

International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; 10(9): 37-45
www.biochemjournal.com
Received: 17-06-2026
Accepted: 20-07-2026

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Exploration of antibiofilm and antibacterial activity of eugenol and cinnamic acid on *Pseudomonas aeruginosa* isolates from dogs

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i9a.9824>

Abstract

Pseudomonas aeruginosa is an important opportunistic pathogen associated with recurrent otitis, pyoderma and wound infections in dogs. The combination of antimicrobial resistance and biofilm formation can complicate treatment and contribute to persistence of infection. The present investigation aimed to characterize *P. aeruginosa* isolates obtained from canine clinical samples and to assess the *in vitro* antibacterial and antibiofilm activities of eugenol and cinnamic acid. Ninety-seven clinical samples collected from dogs with otitis, pyoderma and chronic wounds were examined, from which 35 isolates were confirmed as *P. aeruginosa* by PCR. Antimicrobial susceptibility testing demonstrated that all 35 (100%) isolates were classified as multidrug-resistant and 30 (85.71%) exhibiting an extensively drug-resistant phenotype. Phenotypic testing identified 21 (60%) isolates as ESBL producers. Biofilm production was detected in all 35 isolates by the microtiter plate assay, and the biofilm-associated genes *ppvR* and *pslA* were detected in 85.71% and 77.14% of isolates, respectively. Both phytochemicals demonstrated activity against the tested isolates. Eugenol showed MIC values ranging from 6.41 to 51.31 µg/mL, whereas the MIC range for cinnamic acid was 31.25-500 µg/mL. In the biofilm inhibition assay, mean inhibition values for eugenol were 43.144±2.638% and 20.983±2.010% at 0.4 and 0.2 mg/mL, respectively. Cinnamic acid produced mean inhibition values of 55.873±2.050% and 47.625±2.146% at 2.5 and 1.25 mg/mL, respectively. The findings demonstrated that eugenol and cinnamic acid exhibited measurable antibacterial and antibiofilm activities under the conditions evaluated, supporting their further investigation as potential phytochemical-based approaches against resistant *P. aeruginosa*.

Keywords: Biofilm, minimum inhibitory concentration, multi- drug resistant, phytochemicals

Introduction

In canine clinical practice, *Pseudomonas aeruginosa* is an important opportunistic pathogen associated with persistent infections of the ear, skin and wounds. It has been associated with several chronic and difficult-to-manage infections, including deep pyoderma, otitis externa, wound infections and urinary tract infections (Hillier *et al.*, 2006; Hariharan *et al.*, 2006; Mekic *et al.*, 2011) [13, 15, 22]. The therapeutic management of infections caused by this organism is complicated by its ability to withstand antimicrobial treatment through intrinsic, acquired and mutational mechanisms of resistance (Livermore, 2002; Linder *et al.*, 2005) [19, 20]. The increasing occurrence of Multidrug-Resistant (MDR) *P. aeruginosa* highlights the need to investigate alternatives or complementary approaches to conventional antimicrobial therapy. This concern becomes particularly relevant in biofilm-associated infections, where the presence of a biofilm can contribute to persistence and reduced susceptibility to antimicrobial treatment. Consequently, compounds derived from plants have attracted considerable attention as potential sources of antimicrobial agents. Plants contain numerous biologically active secondary metabolites, including flavonoids, alkaloids, terpenoids and tannins, several of which have demonstrated antimicrobial activity *in vitro* (Cowan *et al.*, 1999; Lewis *et al.*, 2006) [6, 18]. The antibacterial potential of plant-derived compounds has been investigated for many years, following the identification of naturally occurring compounds with therapeutic properties (Schmitz, 1985) [33]. Such phytochemicals may therefore provide useful candidates for investigation against resistant bacterial pathogens.

In our earlier investigations, the same canine clinical isolate collection was characterized with respect to antimicrobial resistance and ESBL production (Swathi *et al.*, 2024) [36, 37]. The present study extends this work by examining the biofilm-forming characteristics of these isolates and evaluating the *in-vitro* antibacterial and antibiofilm activities of eugenol and cinnamic acid.

Materials & Method

Sample collection

A total of 97 clinical samples were obtained from dogs presenting with different clinical conditions. Ear swabs were collected from cases of otitis externa, while skin swabs were obtained from dogs with superficial pyoderma or chronic wounds. Sterile cotton-tipped swabs were used for sample collection. The clinical samples used in the present investigation were derived from the same canine sample collection previously described (Swathi *et al.* 2024a, b) [36, 37].

Isolation and characterization of *Pseudomonas aeruginosa*

The collected ear, skin and wound swabs were directly inoculated into Brain Heart Infusion (BHI) broth and incubated at 37 °C for 24 h. Following incubation, cultures showing turbidity were subsequently streaked onto Pseudomonas Isolation Agar (PIA) and maintained at 37 °C for 24-48 h. Colonies exhibiting the characteristic translucent appearance with green pigmentation were provisionally considered *Pseudomonas* spp. based on their colonial morphology. These colonies were further examined by Gram staining. Species-level confirmation of the *P. aeruginosa* isolates was subsequently performed by PCR using oligonucleotide primers targeting species-specific genes (Spiker *et al.*,). The isolation and preliminary characterization of the canine *P. aeruginosa* isolates have been described previously (Swathi *et al.*, 2024a, b) [36, 37].

Antibiotic sensitivity test

The antimicrobial susceptibility profile of the confirmed *P. aeruginosa* isolates was assessed by the Kirby-Bauer disc diffusion technique (Bauer *et al.*, 1966) [1]. Ten antibiotics commonly used in veterinary practice were included in the test panel. The diameter of the inhibition zones produced around the antibiotic discs was measured and used for interpretation of the susceptibility or resistance pattern of individual isolates.

Multiple antibiotic resistance index

The multiple antibiotic resistance (MAR) index was calculated as the proportion of antibiotics to which an isolate was resistant relative to the total number of antibiotics tested (Osundiya *et al.*, 2013) [24].

MAR index = a/b

Where **a** represents the number of antibiotics against which the isolate exhibited resistance and **b** represents the total number of antibiotics to which the isolate was exposed.

Detection of ESBL production

Phenotypic screening for extended-spectrum β -lactamase (ESBL) production was initially carried out using the Phenotypic Screening Test (PST). Isolates identified as suspected ESBL producers were subsequently evaluated by

the Double Disk Synergy Test (DDST), following the CLSI (2020) guidelines. The confirmed *P. aeruginosa* isolates were further examined by multiplex PCR (m-PCR) for the ESBL-associated genes *blaTEM*, *blaSHV* and *blaOXA* (Spiker *et al.*,). The phenotypic and molecular ESBL detection procedures were based on the methodology previously described by Swathi *et al.* (2024b) [37].

Detection of biofilm formation

Biofilm-forming ability was assessed using three phenotypic approaches: the tube method (Christensen *et al.*, 1985) [2], Congo Red Agar (CRA) method (Freeman *et al.*, 1989) [9] and Microtiter Plate (MTP) assay, with modifications based on Robinson *et al.* (2019) [32]. The presence of the biofilm-associated genes *ppvR* and *pslA* was investigated according to the procedure described by Pournajaf *et al.* (2018) [27].

Microtiter plate assay

For quantitative assessment of biofilm formation, 180 μ L of Tryptic Soy Broth (TSB) was dispensed into the wells of the first row of a microtiter plate, followed by addition of 20 μ L of bacterial suspension adjusted to 0.5 McFarland turbidity. Three wells containing sterile TSB without bacterial inoculum were included as negative controls. The plates were incubated at 37 °C for 24 h.

After incubation, the culture contents were removed and each well was washed three times with 250 μ L phosphate-buffered saline (PBS; pH 7.2). The plates were then inverted and heat-fixed at 60 °C for 60 min. Subsequently, 200 μ L methanol was added to each well and allowed to remain for 15 min at room temperature to fix the adherent cells. The wells were then stained with 150 μ L of 0.1% crystal violet for 15 min at room temperature.

Following staining, excess dye was discarded and the wells were rinsed with sterile distilled water. The plates were inverted and allowed to dry at room temperature for 15-30 min. Biofilm-associated crystal violet was subsequently eluted by adding 150 μ L of 95% ethanol to each well, followed by incubation for 30 min at room temperature. The Optical Density (OD) was measured at 570 nm using a Multiskan GO ELISA plate reader (Thermo Scientific).

Interpretation of the MTP assay

The cut-off Optical Density (OD_c) was calculated as three standard deviations above the mean OD of the negative-control wells. Based on the measured OD values, isolates were categorized as follows

- Non-biofilm producers: $OD \leq OD_c$
- Weak biofilm producers: $OD_c < OD \leq 2 \times OD_c$
- Moderate biofilm producers: $2 \times OD_c < OD \leq 4 \times OD_c$
- Strong biofilm producers: $OD > 4 \times OD_c$

Biofilm inhibition study

Biofilms were first established in microtiter plates using the procedure described above. For evaluation of biofilm inhibition, the established biofilms were exposed separately to cinnamic acid and eugenol by adding 200 μ L of the respective phytochemical preparation to the wells and incubating for 1 h (Coelho *et al.*, 2013) [3]. Untreated wells containing 180 μ L TSB and 20 μ L bacterial suspension were maintained for the same duration as positive controls.

Following treatment, the liquid from each well was removed and the biofilms were washed twice with sterile water. The extent of inhibition was determined by comparing the OD

obtained from untreated control wells with that of phytochemical-treated wells. Absorbance was recorded at 630 nm using a Multiskan GO microtiter plate reader (Thermo Scientific).

The following controls were included

- **Extract control:** TSB containing the respective phytochemical extract.
- **Positive control:** TSB containing the bacterial suspension.
- **Negative control:** TSB containing sterile distilled water.
- **Media control:** TSB alone.

The percentage inhibition of biofilm formation was calculated using

$$\% \text{ inhibition} = [(OD \text{ of control} - OD \text{ of sample}) / OD \text{ of control}] \times 100$$

Determination of minimum inhibitory concentration of phytochemicals

The Minimum Inhibitory Concentrations (MICs) of eugenol and cinnamic acid were determined using the resazurin-based turbidometric assay described by Teh *et al.* (2017)^[38], with minor modifications. Resazurin was used as an oxidation-reduction indicator for assessing bacterial growth.

Preparation of resazurin solution

Resazurin was prepared in sterile distilled water at a concentration of 6.75 mg/mL. The dye is initially blue and, following reduction by oxidoreductase activity in viable cells, undergoes a colour transition through pink to colourless resorufin-related products.

Preparation of bacterial inoculum

Pure cultures of the *P. aeruginosa* isolates were inoculated into 5 mL Mueller-Hinton Broth (MHB) and incubated for 24 h at 37 °C. The resulting suspensions were adjusted to the turbidity of a 0.5 McFarland standard using sterile MHB. Subsequently, 10 µL of the adjusted suspension was dispensed into each well of U-bottom 96-well microtiter plates.

Preparation of phytochemical solutions

For cinnamic acid, a stock solution of 20 mg/mL was prepared by dissolving 20 mg of cinnamic acid in 1 mL Dimethyl Sulfoxide (DMSO). The eugenol stock preparation was obtained by diluting 1 mL of 98% (v/v) eugenol with a small quantity of Tween 80, maintaining the final Tween 80 concentration below 0.5%. The resulting preparation had a concentration of 16,420 µg/mL, based on a molecular weight of 164.2 g/mol, and the final volume was adjusted to 10 mL using sterile distilled water.

Preparation of antibiotic solution

Amoxicillin was used as the reference antibiotic. Its stock solution was prepared by dissolving 10 mg of the antibiotic in 1 mL sterile distilled water.

MIC assay

The assay was performed in U-bottom 96-well microtiter plates (Tarsons, Kolkata). Each well in the first row initially received 100 µL MHB. An equal volume (100 µL) of either cinnamic acid or eugenol preparation was then introduced

into the first well. Two-fold serial dilution was performed sequentially by transferring 100 µL from one well to the next, continuing through the twelfth well. After completion of the dilution series, 100 µL was discarded from the final well.

A 10 µL aliquot of the 0.5 McFarland-adjusted bacterial suspension was added to each well and mixed thoroughly. Each isolate was tested in duplicate. The plates were incubated overnight at 37 °C.

Following incubation, 10 µL of resazurin solution (6.75 mg/mL) was added to every well, and the plates were incubated for a further 4 h at 37 °C. The wells were examined visually for a colour change from purple through pink to colourless. The lowest concentration of phytochemical associated with the observed colour change was recorded as the MIC.

Controls

The following controls were included in the assay:

- **Extract control:** MHB containing the respective phytochemical extract.
- **Antibiotic control:** MHB containing amoxicillin.
- **Growth control:** MHB containing the bacterial suspension.
- **Sterility control:** MHB containing sterile distilled water.
- **Media control:** MHB alone.

Results & Discussion

Sampling, isolation and characterization of *P. aeruginosa*: In this study, a total of 97 samples were collected, of which 45 samples were from otitis cases and 52 samples were from pyoderma and chronic wounds of dogs. Out of 97 samples enriched using BHI broth, 35 samples predominantly produced characteristic green pigmentation on PIA and also have shown pink colour rods on Gram's staining. All these 35 samples were subjected to species specific confirmation by PCR using 16S *rRNA* and yielded a specific PCR product at 956 bp. This present study correlates with the published results on the prevalence of *P. aeruginosa* which reported to be ranging from 6.5 % to 43 % (Foster and DeBoer 1998; Colombini *et al.*, 2000; Quinn *et al.*, 2011; Falodun and Musa 2020)^[4, 8, 20, 28].

Antibiotic sensitivity profile of *P. aeruginosa*

The antimicrobial susceptibility profile of the 35 confirmed *P. aeruginosa* isolates, previously characterized by our group (Swathi *et al.*, 2024a)^[36], was considered as the baseline antimicrobial-resistance profile for the present investigation. Antimicrobial susceptibility testing was carried out for all 35 PCR-confirmed *P. aeruginosa* isolates using the Kirby-Bauer disc diffusion method with ten antibiotics. The greatest proportion of resistant isolates was recorded for tetracycline (97.14%), followed by ampicillin (94.28%). Resistance to aztreonam, streptomycin and enrofloxacin was 91.42% for each antibiotic, while resistance to co-trimoxazole, amoxiclav and gentamicin was 88.57%, 80% and 74.28%, respectively. Ceftriaxone showed resistance in 65.71% of isolates, whereas imipenem exhibited the lowest resistance rate (11.42%). Importantly, all 35 isolates (100%) fulfilled the criteria for Multidrug Resistance (MDR), while 30 isolates (85.71%) were classified as Extensively Drug-Resistant (XDR). All isolates also showed MAR index values ≥ 0.2 . These baseline

resistance characteristics were previously reported for this isolate collection (Swathi *et al.*, 2024a) [36].

The low resistance observed against imipenem (11.42%) was comparable with the 14% reported by Plokarz *et al.* (2023) [25]. In contrast, the resistance rates for enrofloxacin and gentamicin in the present investigation were considerably higher than those reported by Ramanarayana (2022) [30, 31], who recorded resistance of 9.61% and 21.15%, respectively. Such differences between studies may reflect variation in antimicrobial-use practices and treatment protocols across geographical regions. The proportion of MDR isolates observed in the present study was also comparable with the 97.56% MDR reported by Costa *et al.* (2022) [5]. Furthermore, the MAR findings were consistent with Ramanarayana (2022) [30, 31], where all 55 *P. aeruginosa* isolates had MAR indices above 0.2.

ESBL profile of *P. aeruginosa* isolates

The ESBL characteristics of the 35 *P. aeruginosa* isolates used in the present study have been reported previously by our group (Swathi *et al.*, 2024b) [37]. Briefly, phenotypic confirmation by DDST identified 21 (60%) isolates as ESBL producers.

All 35 confirmed *P. aeruginosa* isolates were initially evaluated by phenotypic screening for ESBL production. Resistance to at least one of the tested cephalosporins resulted in their classification as suspected ESBL producers. The DDST was subsequently performed using cefotaxime, ceftazidime, ceftriaxone and aztreonam positioned around a central amoxiclav disc. A positive synergy or “ghost” phenomenon was observed in 21 isolates, corresponding to 60% of the tested population. The 35 PCR-confirmed isolates were further examined for the presence of *blaSHV*, *blaOXA* and *blaTEM*. Amplification products of 564 bp, 713 bp and 800 bp were obtained for *blaSHV*, *blaOXA* and *blaTEM*, respectively. At least one of the investigated ESBL genes was detected in 15 isolates (42.85%). Among the individual genes, *blaOXA* was detected most frequently, occurring in 11 isolates (31.42%), followed by *blaSHV* in nine isolates (25.71%) and *blaTEM* in seven isolates (20%).

The phenotypic ESBL frequency observed here was comparable with the 70.15% reported by Laudy *et al.* (2017) [16]. The frequencies of *blaSHV* and *blaTEM* were also considered in relation to earlier reports by Shacheraghi *et al.* (2010) and Ramanarayana *et al.* (2022) [30, 31]. Differences in the distribution of individual ESBL genes among studies may be associated with geographical variation, as suggested in the original manuscript. The phenotypic and genotypic ESBL findings were consistent with our earlier report based on this isolate collection (Swathi *et al.*, 2024b) [37].

Biofilm formation by *P. aeruginosa* isolates

The three phenotypic assays used for biofilm detection tube assay, Congo Red Agar (CRA) method and microtiter plate (MTP) assay showed differences in the distribution of biofilm-producing isolates.

Using the tube method, 3 isolates (8.57%) were classified as non-adherent, 8 (22.85%) as weak producers, 14 (40%) as moderate producers and 10 (28.57%) as strong producers. These findings differed from those of Raheem *et al.* (2023) [29], who reported 5% non-adherent, 5% weak and 90% strong biofilm-forming isolates. Differences in categorization may partly arise from the subjective interpretation associated with visually assessing the intensity of biofilm formation.

The CRA assay classified all 35 isolates (100%) as biofilm producers. Within this group, 17.14% were weak, 45.71% moderate and 37.14% strong producers. The observation that all isolates produced biofilm was comparable with the findings of Locatelli *et al.* (2004) [21] and Freitas and Barth (2002) [10], who also reported biofilm production among all *P. aeruginosa* strains examined. Quantitative assessment using the MTP assay similarly identified all 35 isolates as biofilm producers. Of these, 8.57% were weak, 62.85% moderate and 28.57% strong producers. The manuscript notes that 1% glucose was incorporated into TSB to promote biofilm development, consistent with the approach reported by Hassan *et al.* (2011) [14]. The 100% biofilm-positive finding obtained by CRA and MTP was also comparable with the report of Ramanarayana (2022) [30, 31].

Table 1: Quantification of biofilm production by *P. aeruginosa* isolates using MTP assay

S. No	Sample No.	MTP	
		Adherence	OD
1	O1	S	1.445
2	O2	M	1.155
3	O3	M	0.994
4	O5	M	0.819
5	O9	M	0.850
6	O10	M	0.651
7	O11	M	0.701
8	O12	S	1.277
9	O13	S	1.689
10	O14	S	1.329
11	O15	M	0.646
12	O21	W	0.507
13	O22	M	0.834
14	O25	W	0.610
15	O26	M	0.742
16	O27	M	1.071
17	O34	M	1.029
18	O38	S	1.342
19	W1	M	0.994
20	W13	M	1.067
21	W15	W	0.580

22	W16	M	0.880
23	W31	M	1.122
24	W32	M	0.852
25	W33	M	0.992
26	W34	M	1.031
27	W35	S	1.598
28	W40	M	0.946
29	W42	M	1.201
30	W43	S	1.338
31	W44	M	1.041
32	W48	M	1.217
33	W49	S	1.496
34	W50	S	1.625
35	W51	S	1.461

S- strong; M- moderate; W- weak

Detection of biofilm-associated genes

Molecular examination of the 35 *P. aeruginosa* isolates demonstrated the presence of the biofilm-associated genes *ppyR* and *pslA*. The *ppyR* gene was detected in 30 isolates (85.71%), whereas *pslA* was identified in 27 isolates (77.14%). The frequencies observed in the present investigation fall within the ranges reported in earlier studies. Published reports have described *ppyR* frequencies of approximately 94-99% and *pslA* frequencies ranging from 60.8-89.5%. Thus, the values obtained in the present study were within the reported ranges (Ghadaksaz *et al.*, 2015; Pournajaf *et al.*, 2018; Ocak and Turkeyilmaz, 2022; Plokarz *et al.*, 2022) [15, 23, 26, 27].

Biofilm inhibition by eugenol and cinnamic acid

All 35 *P. aeruginosa* isolates identified as biofilm producers by the phenotypic assays were subsequently evaluated for inhibition of established biofilms using the microtiter plate assay. Both eugenol and cinnamic acid demonstrated inhibitory activity, although the magnitude of inhibition varied with the compound and concentration used.

Eugenol produced mean inhibition rates of $43.144 \pm 2.638\%$ at 0.4 mg/mL and $20.983 \pm 2.010\%$ at 0.2 mg/mL. Cinnamic acid produced corresponding mean inhibition rates of $55.873 \pm 2.050\%$ at 2.5 mg/mL and $47.625 \pm 2.146\%$ at 1.25 mg/mL. Thus, increasing the concentration of either phytochemical resulted in greater mean biofilm inhibition under the conditions evaluated.

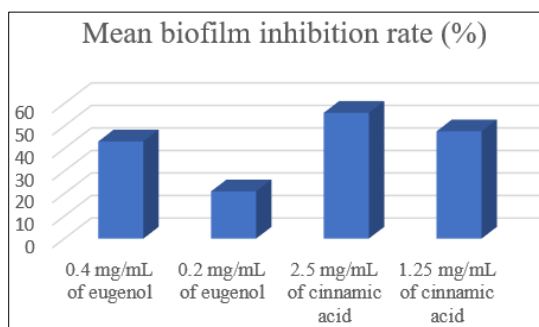


Fig 1: The comparison of biofilm inhibition rates of eugenol and cinnamic acid at different concentrations on *P. aeruginosa* isolates

Although cinnamic acid produced a higher absolute inhibition at the concentrations tested, eugenol achieved a comparable level of inhibition at substantially lower concentrations. This observation suggests that eugenol may have greater potency on a concentration basis in the present experimental system. However, this interpretation should be

presented as a comparative observation from the tested concentrations, rather than as a definitive claim of superior antibiofilm activity. The present findings are comparable with those of Zhou *et al.* (2013) [40], who reported approximately 43% inhibition of *P. aeruginosa* biofilm formation with eugenol at a sub-inhibitory concentration. In the present study, a similar inhibition percentage was obtained, although at a higher concentration. The difference may reflect variation in the characteristics of the bacterial isolates and experimental conditions.

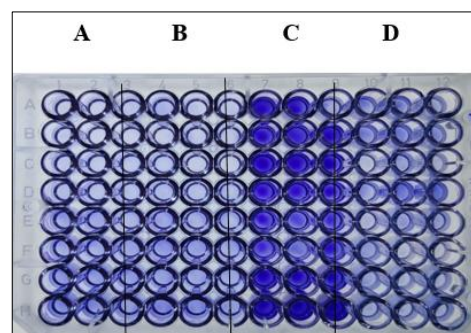


Fig 2: Biofilm inhibition studies of *P. aeruginosa* using MTP

A- 1.25 mg/mL CA C- 0.2 mg/mL eugenol
B- 2.5 mg/mL CA D- 0.4 mg/mL eugenol

The study by Wijesinghe *et al.* (2021) [39] also investigated the antibiofilm activity of *Cinnamomum verum* essential oil and reported a 50% reduction in an established 24-h bacterial biofilm at 1 mg/mL. The findings from the present study show a comparable level of inhibition with the tested phytochemicals, although direct comparison should be made cautiously because the experimental preparations and conditions were not identical. Letsididi *et al.* (2018) [17] reported $\geq 50\%$ inhibition of biofilm formation using trans-cinnamic-acid nanoemulsion at 0.39 mg/mL. In comparison, the present study required 2.5 mg/mL cinnamic acid to achieve a mean inhibition exceeding 50%. The greater activity reported for the nanoemulsion preparation may be related to the formulation and physicochemical characteristics of the delivery system; however, this should be regarded as a possible explanation rather than a demonstrated mechanism in the present study.

Minimum inhibitory concentration of the phytochemicals

The MIC values demonstrated variation among the *P. aeruginosa* isolates. Eugenol showed MIC values ranging

from 6.41 to 51.31 $\mu\text{g/mL}$, whereas the corresponding values for cinnamic acid ranged from 31.25 to 500 $\mu\text{g/mL}$ based on the individual isolate results presented in Table 2.

The reference antibiotic, amoxicillin, showed MIC values ranging from 15.625 to 1000 $\mu\text{g/mL}$ in the tabulated results.

Table 2: Results of Minimum Inhibitory Concentration (MIC) on *P. aeruginosa* isolates

S.No	Sample No.	Eugenol (16420 $\mu\text{g/mL}$)	Cinnamic acid (20000 $\mu\text{g/mL}$)	Amoxicillin (10000 $\mu\text{g/mL}$)
1.	O1	25.65	500	1000
2.	O2	25.65	500	1000
3.	O3	12.82	500	250
4.	O5	12.82	500	1000
5.	O9	25.65	500	500
6.	O10	12.82	500	1000
7.	O11	12.82	500	250
8.	O12	25.65	500	500
9.	O13	6.41	125	1000
10.	O14	12.82	125	1000
11.	O15	12.82	500	500
12.	O21	25.65	500	500
13.	O22	12.82	125	250
14.	O25	25.65	31.25	7.81
15.	O26	25.65	500	250
16.	O27	25.65	500	31.25
17.	O34	12.82	125	500
18.	O38	25.65	500	250
19.	W1	25.65	500	125
20.	W13	12.82	500	250
21.	W15	12.82	125	250
22.	W16	25.65	125	250
23.	W31	25.65	125	125
24.	W32	25.65	125	1000
25.	W33	25.65	125	250
26.	W34	12.82	500	1000
27.	W35	12.82	500	500
28.	W40	6.41	500	1000
29.	W42	6.41	500	1000
30.	W43	51.31	125	250
31.	W44	25.65	125	250
32.	W48	12.82	125	125
33.	W49	25.65	125	31.25
34.	W50	25.65	125	62.5
35.	W51	12.82	125	31.25

The lower MIC values observed with eugenol compared with cinnamic acid indicate greater inhibitory activity of eugenol against the tested isolates under the conditions of this assay. This observation is particularly noteworthy because all isolates evaluated in the present study were characterized as MDR. The finding is consistent with the report of Costa *et al.* (2022) [5], who demonstrated antimicrobial activity of eugenol against MDR *P. aeruginosa* isolates.

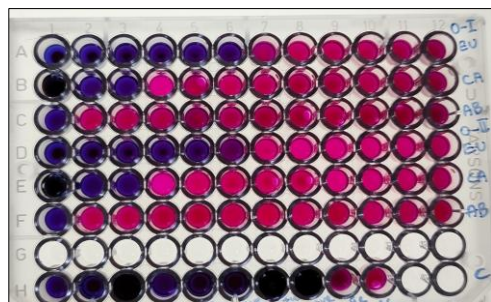


Fig 3: MIC of Eugenol, Cinnamic acid (CA) and amoxicillin (antibiotic) against *P. aeruginosa* (Sample no. O1, O2)

- A row- Serial dilution of eugenol against O1
- B row- Serial dilution of cinnamic acid against O1
- C row- Serial dilution of amoxicillin against O1
- D row- Serial dilution of eugenol against O2
- E row- Serial dilution of cinnamic acid against O2
- F row- Serial dilution of amoxicillin against O2
- H row- Controls
- H₁- Sterility control (Only MH broth medium);
- H₂- Eugenol phytochemical control (MH broth+ Eugenol)
- H₃- CA phytochemical control (MH broth+ cinnamic acid)
- H₄- Antibiotic control (MHB broth+ amoxycillin)
- H₅- Positive control of O1 with eugenol (MHB+ eugenol+ O1 isolate)
- H₆- Positive control of O1 with CA (MHB+ CA+ O1 isolate)
- H₇- Positive control of O2 with eugenol (MHB+ eugenol+ O2 isolate)
- H₈- Positive control of O2 with CA (MHB+ CA+ O2 isolate)
- H₉- Growth control of O1 (MHB+ O1 isolate)
- H₁₀- Growth control of O2 (MHB+ O2 isolate)

Table 3: Details of biofilm inhibition studies in *P. aeruginosa* by Microtiter Plate assay

Samp le No	OD of sample	OD of sample treated with 0.4 mg/ml of eugenol	Inhibi tion %	OD of sample treated with 0.2 mg/ml	Inhibi tion %	OD of sample treated with CA- 2.5 mg/mL	Inhibi tion %	OD of sample treated with CA-1.25 mg/mL	Inhibi tion %
O1	1.445	0.83	42.5	1.186	17.92	0.608	57.92	0.903	37.5
O2	1.155	0.696	39.74	1.01	12.5	0.593	48.65	0.669	42
O3	0.994	0.592	40.4	0.798	19.7	0.479	51.8	0.512	48.4
O5	0.819	0.528	35.5	0.726	11.3	0.275	66.4	0.368	55
O9	0.85	0.503	40.8	0.714	16	0.314	63	0.342	59.7
O10	0.651	0.516	20.7	0.629	3.3	0.365	43.9	0.454	50.2
O11	0.701	0.565	19.4	0.651	7.1	0.494	29.5	0.526	24.9
O12	1.277	0.862	32.4	0.905	29.1	0.647	49.3	0.722	43.4
O13	1.689	0.969	42.6	1.214	28.1	0.619	63.3	0.916	45.7
O14	1.329	0.817	38.5	1.014	23.7	0.607	54.3	0.704	47
O15	0.646	0.414	35.9	0.598	7.4	0.224	65.3	0.494	23.5
O21	0.507	0.419	17.3	0.497	1.9	0.209	58.7	0.302	40.4
O22	0.834	0.594	28.7	0.712	14.6	0.324	61.1	0.356	57.3
O25	0.61	0.419	31.3	0.499	18.1	0.289	52.6	0.312	48.8
O26	0.742	0.522	29.6	0.659	11.1	0.328	55.7	0.374	49.5
O27	1.071	0.317	70.4	0.542	49.3	0.366	65.8	0.462	56.8
O34	1.029	0.717	30.3	0.829	19.4	0.576	44	0.679	34
O38	1.342	0.819	38.9	1.12	16.5	0.624	53.5	0.712	46.9
W1	0.994	0.648	34.8	0.712	28.3	0.448	54.9	0.504	49.2
W13	1.067	0.716	32.8	0.83	22.2	0.667	37.4	0.74	30.6
W15	0.58	0.413	28.7	0.492	15.1	0.28	51.7	0.378	34.8
W16	0.88	0.442	49.7	0.671	23.7	0.255	71	0.267	69.6
W31	1.122	0.465	58.5	0.851	24.1	0.265	76.3	0.303	72.9
W32	0.852	0.413	51.5	0.62	27.2	0.319	62.5	0.343	59.7
W33	0.992	0.462	53.4	0.851	14.2	0.43	56.6	0.524	47.1
W34	1.031	0.594	42.3	0.732	29	0.59	42.7	0.603	41.5
W35	1.598	0.62	61.2	1.337	16.3	0.524	67.2	0.72	54.9
W40	0.946	0.369	60.9	0.914	3.3	0.766	19	0.775	18
W42	1.201	0.411	65.8	0.678	43.5	0.397	66.9	0.412	65.6
W43	1.338	0.273	79.5	0.826	38.2	0.362	72.9	0.437	57.3
W44	1.041	0.67	35.6	0.987	5.1	0.512	50.8	0.619	40.5
W48	1.217	0.792	34.9	0.887	27.1	0.641	47.3	0.742	39
W49	1.496	0.698	53.3	0.942	37	0.512	65.7	0.621	58.4
W50	1.625	0.721	55.6	0.961	40.8	0.579	64.3	0.632	61.1
W51	1.461	0.695	76.6	0.989	32.3	0.531	63.6	0.647	55.7
	Mean±SE		43.144 ±2.638		20.983 ± 2.010		55.873 ± 2.050		47.625 ± 2.146

Galvao *et al.* (2022)^[11] investigated the action of isoeugenol against *P. aeruginosa* and reported significant antibacterial activity with MIC of 64 µg/mL which is in correlation with the upper range value of MIC for eugenol obtained in the current study. Wijesinghe *et al.* (2021)^[39] reported the MIC value of Cinnamon essential oil on *P. aeruginosa* was 0.5 mg/ mL (i.e., 500 µg/ mL) which was similar to the present study.

Due to the increased prevalence of MDR and XDR *P. aeruginosa* isolates, the need of novel agents for the treatment and management of biofilm associated infections is high. As one of the reasons for the increased MDR and XDR strains is due to the biofilm forming ability of the isolates, targeting them with the agents that have the potential antibacterial as well as the anti-biofilm activity helps in reduction of the persistent infections. Addition of eugenol and cinnamic acid to destabilize and inhibit biofilm formation is a promising approach to treat chronic infections. However, further studies are required for the clinical use of these agents.

Conclusion

The present study identified a high frequency of antimicrobial resistance and biofilm-forming ability among

P. aeruginosa isolates obtained from canine clinical infections. All confirmed isolates exhibited multidrug resistance, while a substantial proportion showed an extensively drug-resistant phenotype and phenotypic ESBL production. The detection of biofilm-associated genes further supports the ability of these isolates to persist in clinical environments.

Both eugenol and cinnamic acid demonstrated antibacterial and antibiofilm activity against the tested isolates. Cinnamic acid produced greater mean biofilm inhibition at the concentrations evaluated, whereas eugenol showed lower MIC values in the present assay. These findings indicate that both phytochemicals warrant further investigation as potential adjunct or alternative approaches for controlling resistant and biofilm-associated *P. aeruginosa* infections in dogs. Further studies using larger isolate collections and appropriate *in-vivo* models are required before their therapeutic application can be established.

Funding source of research

The authors are very much grateful to Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India, for providing the facilities to carry out the work.

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