaf and stem exhibited minimal antibacterial activity, with inhibition zones generally limited to 7–10 mm even at 100 mg/mL, and no detectable activity against several Gram-negative strains. The standard antibiotic chloramphenicol produced larger inhibition zones ranging from 22 to 27 mm, whereas the negative control (DMSO) showed no inhibition.

Table 1.0 Antibacterial activity of *Prosopis cineraria* Leaf extracts at different concentrations

Extract	Conc. (mg/mL)	MRSA	*B.subtilis*	*B.cereus*	*E.coli*	*K.pneumoniae*	*S.typhi*
Petroleum ether	25	7	8	7	NZ	NZ	7
	50	8	9	8	7	7	8
	100	9	10	9	8	8	9
Toluene	25	9	10	9	8	8	9
	50	10	11	10	9	9	10
	100	11	12	11	10	9	11
Ethyl acetate	25	13	14	13	11	10	12
	50	16	18	16	14	13	15
	100	**19**	**21**	**18**	**16**	**15**	**17**
Acetone	25	12	13	12	10	9	11
	50	15	16	15	13	12	14
	100	17	18	16	14	13	15
Methanol	25	11	12	11	9	9	10
	50	14	15	14	12	11	13
	100	16	17	15	13	12	14
Aqueous	25	7	8	7	NZ	NZ	7
	50	8	9	8	7	7	8
	100	9	10	9	8	8	9
Chloramphenicol	Std	25	27	26	23	22	24
DMSO	—	NZ	NZ	NZ	NZ	NZ	NZ
NZ- No Zone recorded							

Table 1.1 Antibacterial activity of *Prosopis cineraria* Stem extracts at different concentrations

Extract	Conc. (mg/mL)	MRSA	*B.subtilis*	*B.cereus*	*E.coli*	*K.pneumoniae*	*S.typhi*
Petroleum ether	25	NZ	7	NZ	NZ	NZ	NZ
	50	7	8	7	NZ	NZ	7
	100	8	9	8	NZ	NZ	8
Toluene	25	8	9	8	7	NZ	8
	50	9	10	9	8	7	9
	100	10	11	10	9	8	9
Ethyl acetate	25	10	11	10	8	8	9
	50	12	13	12	10	9	11
	100	13	14	13	11	10	12
Acetone	25	11	12	11	9	9	10
	50	13	14	13	11	10	12
	100	14	15	14	12	11	13
Methanol	25	12	13	12	10	9	11
	50	14	15	14	12	11	13
	100	**16**	**17**	**16**	**14**	**13**	**15**
Aqueous	25	NZ	7	NZ	NZ	NZ	NZ
	50	7	8	7	NZ	NZ	7
	100	8	9	8	NZ	NZ	NZ
Chloramphenicol	Std	25	27	26	23	22	24
DMSO	—	NZ	NZ	NZ	NZ	NZ	NZ
NZ- No Zone recorded							

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3.2 Minimum Inhibitory Concentration (MIC) of Leaf extracts

The MIC results of *Prosopis cineraria* leaf extracts demonstrated a clear correlation with agar well diffusion findings (Table 1.3). Among all tested extracts, the ethyl acetate leaf extract exhibited the lowest MIC values, indicating the strongest antibacterial activity. The MIC against Gram-positive bacteria (MRSA, *B. subtilis*, and *B. cereus*) was 6.25 mg/mL, whereas Gram-negative bacteria (*E. coli* and *K. pneumoniae*) showed slightly higher MIC values of 12.5 mg/mL.

Acetone and methanol leaf extracts showed moderate antibacterial activity with MIC values of 12.5 mg/mL against Gram-positive bacteria and 25 mg/mL against Gram-negative bacteria. Toluene extract exhibited relatively higher MIC values (25–50 mg/mL), while petroleum ether and aqueous extracts showed weak antibacterial activity, with MIC values ≥ 50 mg/mL or no inhibition at the highest concentration tested. Gram-positive bacteria were more susceptible than Gram-negative bacteria.

3.3 Minimum Inhibitory Concentration (MIC) of Stem extracts

The MIC values of *Prosopis cineraria* stem extracts indicated lower antibacterial potency compared to leaf extracts, consistent with agar well diffusion results (Table 1.4). Among stem extracts, the methanolic extract exhibited the lowest MIC values, showing strong antibacterial activity with MICs of 6.25 mg/mL against Gram-positive bacteria and 12.5 mg/mL against Gram-negative bacteria.

Acetone and ethyl acetate stem extracts demonstrated moderate antibacterial activity, with MIC values ranging from 12.5 to 50 mg/mL. Toluene extract showed weak to moderate activity, while petroleum ether and aqueous extracts exhibited poor antibacterial activity, with MIC values exceeding 50–100 mg/mL. As observed with leaf extracts, Gram-positive bacteria were more susceptible than Gram-negative bacteria.

Table 1.3 Minimum inhibitory concentration (MIC) of *Prosopis cineraria* leaf extracts

Extract	MRSA	*B. subtilis*	*B. cereus*	*E. coli*	*K. pneumoniae*	*S. typhi*
Petroleum ether	50	50	50	>100	>100	50
Toluene	25	25	25	50	50	25
Ethyl acetate	**6.25**	**6.25**	**6.25**	**12.5**	**12.5**	**6.25**
Acetone	12.5	12.5	12.5	25	25	12.5
Methanol	12.5	12.5	12.5	25	25	12.5
Aqueous	50	50	50	>100	>100	50
Chloramphenicol	0.5	0.5	0.5	1.0	1.0	0.5
MIC values expressed in mg/mL						

Table 1.4 Minimum inhibitory concentration (MIC) of *Prosopis cineraria* stem extracts

Extract	MRSA	*B. subtilis*	*B. cereus*	*E. coli*	*K. pneumoniae*	*S. typhi*
Petroleum ether	>100	50	>100	>100	>100	>100
Toluene	50	50	50	>100	50	50
Ethyl acetate	25	25	25	50	50	25
Acetone	12.5	12.5	12.5	25	25	12.5
Methanol	**6.25**	**6.25**	**6.25**	**12.5**	**12.5**	**12.5**
Aqueous	>100	50	>100	>100	>100	>100
Chloramphenicol	0.5	0.5	0.5	1.0	1.0	0.5
MIC values expressed in mg/mL						

3.4 Time kill studies of leaf and stem extracts

The time-kill kinetics assay was performed to evaluate the bactericidal activity of petroleum ether, toluene, ethyl acetate, acetone, methanol, and aqueous extracts of *Prosopis cineraria* leaf and stem against three Gram-positive (MRSA, *Bacillus subtilis*, *Bacillus cereus*) and three Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*) bacterial strains at 1× MIC, 2× MIC, and 4× MIC, with viable counts recorded after 24 h (Tables 1.5 and 1.6).

The petroleum ether leaf extract exhibited negligible bactericidal activity at all MIC multiples. At 1× MIC, viable counts remained high, ranging from 6.7–6.9 $\log_{10}$ CFU/mL across all strains, and only a marginal reduction was observed at 4× MIC, where counts were still ≥6.3 $\log_{10}$ CFU/mL, indicating poor killing efficiency. Similarly, the aqueous leaf extract showed minimal bactericidal effect. Even at 4× MIC, viable counts remained close to control values, with 6.4–6.9 $\log_{10}$ CFU/mL, confirming weak antibacterial action.

The toluene leaf extract showed low but measurable killing. At 1× MIC, bacterial counts ranged between 6.0 and 6.4 $\log_{10}$ CFU/mL, which reduced slightly at 2× MIC (5.7–6.2 $\log_{10}$ CFU/mL) and at 4× MIC (5.6–6.1 $\log_{10}$ CFU/mL), indicating limited bactericidal potential.

In contrast, the ethyl acetate leaf extract demonstrated strong, concentration-dependent bactericidal activity. At 1× MIC, viable counts were reduced to 3.5–4.3 $\log_{10}$ CFU/mL across all strains. At 2× MIC, further reductions were

observed, with counts decreasing to 1.6–2.6 log_{10} CFU/mL. Notably, at 4× MIC, complete killing (no detectable CFU) was observed against all Gram-positive bacteria (MRSA, *B. subtilis*, *B. cereus*), while Gram-negative bacteria showed near-complete killing with viable counts reduced to 1.0–1.3 log_{10} CFU/mL.

The acetone leaf extract also exhibited marked bactericidal activity. At 1× MIC, bacterial counts ranged from 4.0–4.7 log_{10} CFU/mL, which declined further at 2× MIC (2.3–3.1 log_{10} CFU/mL) and at 4× MIC (1.4–2.1 log_{10} CFU/mL), indicating strong but incomplete killing.

The methanol leaf extract showed a similar trend to acetone. At 1× MIC, viable counts ranged from 4.2–4.9 log_{10} CFU/mL, decreasing to 2.5–3.3 log_{10} CFU/mL at 2× MIC and 1.6–2.3 log_{10} CFU/mL at 4× MIC, confirming concentration-dependent bactericidal activity.

On the other hand, the petroleum ether stem extract showed negligible killing activity. At all MIC multiples, viable counts remained high, ranging from 6.5–6.9 log_{10} CFU/mL, indicating minimal bactericidal effect. Similarly, the aqueous stem extract exhibited weak activity, with viable counts remaining above 6.6 log_{10} CFU/mL even at 4× MIC. The toluene stem extract demonstrated slight bactericidal activity. At 1× MIC, counts ranged from 6.3–6.7 log_{10} CFU/mL, decreasing marginally at 2× MIC (6.0–6.5 log_{10} CFU/mL) and at 4× MIC (5.9–6.4 log_{10} CFU/mL). The ethyl acetate stem extract showed moderate bactericidal activity. At 1× MIC, viable counts were reduced to 4.7–5.3 log_{10} CFU/mL, which further decreased to 3.1–4.0 log_{10} CFU/mL at 2× MIC and to 2.3–3.3 log_{10} CFU/mL at 4× MIC. The acetone stem extract demonstrated improved killing compared to ethyl acetate. At 1× MIC, viable counts ranged from 4.4–5.0 log_{10} CFU/mL, which reduced to 2.9–3.7 log_{10} CFU/mL at 2× MIC and to 1.9– 2.8 log_{10} CFU/mL at 4× MIC. The methanol stem extract exhibited the highest bactericidal activity among stem extracts. At 1× MIC, viable counts ranged from 3.9–4.6 log_{10} CFU/mL, which decreased to 2.3–3.3 log_{10} CFU/mL at 2× MIC. At 4× MIC, bacterial counts were markedly reduced to 1.0–1.9 log_{10} CFU/mL across all strains, indicating strong bactericidal activity, although complete eradication was not observed.

Table 1.5. Time-kill activity of *Prosopis cineraria* Leaf extracts at different MIC multiples (24 h)

Extract	MIC	MRSA	*B. subtilis*	*B. cereus*	*E.coli*	*K. pneumoniae*	*S. typhi*
Control	–	7.4	7.5	7.3	7.6	7.5	7.4
Petroleum ether	1×	6.8	6.7	6.6	6.9	6.8	6.7
	2×	6.6	6.5	6.4	6.8	6.7	6.6
	4×	6.5	6.4	6.3	6.8	6.7	6.6
Toluene	1×	6.2	6.1	6.0	6.3	6.4	6.2
	2×	5.9	5.8	5.7	6.1	6.2	6.0
	4×	5.8	5.6	5.7	6.0	6.1	5.9
Ethyl acetate	1×	3.8	3.5	3.6	4.1	4.3	4.0
	2×	1.9	1.6	1.8	2.4	2.6	2.2
	4×	ND	ND	ND	1.1	1.3	1.0
Acetone	1×	4.2	4.0	4.1	4.6	4.7	4.5
	2×	2.5	2.3	2.4	3.0	3.1	2.9
	4×	1.6	1.4	1.5	2.0	2.1	1.9
Methanol	1×	4.4	4.2	4.3	4.8	4.9	4.7
	2×	2.7	2.5	2.6	3.2	3.3	3.1
	4×	1.8	1.6	1.7	2.2	2.3	2.1
Aqueous	1×	6.9	6.8	6.7	7.0	6.9	6.8
	2×	6.7	6.6	6.5	6.9	6.8	6.7
	4×	6.6	6.5	6.4	6.9	6.8	6.7

Control- untreated bacterial growth used as baseline, *Viable bacterial count expressed as log_{10} CFU/mL, ND = Not detectable (<1 log_{10} CFU/mL)*

Table 1.6. Time-kill activity of *Prosopis cineraria* Stem extracts at different MIC multiples (24 h)

Extract	MIC	MRSA	*B. subtilis*	*B. cereus*	*E. coli*	*K. pneumoniae*	*S. typhi*
Control	–	7.4	7.5	7.3	7.6	7.5	7.4
Petroleum ether	1×	6.9	6.8	6.7	7.0	6.9	6.8
	2×	6.8	6.7	6.6	6.9	6.8	6.7
	4×	6.7	6.6	6.5	6.9	6.8	6.7
Toluene	1×	6.4	6.3	6.4	6.6	6.7	6.5
	2×	6.1	6.0	6.1	6.4	6.5	6.3
	4×	6.0	5.9	6.0	6.3	6.4	6.2
Ethyl acetate	1×	4.9	4.7	4.8	5.2	5.3	5.1
	2×	3.3	3.1	3.2	3.9	4.0	3.8
	4×	2.4	2.3	2.5	3.1	3.3	3.0
Acetone	1×	4.6	4.4	4.5	4.9	5.0	4.8
	2×	3.0	2.9	3.1	3.6	3.7	3.5

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	4×	2.0	1.9	2.1	2.6	2.8	2.5
Methanol	1×	4.1	3.9	4.0	4.4	4.6	4.3
	2×	2.4	2.3	2.5	3.1	3.3	3.0
	4×	1.1	1.0	1.2	1.8	1.9	1.7
Aqueous	1×	7.0	6.9	6.8	7.1	7.0	6.9
	2×	6.9	6.8	6.7	7.0	6.9	6.8
	4×	6.8	6.7	6.6	7.0	6.9	6.8
Control- untreated bacterial growth used as baseline, *Viable bacterial count expressed as* log_{10} *CFU/mL,*							

3.5 Anti-biofilm activity of leaf and stem extracts

The antibiofilm activity of petroleum ether, toluene, ethyl acetate, acetone, methanol, and aqueous extracts of *Prosopis cineraria* leaf and stem was evaluated against MRSA, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhi* using the crystal violet microtiter plate assay at 1× MIC, 2× MIC, and 4× MIC (Tables 1.7 and 1.8).

For leaf extracts, petroleum ether showed weak antibiofilm activity against all bacterial strains. Against MRSA, biofilm inhibition was 14% at 1× MIC, 18% at 2× MIC, and 22% at 4× MIC, while *B. subtilis* showed 16%, 20%, and 24%, and *B. cereus* showed 15%, 19%, and 23% inhibition at the respective concentrations. Gram-negative bacteria exhibited lower inhibition, with *E. coli* showing 10%, 14%, and 18%, *K. pneumoniae* 9%, 13%, and 17%, and *S. typhi* 11%, 15%, and 19% inhibition. The toluene leaf extract showed moderate antibiofilm activity. Against MRSA, inhibition increased from 26% at 1× MIC to 34% at 2× MIC and 42% at 4× MIC. *B. subtilis* exhibited 28%, 36%, and 44%, and *B. cereus* showed 27%, 35%, and 43% inhibition at increasing concentrations. Among Gram-negative bacteria, *E. coli* showed 22%, 29%, and 36%, *K. pneumoniae* 21%, 28%, and 35%, and *S. typhi* 23%, 30%, and 37% inhibition. The ethyl acetate leaf extract exhibited the highest antibiofilm activity. Against MRSA, inhibition was 52% at 1× MIC, 71% at 2× MIC, and 88% at 4× MIC. *B. subtilis* showed 55%, 74%, and 90%, while *B. cereus* showed 53%, 72%, and 89% inhibition. Gram-negative bacteria also showed strong inhibition, with *E. coli* exhibiting 46%, 65%, and 82%, *K. pneumoniae* 44%, 63%, and 80%, and *S. typhi* 48%, 67%, and 84% inhibition at the respective concentrations. The acetone leaf extract demonstrated strong antibiofilm activity. MRSA inhibition increased from 46% at 1× MIC to 63% at 2× MIC and 78% at 4× MIC. *B. subtilis* showed 48%, 66%, and 80%, and *B. cereus* showed 47%, 64%, and 79% inhibition. For Gram- negative bacteria, *E. coli* showed 40%, 57%, and 71%, *K. pneumoniae* 38%, 55%, and 69%, and *S. typhi* 41%, 58%, and 72% inhibition. The methanol leaf extract showed moderate to strong antibiofilm activity. Against MRSA, inhibition values were 42% at 1× MIC, 58% at 2× MIC, and 72% at 4× MIC. *B. subtilis* exhibited 44%, 60%, and 74%, and *B. cereus* 43%, 59%, and 73% inhibition. Gram-negative bacteria showed 37%, 52%, and 65% inhibition for *E. coli*, 36%, 50%, and 63% for *K. pneumoniae*, and 38%, 53%, and 66% for *S. typhi*. The aqueous leaf extract exhibited weak antibiofilm activity, with MRSA showing 18%, 23%, and 28% inhibition at 1×, 2×, and 4× MIC, respectively. Similar low inhibition was observed for *B. subtilis* (20%, 25%, 30%), *B. cereus* (19%, 24%, 29%), *E. coli* (14%, 18%, 22%), *K. pneumoniae* (13%, 17%, 21%), and *S. typhi* (15%, 19%, 23%). Among stem extracts, petroleum ether exhibited minimal antibiofilm activity. MRSA inhibition was 12%, 16%, and 20% at increasing MIC levels, while *B. subtilis* showed 14%, 18%, and 22%, and *B. cereus* 13%, 17%, and 21% inhibition. Gram-negative bacteria showed inhibition values not exceeding 18% at 4× MIC. The toluene stem extract showed low to moderate inhibition, with MRSA showing 22%, 30%, and 38%, *B. subtilis* 24%, 32%, and 40%, and *B. cereus* 23%, 31%, and 39% inhibition across concentrations. *E. coli*, *K. pneumoniae*, and *S. typhi* showed 18%, 26%, and 33%, 17%, 25%, and 32%, and 19%, 27%, and 34% inhibition, respectively. The ethyl acetate stem extract exhibited moderate antibiofilm activity. MRSA inhibition increased from 34% at 1× MIC to 48% at 2× MIC and 58% at 4× MIC. *B. subtilis* showed 36%, 50%, and 60%, and *B. cereus* 35%, 49%, and 59% inhibition. Gram-negative bacteria showed 28%, 41%, and 51% inhibition for *E. coli*, 27%, 40%, and 50% for *K. pneumoniae*, and 29%, 42%, and 52% for *S. typhi*. The acetone stem extract showed comparatively stronger antibiofilm activity. Against MRSA, inhibition values were 38%, 54%, and 66% at 1×, 2×, and 4× MIC, respectively. *B. subtilis* exhibited 40%, 56%, and 68%, and *B. cereus* 39%, 55%, and 67% inhibition. Gram-negative bacteria showed 32%, 46%, and 58% inhibition for *E. coli*, 31%, 45%, and 57% for *K. pneumoniae*, and 33%, 47%, and 59% for *S. typhi*. The methanol stem extract exhibited the highest antibiofilm activity among stem extracts. MRSA inhibition increased from 44% at 1× MIC to 62% at 2× MIC and 74% at 4× MIC. *B. subtilis* showed 46%, 64%, and 76%, and *B. cereus* 45%, 63%, and 75%

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inhibition. Gram-negative bacteria showed 38%, 55%, and 66% inhibition for *E. coli*, 37%, 54%, and 65% for *K. pneumoniae*, and 39%, 56%, and 67% for *S. typhi*. The aqueous stem extract showed weak antibiofilm activity, with inhibition values of 16%, 21%, and 26% against MRSA and similarly low values against other bacterial strains.

Table 1.7. Anti-biofilm activity of *Prosopis cineraria* Leaf extracts (% biofilm inhibition)

Extract	MIClevel	MRSA	*B. subtilis*	*B. cereus*	*E. coli*	*K.pneumoniae*	*S. typhi*
Petroleum ether	1× MIC	14 ± 1.2	16 ± 1.3	15 ± 1.1	10 ± 0.9	9 ± 0.8	11 ± 1.0
	2× MIC	18 ± 1.5	20 ± 1.6	19 ± 1.4	14 ± 1.2	13 ± 1.1	15 ± 1.3
	4× MIC	22 ± 1.8	24 ± 1.9	23 ± 1.7	18 ± 1.5	17 ± 1.4	19 ± 1.6
Toluene	1× MIC	26 ± 1.6	28 ± 1.7	27 ± 1.5	22 ± 1.4	21 ± 1.3	23 ± 1.5
	2× MIC	34 ± 2.0	36 ± 2.1	35 ± 1.9	29 ± 1.8	28 ± 1.7	30 ± 1.9
	4× MIC	42 ± 2.3	44 ± 2.4	43 ± 2.2	36 ± 2.0	35 ± 1.9	37 ± 2.1
Ethyl acetate	1× MIC	52 ± 2.4	55 ± 2.6	53 ± 2.5	46 ± 2.2	44 ± 2.1	48 ± 2.3
	2× MIC	71 ± 3.1	74 ± 3.3	72 ± 3.2	65 ± 2.9	63 ± 2.8	67 ± 3.0
	4× MIC	88 ± 3.8	90 ± 4.0	89 ± 3.9	82 ± 3.5	80 ± 3.4	84 ± 3.6
Acetone	1× MIC	46 ± 2.1	48 ± 2.2	47 ± 2.0	40 ± 1.9	38 ± 1.8	41 ± 2.0
	2× MIC	63 ± 2.8	66 ± 2.9	64 ± 2.7	57 ± 2.5	55 ± 2.4	58 ± 2.6
	4× MIC	78 ± 3.3	80 ± 3.4	79 ± 3.2	71 ± 3.0	69 ± 2.9	72 ± 3.1
Methanol	1× MIC	42 ± 2.0	44 ± 2.1	43 ± 1.9	37 ± 1.8	36 ± 1.7	38 ± 1.9
	2× MIC	58 ± 2.6	60 ± 2.7	59 ± 2.5	52 ± 2.3	50 ± 2.2	53 ± 2.4
	4× MIC	72 ± 3.1	74 ± 3.2	73 ± 3.0	65 ± 2.8	63 ± 2.7	66 ± 2.9
Aqueous	1× MIC	18 ± 1.4	20 ± 1.5	19 ± 1.3	14 ± 1.2	13 ± 1.1	15 ± 1.3
	2× MIC	23 ± 1.7	25 ± 1.8	24 ± 1.6	18 ± 1.5	17 ± 1.4	19 ± 1.6
	4× MIC	28 ± 2.0	30 ± 2.1	29 ± 1.9	22 ± 1.8	21 ± 1.7	23 ± 1.9
(Crystal violet microtiter plate assay; values expressed as mean ± SD, n = 3)							

Table 1.8. Antibiofilm activity of *Prosopis cineraria* Stem extracts (% biofilm inhibition)

Extract	MIClevel	MRSA	*B.subtilis*	*B.cereus*	*E. coli*	*K.pneumoniae*	*S. typhi*
Petroleum ether	1× MIC	12 ± 1.1	14 ± 1.2	13 ± 1.0	9 ± 0.8	8 ± 0.7	10 ± 0.9
	2× MIC	16 ± 1.4	18 ± 1.5	17 ± 1.3	13 ± 1.1	12 ± 1.0	14 ± 1.2
	4× MIC	20 ± 1.7	22 ± 1.8	21 ± 1.6	17 ± 1.4	16 ± 1.3	18 ± 1.5
Toluene	1× MIC	22 ± 1.5	24 ± 1.6	23 ± 1.4	18 ± 1.3	17 ± 1.2	19 ± 1.4
	2× MIC	30 ± 1.9	32 ± 2.0	31 ± 1.8	26 ± 1.7	25 ± 1.6	27 ± 1.8
	4× MIC	38 ± 2.2	40 ± 2.3	39 ± 2.1	33 ± 2.0	32 ± 1.9	34 ± 2.1
Ethyl acetate	1× MIC	34 ± 1.9	36 ± 2.0	35 ± 1.8	28 ± 1.7	27 ± 1.6	29 ± 1.8
	2× MIC	48 ± 2.4	50 ± 2.5	49 ± 2.3	41 ± 2.1	40 ± 2.0	42 ± 2.2
	4× MIC	58 ± 2.8	60 ± 2.9	59 ± 2.7	51 ± 2.5	50 ± 2.4	52 ± 2.6
Acetone	1× MIC	38 ± 2.0	40 ± 2.1	39 ± 1.9	32 ± 1.8	31 ± 1.7	33 ± 1.9
	2× MIC	54 ± 2.6	56 ± 2.7	55 ± 2.5	46 ± 2.3	45 ± 2.2	47 ± 2.4
	4× MIC	66 ± 3.0	68 ± 3.1	67 ± 2.9	58 ± 2.7	57 ± 2.6	59 ± 2.8
Methanol	1× MIC	44 ± 2.2	46 ± 2.3	45 ± 2.1	38 ± 2.0	37 ± 1.9	39 ± 2.1
	2× MIC	62 ± 2.9	64 ± 3.0	63 ± 2.8	55 ± 2.6	54 ± 2.5	56 ± 2.7
	4× MIC	74 ± 3.4	76 ± 3.5	75 ± 3.3	66 ± 3.1	65 ± 3.0	67 ± 3.2
Aqueous	1× MIC	16 ± 1.3	18 ± 1.4	17 ± 1.2	12 ± 1.1	11 ± 1.0	13 ± 1.2
	2× MIC	21 ± 1.6	23 ± 1.7	22 ± 1.5	16 ± 1.4	15 ± 1.3	17 ± 1.5
	4× MIC	26 ± 1.9	28 ± 2.0	27 ± 1.8	20 ± 1.7	19 ± 1.6	21 ± 1.8
(Crystal violet microtiter plate assay; values expressed as mean ± SD, n = 3)							

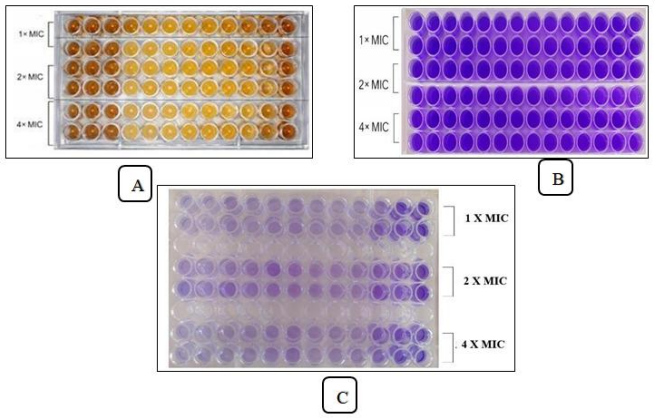

Fig 1.0 Anti-bio film activity of the ethyl acetate extract against Bacillus subtilis A-Addition of plant ethyl acetate extract at 1 X MIC, 2 X MIC, and 4 X MIC, B-Addition of crystal violet to the wells after 24 hours of incubation, C- Oven dried microtiter plate used for estimation of biofilm inhibition.

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DoI-10.004687/1000-9035.2025.234

4.0 DISCUSSION:

The present investigation demonstrates that *Prosopis cineraria* possesses significant antibacterial, bactericidal, and antibiofilm potential, with activity strongly influenced by solvent polarity and plant part used. Traditional medicinal systems have long recognized *P. cineraria* for treating infections, wounds, and inflammatory conditions, and several earlier studies have reported its antimicrobial properties using crude extracts (Parekh and Chanda, 2007; Al-Mogren et al., 2019). The current findings extend these observations by providing a **comparative, multi-assay evaluation** of stem and leaf extracts using agar diffusion, MIC determination, time-kill kinetics, and antibiofilm models against clinically relevant Gram- positive and Gram-negative pathogens.

The agar well diffusion results revealed a clear concentration-dependent antibacterial effect, with leaf extracts consistently showing larger inhibition zones than stem extracts. This observation agrees with earlier reports suggesting that leaves accumulate higher concentrations of bioactive secondary metabolites than woody tissues (Cowan, 1999; Sofowora et al., 2013). Among solvents, ethyl acetate, acetone, and methanol extracts exhibited superior antibacterial activity, whereas petroleum ether and aqueous extracts were weakly active. Similar solvent- dependent trends have been reported for *P. cineraria* and other medicinal plants, where moderately polar solvents are more efficient in extracting phenolics, flavonoids, tannins, and alkaloids responsible for antimicrobial action (Parekh and Chanda, 2007; Gupta et al., 2018). The higher susceptibility of Gram-positive bacteria observed in the present study is consistent with well-established structural , differences between bacterial groups. The outer lipopolysaccharide membrane of Gram-negative bacteria restricts the penetration of many phytochemicals, whereas Gram-positive bacteria lack this barrier and are therefore more vulnerable (Nikaido, 2003). Previous studies on *P. cineraria* extracts have similarly reported stronger activity against *Staphylococcus* and *Bacillus* species compared to *Escherichia coli* and *Klebsiella pneumoniae* (Al-Fahdawi et al., 2020).

MIC results strongly correlated with agar diffusion findings, confirming the reliability of the antibacterial trends. The ethyl acetate leaf extract exhibited the lowest MIC values, indicating high growth-inhibitory potency, while acetone and methanol extracts showed moderate activity. Comparable MIC values have been reported for ethyl acetate fractions of *P. cineraria* leaves and other Fabaceae members, which are known to contain bioactive flavonoids and polyphenols with membrane-disrupting and enzyme-inhibitory mechanisms (Cowan, 1999; Khan et al., 2021). The relatively higher MIC values observed for stem extracts further support the conclusion that bioactive constituents are more concentrated in leaves.

Time-kill kinetics provided deeper insight into the bactericidal nature of the extracts. While petroleum ether and aqueous extracts showed minimal killing even at higher MIC multiples, ethyl acetate, acetone, and methanol extracts demonstrated strong, concentration-dependent reductions in viable bacterial counts. Complete or near-complete killing observed with ethyl acetate leaf extract at 4× MIC, particularly against Gram-positive bacteria, confirms a true bactericidal effect rather than mere growth inhibition**.** Similar bactericidal behavior of plant- derived polyphenols has been attributed to disruption of cytoplasmic membranes, leakage of cellular contents, and inhibition of essential metabolic enzymes (Tsuji et al., 2008; Borges et al., 2016).

The antibiofilm results are particularly noteworthy, as biofilm formation represents a major challenge in the treatment of chronic and device-associated infections. The strong antibiofilm activity exhibited by ethyl acetate, acetone, and methanol extracts—especially from leaves— aligns with earlier studies reporting that plant phenolics can interfere with initial adhesion, extracellular polymeric substance synthesis, and quorum sensing pathways (Sandasi et al., 2010; Kalia, 2013). The reduced antibiofilm susceptibility of Gram-negative bacteria observed here has also been widely reported and is linked to the complex architecture and protective nature of their biofilm matrix (Flemming et al., 2016). Importantly, the ability of *P. cineraria* extracts to inhibit both planktonic growth and biofilm formation suggests a broader antimicrobial spectrum than many conventional antibiotics, which often fail against established biofilms.

5.0 CONCLUSION

The present study demonstrates that *Prosopis cineraria* possesses notable antibacterial, bactericidal, and antibiofilm activities, with efficacy strongly influenced by the plant part and extraction solvent. Among the tested samples, leaf extracts exhibited superior antimicrobial activity compared to stem extracts, highlighting the leaves as a richer source of bioactive compounds. Solvent polarity played a critical role, with ethyl acetate, acetone, and methanol extracts showing pronounced activity, whereas petroleum ether and aqueous extracts were largely ineffective. The antibacterial effects observed in agar well diffusion

assays were supported by low MIC values, concentration-dependent bactericidal activity in time-kill studies, and significant inhibition of biofilm formation, particularly against Gram-positive bacteria. The ethyl acetate leaf extract consistently emerged as the most potent, demonstrating strong growth inhibition, rapid bacterial killing, and effective suppression of biofilm formation. The greater susceptibility of Gram-positive bacteria compared to Gram-negative bacteria underscores the influence of bacterial cell wall architecture on extract efficacy.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this research work.

ACKNOWLEDGEMENT

The authors sincerely express their gratitude to the management of Chaitanya (Deemed to be University), Himayathnagar, Telangana, for providing the necessary laboratory facilities and support to carry out this research work.

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