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Natural Compounds as Novel Biofilm Inhibitors: Targeting Multidrug-Resistant Bacterial Pathogenesis

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ABSTRACT

Introduction: Biofilm formation by multidrug-resistant (MDR) bacteria confers increased antimicrobial tolerance and contributes to persistent infections, presenting significant therapeutic challenges. These challenges have driven research into natural compounds that may target key processes, including efflux pump activity, quorum sensing, bacterial adhesion, and biofilm development. This study investigates the anti-biofilm efficacy of six naturally occurring compounds—berberine, chitosan, curcumin, eugenol, linoleic acid, and reserpine—against clinically relevant aerobic MDR bacterial pathogens. **Methods**: Biofilm formation was evaluated in 200 MDR clinical isolates, including isolates of *Escherichia coli* (n=49), *Klebsiella pneumoniae* (n=46), *Acinetobacter baumannii* (n=24), *Pseudomonas aeruginosa* (n=29), *Staphylococcus aureus* (n=25), and *Staphylococcus epidermidis* (n=27), sourced from various clinical specimens, including pus, urine, blood, and sputum. Biofilm production was quantified using the modified tissue culture plate (MTCP) method. From these isolates, 36 strong biofilm-forming isolates were selected, and the minimum biofilm inhibitory concentration (MBIC) of each compound was determined via a microtiter plate assay with two-fold serial dilutions. **Results:** Of 200 isolates, 101 (50.5%) exhibited biofilm formation. MBIC values ranged from 0.0156 mg/mL (lowest for eugenol against *E. coli* and reserpine against *E. coli*, *K. pneumoniae*, and *S. aureus*) to 1 mg/mL (for curcumin against *P. aeruginosa* and *A. baumannii*). Eugenol and reserpine showed significantly lower MBICs compared to curcumin ($P < 0.05$). Eugenol displayed the lowest mean MBIC (0.049 mg/mL) across the 36 selected strong biofilm-forming isolates, followed by reserpine (0.070 mg/mL), while curcumin exhibited the highest mean MBIC (0.583 mg/mL). Linoleic acid demonstrated its lowest MBIC (0.0312 mg/mL) against *K. pneumoniae*. **Conclusion:** The tested compounds exhibited variable anti-biofilm potency, with eugenol and reserpine demonstrating the greatest efficacy and curcumin the least, suggesting limited anti-biofilm efficacy at tested concentrations. These findings underscore the potential of eugenol, reserpine, linoleic acid, berberine, and chitosan as promising *in vitro* anti-biofilm candidates for managing biofilm-associated infections caused by MDR bacteria; however, *in vivo* efficacy, pharmacokinetics, and safety warrant further investigation in animal models and clinical trials.

INTRODUCTION

Bacterial biofilms are structured, surface-attached communities that confer significant survival advantages, including increased tolerance to antibiotics and host immune defenses. Biofilm-associated growth contributes to chronic, difficult-to-treat infections, particularly in the context of MDR pathogens. The treatment of these infections is hindered by the reduced efficacy of conventional antibiotics. This reduced efficacy is

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attributable to limited penetration of the biofilm matrix, decreased metabolic activity of embedded cells, and the presence of persister cells [1]. Biofilm-associated infections result in increased treatment failure, prolonged hospitalization, elevated morbidity, and—in severe cases—mortality. Even antibiotics of last resort exhibit limited efficacy against biofilms. For example, vancomycin, used against MDR Gram-positive pathogens such as *S. aureus*, and meropenem or colistin, used against MDR Gram-negative pathogens such as *P. aeruginosa*, often fail to eradicate biofilm-associated infections, highlighting a critical clinical challenge [2, 3]. Achieving therapeutic concentrations within biofilms often requires high antibiotic doses, increasing the risk of systemic toxicity and other adverse effects [4]. Consequently, novel therapeutic strategies, including alternative anti-biofilm agents, are urgently needed to address the challenge of biofilm-associated MDR infections.

Natural compounds derived from plants, microbes, and marine organisms have emerged as promising candidates for anti-biofilm therapy development. Many of these compounds exhibit diverse biological activities, such as antibacterial, antifungal and antiviral effects, as well as the ability to inhibit biofilm formation or disrupt pre-formed biofilms [5]. For instance, plant-derived compounds such as eugenol (from clove, *Syzygium aromaticum*) and curcumin (from turmeric, *Curcuma longa*), as well as fatty acids such as linoleic acid, have shown antibacterial activity against planktonic bacteria [6]. However, their specific anti-biofilm effects have not been systematically investigated against MDR pathogens. Importantly, the mechanisms underlying anti-biofilm activity—including quorum sensing inhibition, efflux pump modulation, and adhesion interference—often differ from those targeting planktonic growth, such as cell wall disruption. This distinction necessitates targeted investigation of anti-biofilm mechanisms.

Several natural compounds, including berberine, chitosan, curcumin, eugenol, linoleic acid, and reserpine, have exhibited promising anti-biofilm activity *in vitro* against antibiotic-susceptible strains and some clinical isolates [7–13]. The selection of these compounds was based on (i) documented antibacterial, antifungal, or anti-biofilm properties, (ii) traditional medicinal use for infection treatment, and (iii) preliminary evidence of efficacy against MDR bacteria. Mechanisms proposed in the literature include inhibition of efflux pumps, disruption of quorum sensing pathways, and suppression of bacterial adhesion and biofilm development, among others. The diversity of these mechanisms may lower the selective pressure for resistance emergence compared to conventional antibiotics. Despite this potential, comprehensive studies systematically evaluating their anti-biofilm activity against a broad range of clinically relevant MDR pathogens, including *P. aeruginosa*, *S. aureus*, and other clinically significant species, remain limited.

This study evaluates the *in vitro* anti-biofilm activity of six natural compounds—berberine, chitosan, curcumin, eugenol, linoleic acid, and reserpine—against a panel of strong biofilm-forming MDR bacterial isolates. Clinical isolates were obtained from patients at a tertiary care hospital, from patients with urinary tract infections, wound infections, pneumonia, and bacteremia. The panel included MDR strains of *K. pneumoniae*, *E. coli*, *P. aeruginosa*, *A. baumannii*, *S. aureus*, and *S. epidermidis*, reflecting the clinical significance of these pathogens. Accordingly, this study aimed to: (i) quantify biofilm formation among the tested aerobic MDR clinical isolates and (ii) determine the minimum biofilm inhibitory concentration (MBIC) of each compound against them.

MATERIAL AND METHODS

Bacterial isolates and ethical considerations. This prospective study was conducted at the Department of Microbiology, Postgraduate Institute of Medical Sciences (PGIMS), Rohtak, a tertiary care hospital, from July 2017 to October 2017. The study was approved by the Institutional Ethics Committee and adhered to the Declaration of Helsinki and institutional ethical guidelines. Two hundred non-duplicate (one isolate per patient) MDR bacterial isolates were collected from patients presenting with clinical infections. The isolates comprised *E. coli* (n=49), *K. pneumoniae* (n=46), *P. aeruginosa* (n=29), *A. baumannii* (n=24), *S. aureus* (n=25), and *S. epidermidis* (n=27). Clinical specimens included pus (n=109), urine (n=39), blood (n=29), and sputum (n=23).

Sample processing and identification. Clinical specimens were aseptically inoculated onto 5% sheep blood agar and MacConkey agar plates, then incubated aerobically at 37°C for 18–24 h. Bacterial growth was identified using standard microbiological techniques, including Gram staining and biochemical tests (oxidase, catalase, coagulase, indole, methyl red, Voges-Proskauer, citrate utilization, urease, and triple sugar iron agar) [14]. Antibiotic susceptibility was determined via the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (MHA) plates, following Clinical and Laboratory Standards Institute (CLSI) guidelines [15].

For Gram-positive bacteria, tested antibiotics included erythromycin (15 µg), penicillin (10 U), cefoxitin (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), linezolid (30 µg), doxycycline (30 µg), clindamycin (2 µg), amoxicillin/clavulanic acid (20/10 µg), ciprofloxacin (5 µg), gentamicin (10 µg), nitrofurantoin (300 µg), and norfloxacin (10 µg). For Gram-negative bacteria, tested antibiotics included gentamicin (10 µg), amikacin (30 µg), piperacillin/tazobactam (100/10 µg), cefepime (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), meropenem (10 µg), and ceftazidime (30 µg). Plates were incubated at 35±2°C for 16–18 h, and zone diameters were interpreted according to CLSI breakpoints [15].

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Bacterial suspensions were standardized to a 0.5 McFarland standard (approximately 1.5×10^8 colony-forming units [CFU]/mL) using a densitometer. MDR was defined as resistance to at least one agent in three or more antimicrobial classes. Quality control was maintained using *S. aureus* American Type Culture Collection (ATCC) 25923, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922. Sterility controls (uninoculated MHA plates) were incubated alongside test samples. Isolates were stored as nutrient agar stabs at 4°C and subcultured onto MHA plates before experiments.

Biofilm detection. Biofilm formation was quantified using the modified tissue culture plate (MTCP) method [16]. All 200 MDR isolates were inoculated into brain heart infusion (BHI) broth with 2% (*w/v*) sucrose and incubated aerobically at 37°C for 24 h. Cultures were diluted 1:100 in BHI broth, and 200 µL aliquots were transferred to sterile, untreated 96-well flat-bottom polystyrene microtiter plates (HiMedia Laboratories, Mumbai, India). After 24 h incubation at 37°C, wells were washed three times with 200 µL of phosphate-buffered saline (PBS; pH 7.4), with aspiration between washes, to remove non-adherent cells. Wells were then stained with 0.1% (w/v) crystal violet for 30 min at room temperature and rinsed three times with 200 µL of sterile deionized water. Biofilm absorbance was measured at 570 nm using a Bio-Rad iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA, USA), calibrated according to the manufacturer's instructions. Biofilm strength was classified based on optical density (OD) values (Table 1). Isolates with OD ≥ 0.120 (moderately adherent: 0.120 ≤ OD < 0.240; strongly adherent: OD ≥ 0.240) were classified as biofilm-positive; those with OD < 0.120 (non-adherent or weakly adherent) were classified as biofilm-negative. Thirty-six strong biofilm-forming isolates (OD ≥ 0.240; six per species) were selected for further testing.

Table 1. Classification of biofilm formation using the quantitative MTCP method [16]

Mean OD at 570 nm	Adherence category	Biofilm strength
OD < 0.120	Non-adherent or weakly adherent	No/weak biofilm
0.120 ≤ OD < 0.240	Moderately adherent	Moderate biofilm
OD ≥ 0.240	Strongly adherent	Strong biofilm

Natural compounds. Six natural compounds—berberine (B3251; Sigma-Aldrich, St. Louis, MO, USA) and five compounds from HiMedia Laboratories (Mumbai, India): chitosan (PCT0817-25G), curcumin (RM1449-5G), eugenol (RM6992-100G), linoleic acid (RM566-1G), and reserpine (RM1149-1G)—were procured in powdered or solid form. Compounds were stored at 20–25°C, protected from light and moisture, except for linoleic acid and curcumin, which were refrigerated at 2–6°C. Stock solutions (4 mg/mL) were prepared in 10% (*v/v*) dimethyl sulfoxide (DMSO) in water, filter-sterilized using 0.22 µm membrane filters, except for chitosan, which was dissolved in 1% (*v/v*) acetic acid in water. Two-fold serial dilutions were performed to achieve final test concentrations ranging from 0.0078 to 2 mg/mL, freshly prepared for each experiment. This range was selected based on previous studies to identify the MBIC while assessing sub-inhibitory and maximal effective concentrations within ranges reported as non-cytotoxic in the literature. Solvent controls (10% DMSO and 1% acetic acid without compounds) were included to assess potential solvent effects on biofilm formation.

Determination of MBIC. Six strong biofilm-forming isolates per bacterial species were tested (n=36 total). Isolates were cultured overnight in 2 mL BHI broth at 37°C and subsequently diluted to a 0.5 McFarland standard in 7 mL fresh BHI broth. Then, 100 µL of the bacterial suspension was combined with 100 µL of each natural compound in 96-well flat-bottom polystyrene microtiter plates. Final concentrations ranged from 0.0156 to 4 mg/mL across 10 wells via two-fold serial dilutions. A positive growth control well, containing 100 µL of the bacterial suspension and 100 µL of BHI broth (without compound), was included. A sterility control well (negative control), containing 200 µL of sterile BHI broth without bacteria, was also included on each plate. All experiments were performed in triplicate, and mean OD values were calculated. Plates were incubated aerobically in ambient air at 37°C for 24 h without shaking, then washed three times with 200 µL of PBS, fixed with 200 µL of absolute methanol for 15 min, and stained with 0.1% (*w/v*) crystal violet as described above [16]. Bound crystal violet was solubilized with 200 µL of 33% (*v/v*) glacial acetic acid, and absorbance was measured at 570 nm. MBIC was defined as the lowest concentration reducing biofilm OD to < 0.120 at 570 nm, after subtracting the sterility control OD. Figure 1 illustrates the experimental setup and staining results using reserpine as an example.

Data collection and statistical analysis. Data were recorded in Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA) and independently verified by two researchers for data entry accuracy. The primary outcome was the MBIC for each of the 36 tested isolates; the secondary outcome was the mean MBIC ± standard deviation (SD) across isolates. Descriptive statistics (frequencies, percentages, means, and standard deviations) were calculated. Data normality was assessed for MBIC values within each compound group using the Shapiro-Wilk test, which indicated non-normal distributions ($P < 0.05$). Therefore, differences in MBIC values among compounds were analyzed using the non-parametric Kruskal-Wallis test, with Dunn's post hoc test

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and Holm-Bonferroni correction for pairwise comparisons to control for Type I error. Analyses were performed using IBM SPSS Statistics version 20.0 (IBM Corp., Armonk, NY, USA), with $P < 0.05$ (two-tailed) considered statistically significant.

RESULTS

Demographics and isolate characterization. Of the 200 MDR bacterial isolates, the largest proportion was from patients aged 21–30 years (32.0%), followed by 31–40 years (21.5%), ≥50 years (17.0%), 41–50 years (16.0%), 11–20 years (9.5%), and 0–10 years (4.0%). Of the patients, 56% were male and 44% were female.

The isolates were distributed as follows: *E. coli* (n=49; 24.5%), *K. pneumoniae* (n=46; 23.0%), *P. aeruginosa* (n=29; 14.5%), *S. epidermidis* (n=27; 13.5%), *S. aureus* (n=25; 12.5%), and *A. baumannii* (n=24; 12.0%).

Biofilm formation. Of the 200 isolates, biofilm formation was detected in 101 (50.5%), while 99 (49.5%) were non-biofilm-forming. Among the 101 biofilm-forming isolates, 36 (35.6%) exhibited strong adherence, and 65 (64.4%) displayed moderate adherence. The distribution of biofilm formation by patient setting is presented in Table 2. Statistical analysis using the Chi-squared test revealed no significant association between biofilm formation and patient setting (*i.e.*, inpatients versus outpatients; $P = 0.460$). However, biofilm formation varied significantly across bacterial species (Chi-squared test, $P < 0.05$; Table 3) and clinical specimen types (Chi-squared test, $P < 0.05$; Table 4), indicating significant differences in biofilm production among bacterial species and specimen types.

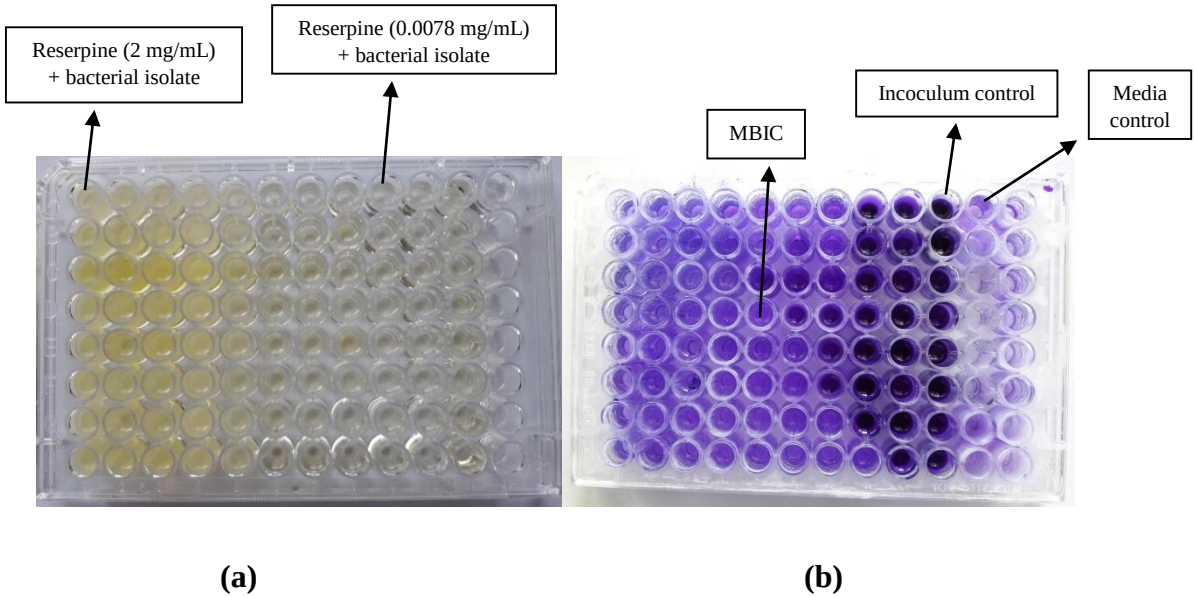

Fig. 1. Experimental setup and anti-biofilm activity assessment. (a) Serial two-fold dilutions of reserpine (0.0078-2 mg/mL) mixed with bacterial culture in brain heart infusion (BHI) broth (adjusted to a 0.5 McFarland standard) in a 96-well flat-bottom polystyrene microtiter plate. Wells 1–9 contain reserpine and culture; Well 10 (positive control) contains culture only; Well 11 (negative control) contains sterile broth. (b) Anti-biofilm activity is visualized after phosphate-buffered saline (PBS) washing, methanol fixation, and crystal violet staining using the modified tissue culture plate (MTCP) method [16].

Table 2. Biofilm formation by patient setting

Patient setting	Biofilm-forming isolates, n (%)	Non-biofilm-forming isolates, n (%)
Inpatients (n=151)	79 (52.3%)	72 (47.7%)
Outpatients (n=49)	22 (44.9%)	27 (55.1%)
Total (n=200)	101 (50.5%)	99 (49.5%)

Note: Percentages are calculated within each row. $P = 0.460$ (Chi-squared test); no significant association between biofilm formation and patient setting.

Antibiotic resistance profiles. Biofilm-forming (BF) isolates generally exhibited higher resistance rates than non-biofilm-forming (NBF) isolates (Tables 5 and 6). For Gram-negative isolates (n=148; Table 5), significant differences in resistance rates between BF and NBF isolates were observed only for amikacin ($P = 0.020$; Chi-squared test). Among Gram-positive isolates, *S. aureus* and *S. epidermidis* (n=52; Table 6) showed significant differences for gentamicin ($P = 0.034$; Chi-squared test) and ciprofloxacin ($P = 0.045$; Chi-squared test).

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Table 3. Biofilm formation by bacterial species

Bacterial species (n)	Strong, n (%)	Moderate, n (%)	Non-Biofilm, n (%)	*P*-value
S. epidermidis (n=27)	6 (22.2)	13 (48.1)	8 (29.6)	0.500
P. aeruginosa (n=29)	6 (20.7)	14 (48.3)	9 (31.0)	0.433
A. baumannii (n=24)	6 (25.0)	8 (33.3)	10 (41.7)	0.776
S. aureus (n=25)	6 (24.0)	6 (24.0)	13 (52.0)	0.411
E. coli (n=49)	6 (12.2)	14 (28.6)	29 (59.2)	0.014
K. pneumoniae (n=46)	6 (13.0)	10 (21.7)	30 (65.2)	0.007
Total (n=200)	36 (18.0%)	65 (32.5%)	99 (49.5%)	—

Note: *P*-values from Chi-squared tests (df=2) comparing the distribution of biofilm categories for each species against the overall distribution; significant differences were observed for *E. coli* and *K. pneumoniae*.

Table 4. Biofilm formation by specimen type

Specimen type (n)	Biofilm-forming, n (%)	Non-biofilm-forming, n (%)	*P*-value
Pus (n=109)	66 (60.6)	43 (39.4)	0.003
Sputum (n=23)	10 (43.5)	13 (56.5)	0.621
Urine (n=39)	15 (38.5)	24 (61.5)	0.134
Blood (n=29)	10 (34.5)	19 (65.5)	0.096
Total (n=200)	101 (50.5%)	99 (49.5%)	—

Note: *P*-values from Chi-squared tests (df=1) comparing biofilm formation proportions for each specimen type against the overall distribution; significant association found for pus samples, which showed higher biofilm formation than expected.

Table 5. Antibiotic resistance in Gram-negative BF and NBF isolates

Antibiotic	Resistant BF, n (%)	Resistant NBF, n (%)	*P*-value
Gentamicin	40 (57.1)	39 (50.0)	0.481
Amikacin	54 (77.1)	45 (57.7)	0.020
Ceftazidime	57 (81.4)	63 (80.7)	1.000
Cefepime	47 (67.1)	48 (61.5)	0.590
Piperacillin-tazobactam	49 (70.0)	48 (61.5)	0.364
Meropenem	36 (51.4)	34 (43.5)	0.430
Imipenem	16 (22.9)	16 (20.5)	0.884
Ciprofloxacin	56 (80.0)	55 (70.5)	0.254

Abbreviations: BF, biofilm-forming; NBF, non-biofilm-forming.
Note: Gram-negative BF and NBF isolates (n=148; BF: n=70; NBF: n=78); *P*-values were calculated using the Chi-squared test or Fisher's exact test as appropriate.

Table 6. Antibiotic resistance in *S. aureus* and *S. epidermidis* BF and NBF isolates

Antibiotic	Resistant BF isolates, n (%)	Resistant NBF isolates, n (%)	*P*-value
Penicillin	31 (100%)	21 (100%)	>0.999
Amoxicillin-clavulanic acid	24 (77.4%)	13 (61.9%)	0.368
Cefoxitin	21 (67.7%)	9 (42.9%)	0.135
Erythromycin	21 (67.7%)	16 (76.2%)	0.728
Clindamycin	16 (51.6%)	7 (33.3%)	0.308
Cotrimoxazole	29 (93.5%)	18 (85.7%)	0.645
Doxycycline	14 (45.2%)	6 (28.6%)	0.359
Linezolid	1 (3.2%)†	0 (0.0%)	>0.999
Gentamicin	28 (90.3%)	13 (61.9%)	0.034*
Ciprofloxacin	31 (100%)	17 (81.0%)	0.045*
Nitrofurantoin	2 (6.5%)	1 (4.8%)	>0.999
Norfloxacin	4 (12.9%)	2 (9.5%)	>0.999

Abbreviations: BF, biofilm-forming; NBF, non-biofilm-forming.
Note: *P*-values were calculated using the Chi-squared test or Fisher's exact test as appropriate. * $P < 0.05$ is statistically significant. †One linezolid-resistant isolate was identified among BF isolates. *S. aureus* and *S. epidermidis* BF and NBF isolates (n=52; BF: n=31; NBF: n=21)

MBIC of natural compounds. MBIC values were determined for six strong biofilm-forming isolates per species (Table 7). Eugenol exhibited the lowest mean MBIC (0.049 mg/mL), with values as low as 0.0156 mg/mL against *E. coli*. Reserpine showed an MBIC of 0.0156 mg/mL against *E. coli*, *K. pneumoniae*, and *S. aureus*. Linoleic acid had an MBIC of 0.0312 mg/mL against *K. pneumoniae*. Curcumin displayed the highest mean MBIC (0.583 mg/mL), reaching 1 mg/mL against *P. aeruginosa* and *A. baumannii*. Berberine and chitosan exhibited intermediate MBICs (0.0625–0.5 mg/mL).

The Kruskal-Wallis test indicated significant differences in MBIC values among the six natural compounds ($P < 0.05$). Post hoc analysis using Dunn's test with Holm-Bonferroni correction (Table 8) revealed that the MBIC of eugenol was significantly lower than that of curcumin ($P = 0.009$), berberine ($P = 0.014$), chitosan ($P = 0.033$), and linoleic acid ($P = 0.047$). The MBIC of

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reserpine was significantly lower than that of curcumin (P = 0.007) and that of chitosan (P = 0.026). The difference between reserpine and eugenol was not statistically significant (P = 0.426); all other non-significant pairwise comparisons are detailed in Table 8. Overall, eugenol and reserpine demonstrated the lowest MBIC values, indicating the highest anti-biofilm potency among the tested compounds.

Table 7. MBIC of natural compounds (mg/mL)

Bacterial species (n)	Berberine	Chitosan	Curcumin	Eugenol	Linoleic Acid	Reserpine
E. coli (n=6)	0.25	0.0625	0.25	0.0156	0.125	0.0156
K. pneumoniae (n=6)	0.125	0.125	0.5	0.0625	0.0312	0.0156
P. aeruginosa (n=6)	0.0625	0.5	1	0.0312	0.25	0.0625
A. baumannii (n=6)	0.25	0.5	1	0.125	0.25	0.25
S. aureus (n=6)	0.125	0.25	0.25	0.0312	0.0625	0.0156
S. epidermidis (n=6)	0.125	0.125	0.5	0.0312	0.125	0.0625
Mean (all species, n=36)	0.156	0.260	0.583	0.049	0.141	0.070

Note: MBIC values represent the lowest concentration inhibiting biofilm formation (OD < 0.120) for each species. Bold values indicate the lowest (most potent) MBIC observed. Statistical comparisons between compounds are presented in Table 8.

Table 8. Post hoc pairwise comparisons of MBIC values

Comparison	*P*-value	Significance
Reserpine *vs.* Eugenol	0.426	NS
Reserpine *vs.* Curcumin	0.007*	S
Reserpine *vs.* Linoleic Acid	0.055	NS
Reserpine *vs.* Chitosan	0.026*	S
Reserpine *vs.* Berberine	0.062	NS
Eugenol *vs.* Curcumin	0.009*	S
Eugenol *vs.* Linoleic Acid	0.047*	S
Eugenol *vs.* Chitosan	0.033*	S
Eugenol *vs.* Berberine	0.014*	S
Curcumin *vs.* Linoleic Acid	0.010*	S
Curcumin *vs.* Chitosan	0.010*	S
Curcumin *vs.* Berberine	0.033*	S
Linoleic Acid *vs.* Chitosan	0.076	NS
Linoleic Acid *vs.* Berberine	0.745	NS
Chitosan *vs.* Berberine	0.296	NS

Abbreviations: S, significant (*P < 0.05 after Holm-Bonferroni correction); NS, non-significant ($P \geq 0.05$); *vs*: versus. **Note:** Kruskal-Wallis test with Dunn's post hoc analysis and Holm-Bonferroni correction. Fifteen pairwise comparisons were performed for six compounds.

DISCUSSION

This study evaluated the *in vitro* anti-biofilm activity of six natural compounds—berberine, chitosan, curcumin, eugenol, linoleic acid, and reserpine—against 36 strong biofilm-forming MDR bacterial isolates, demonstrating variable anti-biofilm efficacy. Eugenol and reserpine exhibited the lowest MBIC values (highest potency), while curcumin showed the highest MBIC values (lowest potency). These findings highlight their potential as novel therapeutic agents. Biofilms significantly contribute to persistent infections, and the limited efficacy of conventional antibiotics against biofilm-associated MDR pathogens underscores the continued need for alternative strategies. Natural compounds, with their diverse mechanisms such as quorum sensing inhibition and efflux pump modulation, represent promising candidates for anti-biofilm therapy development.

Biofilm formation was detected in 50.5% (101/200) of MDR isolates, aligning with the 54.2% prevalence reported by Faaz *et al*. (2012) in a similar clinical setting [17]. Pus samples exhibited the highest biofilm prevalence (60.6%), consistent with their association with wound infections, followed by sputum (43.5%), urine (38.5%), and blood (34.5%), corroborating findings by Asati *et al*. (2017) [18]. This distribution could potentially reflect the frequent use of medical devices in hospitalized patients, a known risk factor for biofilm formation, although this association was not directly assessed in the current study. Regarding species-specific biofilm formation, *S. epidermidis* (70.4%) and *P. aeruginosa* (69.0%) showed the highest biofilm-forming rates, followed by *A. baumannii* (58.3%), *S. aureus* (48.0%), *E. coli* (40.8%), and *K. pneumoniae* (34.8%). The biofilm formation rates observed for *S. aureus* (48.0%) and *S. epidermidis* (70.4%) exceed the 42.7% overall biofilm formation rate for staphylococci reported by Samant *et al*. (2012) [19], possibly due to differences in strain composition, patient populations, biofilm detection methods, or temporal trends in biofilm prevalence. The 58.3% biofilm formation rate for *A. baumannii* aligns with Bala *et al*. (2017) (52%) [20], reinforcing the clinical significance of biofilm-mediated infections caused by this pathogen. Similarly, the biofilm formation rates for *E. coli* (40.8%) and *K. pneumoniae* (34.8%) are consistent with the approximately 40% reported by Faaz *et al*. (2012) [17] and Yang *et al*. (2008) [17, 21]. Consistent with prior studies, BF isolates exhibited generally higher antibiotic resistance than NBF isolates, with statistically significant

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differences observed for amikacin (Gram-negative isolates) and gentamicin and ciprofloxacin (Gram-positive isolates), a trend also noted by Asati *et al*. (2017) [18].

Eugenol demonstrated the lowest mean MBIC (0.049 mg/mL), indicating potent anti-biofilm activity, while curcumin exhibited the highest (0.583 mg/mL). Reserpine was notably effective against *K. pneumoniae*, *E. coli*, and *S. aureus* (MBIC = 0.0156 mg/mL), consistent with prior reports [4], with linoleic acid following at 0.0312 mg/mL against *K. pneumoniae*. Curcumin required higher concentrations (0.25–1 mg/mL), particularly against *P. aeruginosa* and *A. baumannii* (1 mg/mL)**,** which may be attributed to its limited aqueous solubility or biofilm penetration. The MBIC of berberine against *P. aeruginosa* (0.0625 mg/mL) falls within the 0.019–1.25 mg/mL range reported previously [22], indicating comparable anti-biofilm activity, while the MBIC values of chitosan were consistent with those reported by Etemadi *et al*. (2021) [9]. The MBIC of eugenol against *S. aureus* (0.0312 mg/mL) was lower than the 0.05–0.2 mg/mL range reported by Yadav *et al*. (2015) [7], suggesting enhanced potency in the current study, and against *E. coli* (0.0156 mg/mL); it demonstrated greater potency than the 0.05 mg/mL noted by Kim *et al*. (2016) [23]. Post hoc analysis confirmed significant differences in MBIC values ($P < 0.05$), with eugenol showing significantly lower MBIC values than curcumin, berberine, chitosan and linoleic acid, and with reserpine demonstrating significantly lower MBIC values than curcumin and chitosan (Tables 7 and 8). These findings suggest eugenol, reserpine, linoleic acid, and berberine as promising candidates for further anti-biofilm research, while curcumin demonstrated limited potency at the concentrations tested.

Variations in anti-biofilm efficacy across studies are likely attributable to differences in bacterial strains, regional variations in resistance patterns, methodological approaches, and compound-specific mechanisms [24]. Furthermore, the significant MBIC differences among the tested compounds may reflect diverse modes of action, such as quorum sensing inhibition (eugenol [7, 23], reserpine [4]), efflux pump disruption (reserpine [4]), quorum sensing modulation (berberine [10]), or adhesion interference (chitosan [9, 12]), among others. These distinct mechanisms could inform future studies on combination therapies to synergistically enhance anti-biofilm efficacy. While these *in vitro* results are encouraging, they represent a preliminary assessment, and translating effective concentrations to *in vivo* settings remains a challenge due to factors such as bioavailability, compound stability, tissue penetration, and potential host toxicity.

This study complements existing evidence on natural compounds as anti-biofilm agents, and has notable strengths, including the systematic comparison of six compounds against clinically relevant MDR isolates from diverse specimen types. However, the study also has limitations. It focused solely on MBIC, omitting minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assessments for planktonic cells, as well as minimum biofilm eradication concentration (MBEC) determination, which could provide insights into planktonic cell susceptibility and allow comparison of anti-biofilm versus bactericidal activities. Additionally, the study was conducted at a single center, tested a limited number of isolates per species (n=6) for MBIC determination, and did not investigate the molecular mechanisms underlying the observed anti-biofilm effects. Furthermore, the *in vitro* nature of this study limits direct extrapolation of findings to clinical settings. To address these limitations, future research should: (1) elucidate the molecular mechanisms of biofilm inhibition (*e.g*., through gene expression analysis or proteomic studies), (2) assess *in vivo* efficacy using appropriate animal infection models, and (3) conduct cytotoxicity assessments on mammalian cells, followed by clinical trials to evaluate safety and therapeutic potential. Synergy studies combining these natural compounds with conventional antibiotics may also reveal enhanced therapeutic potential. Additionally, developing advanced formulations (*e.g*., nanoparticle encapsulation, liposomal delivery systems) to optimize stability, bioavailability, and targeted delivery are essential for successful clinical translation.

In conclusion, biofilm formation was detected in 50.5% of the 200 MDR isolates tested, with pus samples and staphylococci showing the highest prevalence. This study demonstrated the *in vitro* anti-biofilm activity of six natural compounds (berberine, chitosan, curcumin, eugenol, linoleic acid, and reserpine) against clinically relevant MDR bacteria, with eugenol and reserpine exhibiting the greatest potency (mean MBIC: 0.049 and 0.070 mg/mL, respectively) and curcumin the least (mean MBIC: 0.583 mg/mL). Linoleic acid and berberine also demonstrated notable anti-biofilm activity. These results support further investigation into synergistic combinations with conventional antibiotics, as well as *in vivo* efficacy and safety studies. These findings underscore the therapeutic potential of natural compounds as alternative or adjunctive anti-biofilm agents in the era of increasing antimicrobial resistance.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interests associated with this manuscript.

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AI DISCLOSURE

No artificial intelligence tools or technologies were used in the design, execution, analysis, or writing of this study.

DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

AUTHORS' CONTRIBUTIONS

AJ: Conceptualization; Methodology; Investigation; Data analysis; Writing – review & editing. SA: Data analysis and manuscript review. UC: Supervision and critical review. All authors approved the final version.

ETHICS STATEMENT

This study is based on work originally conducted as part of the MD/MS thesis research of Dr. Ankita Agrawal (Roll No. 367054) in the Department of Microbiology, Pt. B. D. Sharma PGIMS, Rohtak. The thesis, titled "Biofilm Inhibition of Aerobic Bacteria by Natural Compounds" (Examination: May 2018), was officially evaluated and classified as 'Commended' by Pt. B. D. Sharma University of Health Sciences, Rohtak (Reference No. UHSR/Exams./AE-II/24/766).

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