*Indian Journal of Animal Sciences* **95** (6): 497–504, June 2025/Article
https://doi.org/10.56093/ijans.v95i6.169361

# Antibacterial and antibiofilm activity of methanolic Indian propolis extract against ESBL *Pseudomonas* isolated from buffalo mastitis

ASHOK BOORA[1], SARITA YADAV[1], SUNITA YADAV[2], ASHOK BALHARA[1], SUNESH BALHARA[1], NISHU DHAKA[1], NISHA VERMA[1], ANIL KUMAR[3] AND RAJESH KUMAR LOHCHAB[4]✉

*ICAR-Central Institute for Research on Buffaloes, Hisar, Haryana 125 001, India*

Received: 22 July 2025; Accepted: 29 August 2025

ABSTRACT

Increasing antimicrobial resistance poses a major challenge to mastitis treatment in dairy animals, highlighting the need for alternative therapeutics. This study analysed the antibacterial and antibiofilm activity of methanolic propolis extract (MPE) against extended-spectrum β-lactamase (ESBL) positive *Pseudomonas* spp. recovered from mastitis affected Murrah buffaloes. Of the 175 environmental isolates from mastitis-affected buffalo milk (n= 472 animals), *Pseudomonas* spp. accounted for 23.4% (n=41), making it the second most prevalent pathogen after *Escherichia coli* (24.6%). Six clinical ESBL positive *Pseudomonas* isolates were selected for analysis. MPE exhibited antibacterial activity with minimum inhibitory concentration (MIC) in the range of 7.81 to 15.62 mg/mL. Scanning electron microscopy and fluorescence microscopy revealed membrane disruption in treated cells, and flow cytometry further confirmed a high proportion of apoptotic cells post-treatment. All isolates were strong biofilm producers, and MPE showed significant antibiofilm activity with 72.5± 0.39% and 71.2 ± 0.95% biofilm inhibition at 2MIC and MIC, respectively. The findings indicate that Indian propolis is a promising natural alternative with potent antibacterial and antibiofilm activity against *Pseudomonas* associated with bovine mastitis.

**Keywords**: Antibacterial, Antibiofilm, Environmental mastitis ESBL, Propolis, *Pseudomonas*, Minimum inhibitory concentration

*Pseudomonas* is an opportunistic environmental pathogen, causes environmental mastitis in dairy animals, contributing to inflammatory responses ranging from chronic subclinical to severe clinical manifestations. *Pseudomonas aeruginosa* is resistant to multiple antibiotics, making it a persistent challenge in treatment due to repeated therapeutic failures. Controlling *Pseudomonas* mastitis requires culling chronically infected animals and addressing contamination sources, including udder wash water, intramammary antibiotics and milking equipment (Kirk and Bartlett 1984, Erskine *et al.* 1987). Contaminated bedding, manure, contaminated wash-water sources, udder wash water, teat dips and milking equipment, including wash hoses serve as potential infection sources (Daly *et al.* 1999). Additionally, biofilm formation and inadequate hygiene in the milking parlor increase the risk of intramammary infections (Erskine *et al.* 1987). In dairy animals, *Pseudomonas* intramammary infections typically arise due to prolonged exposure to contaminated environmental sources (Schauer *et al.* 2021). Over the recent decades, researchers have investigated novel, economically viable and highly effective antimicrobial therapeutics for combating infections induced by biofilm forming pathogens (Aslam *et al.* 2018). Scientific focus has increasingly shifted towards exploring natural plant-based products for the development of potential potent antimicrobial agents, for treating and preventing infectious diseases (Anand *et al.* 2019). Propolis is derived from the floral and resinous exudates of plants. Honeybees, particularly *Apis mellifera*, collect and modify this gummy substance with the help of secretions from their salivary glands and beeswax, yielding propolis (Erskine *et al.* 1987). Propolis primarily safeguards beehives against humidity and invaders by closing cracks and maintaining a temperature homeostasis. Extensive literature supports the diverse therapeutic properties of propolis, encompassing antimicrobial, antiviral, antiprotozoal, antimycotic and anticancer properties (da Silva *et al.* 2018) and antimicrobial properties of propolis have demonstrated potential efficacy against diverse bacterial species, including both Gram-positive and Gram-negative organisms (Rivera-Yañez *et al.* 2021). This study aimed to evaluate the *in vitro* antimicrobial and biofilm inhibitory activity of Indian propolis against *Pseudomonas* strains isolated from mastitis cases of Murrah buffaloes.

Present address: [1]ICAR-Central Institute for Research on Buffaloes, Hisar 125 001. [2]Chaudhary Charan Singh Haryana Agricultural University, Hisar 125 001. [3]Indian Council of Agricultural Research, New Delhi 110 001. [4*]Guru Jambheshwar University of Science & Technology, Hisar 125 001. ✉Corresponding author email: rajeshlohchab@gmail.com

## MATERIAL AND METHODS

*Bacterial samples*: From 2019 to 2022, composite milk samples were aseptically collected from mastitis buffaloes (n = 472) at the farmers' doorstep in villages covered under the ICAR Farmer FIRST Project (Haryana and Rajasthan) and from organized herds maintained at ICAR-CIRB, Hisar. Bacterial isolation and identification were carried out on the basis of standard procedures (Quinn 1994).

*Determination of antimicrobial susceptibility*: Antimicrobial susceptibility of 41 *Pseudomonas* strains isolated from mastitis milk of buffaloes was performed on Mueller-Hinton agar (MHA) (Himedia) by disc diffusion method in accordance with the guidelines of Clinical Laboratory Standards Institute (CLSI 2012). Five antimicrobial discs (Himedia) from four antimicrobial classes were used: amoxycillin/sulbactam (AMS, 30/15 µg), gentamicin (GEN, 10 µg), cefoperazone/sulbactam (CFS, 75/30 µg), ceftriaxone (CTR, 30 µg) and enrofloxacin (EX, 10 µg).

*Pseudomonas test strains*: Six clinical *Pseudomonas* test isolates (bacterial codes PS_01 to PS_06) were selectively chosen and evaluated for ESBL, MIC, biofilm and antibiofilm assays. These isolates were confirmed by PCR targeting the outer membrane lipoprotein gene *oprI* for genus-level identification of *Pseudomonas* and the *oprL* gene for species-specific detection of *Pseudomonas aeruginosa* yielding amplicons of 249 and 504 bp fragments respectively (De Vos *et al.* 1997).

*Phenotypic identification of extended-spectrum beta-lactamase (ESBL) enzyme*: Initial screening for potential ESBL production was conducted using two antibiotic discs: ceftazidime (CAZ, 30 µg) and cefotaxime (CTX, 30 µg) on Mueller-Hinton Agar (MHA) plates inoculated with bacterial cultures with bacterial culture (0.5 McFarland standard) as per CLSI method. The confirmative combined disc test for ESBL was conducted if the inhibition zone of these antibiotics on initial screening was ≤22 mm and ≤27 mm, respectively as per CLSI ESBL disc screening criteria. Phenotypic confirmation of ESBL producing strains was done by combined disc test using ceftazidime-clavulanate (CAC, 30/10 µg); ceftazidime (CAZ, 30 µg) and cefotaxime-clavulanate (CEC, 30/10 µg); cefotaxime (CTX, 30 µg) disks (Pantha *et al.* 2024). An expansion of ≥5 mm in zone of inhibition observed with either of two: Cefotaxime with clavulanic acid (CEC) or ceftazidime with clavulanic acid (CAC) in contrast to their respective cefotaxime (CTX, 30 µg) or ceftazidime (CAZ, 30 µg) disks alone, confirmed ESBL producing strains.

*Genotypic screening of ESBL genes*: Isolates confirmed as ESBL producers were further screened by PCR for the presence of β-lactamase encoding genes: *blaTEM*, *blaSHV* (Doosti *et al.* 2015) and *blaCTX-M* (Pitout *et al.* 2004).

*Extraction of propolis*: Propolis was manually collected from honeybee colonies by the Entomology department of CCS Haryana Agricultural University, Hisar. The resinous material was stored at -20°C until its processing. Propolis (100 g) was cut into small pieces and ground using a mortar and pestle. Crude propolis was extracted using the maceration method (El-Sakhawy 2023). Briefly, 20 g of dried and finely ground propolis was extracted with 100 mL of respective pure solvent methanol (1:5 w/v) through regular shaking at room temperature for seven days to obtain 20% (w/v) propolis extract (Šuran *et al.* 2021). The extract was clarified by centrifugation at 7,000 rpm, 25°C for 15 min. Extracts were kept for 2-3 hours at 4°C to remove wax and then filtered using Whatman filter paper. The supernatant was collected and evaporated at room temperature for 1-2 weeks to remove the solvent. The resulting dried material was stored at -20°C for further studies. Stock solutions of the extracts were prepared by dissolving 0.25 g of dried extract in 1 mL of the respective pure solvent to obtain a final concentration of 250 mg/mL (25% w/v) and were designated as methanol propolis extract (MPE). MPE was then filtered and the stock solution was serially diluted in a range of concentrations from 250 mg/mL to 0.49 mg/mL for antibacterial and antibiofilm activity assays using micro-broth dilution method.

*Preparation of inoculum*: *Pseudomonas* test strains (n=6) were sub-cultured overnight at 37°C on Mueller-Hilton agar plates. Three to four discrete colonies from agar plates were inoculated into 5 mL tryptic soy broth (TSB) and then incubated for 3-4 h at 37°C. The turbidity of bacterial suspension was standardized with sterile TSB to achieve an optical density $(OD)_{600}$ = 0.8-1 using spectrophotometer and further diluted to 1:20 to get cell count of $10^7$ CFU/mL.

*Antibacterial activity*: Antimicrobial activity of MPE was evaluated against *Pseudomonas* test strains using broth microdilution as recommended by the CLSI (Wayne 2011). In broth microdilution assay, MIC of propolis extract against these strains were determined by serial two-fold dilution of concentrations ranging from 250 mg/mL to 0.49 mg/mL in TSB on a 96-well microtiter plate with a few minor modifications. For negative control, methanol was used in place of MPE. A growth control, comprising only bacterial culture without propolis extract and a positive control using gentamicin antibiotic at 250 mg/mL concentration were used. Additionally, TSB was employed as a sterility control. Plates were incubated overnight at 37°C. After incubation, the bacterial growth was verified by adding 20 µL of 2,3,5-Triphenyltetrazolium chloride (5 mg/mL) to each well and incubating for an additional 15–20 min at room temperature. The bacterium's viability was then shown by the formation of a reddish bacterial button at the bottom of each well. MIC was determined as the lowest propolis concentration capable of inhibiting bacterial growth by preventing the color shift of the solution from yellow to red.

*Scanning electron microscopy (SEM)*: Scanning electron microscopy was done to evaluate morphological alterations in bacterial cells following propolis treatment. For this, 100 µL of propolis solution at the minimum inhibitory concentration (MIC, 15.62 µg/mL), 100 µL of broth and

20 µL of bacterial suspension ($10^7$ CFU/mL) were added to wells of a 6-well plate containing sterile glass coverslips. Control wells received only broth and bacterial suspension. Following incubation at 37°C overnight, non-adherent bacteria were removed by washing the wells three times with PBS. Fixing was done using 2.5% glutaraldehyde in PBS for 4 h at 4°C. A stepwise ethanol gradient (30% to 100%) was used for dehydration, with samples immersed for 5 min in each concentration. The dried samples were submitted to Guru Jambheshwar University of Science and Technology (Hisar, India) for SEM imaging. Gold sputter coating was performed prior to imaging.

*Flow cytometry*: Bacterial viability and membrane integrity post-treatment were further assessed using flow cytometry (Beckman Coulter Cytoflex BB38280) (Rozloznik *et al.* 2020). To quantify live (green) and dead (red) bacterial populations, cells were treated with SYTO 9 and propidium iodide (PI) according to the manufacturer's protocol (Thermo Fisher Scientific, L34856). (Berney *et al.* 2007). This allowed a validation of cell death and membrane damage induced by propolis treatment.

*Live/dead bacterial staining assay*: Fluorescence microscopy was used to visualize bacterial viability. Bacterial cultures ($10^7$ CFU/mL) from control (without propolis) and treatment (exposed to MIC of MPE) groups, maintained in the logarithmic phase, were incubated overnight and centrifuged at 10,000 × g to sediment the cells. Following washing, the samples were reconstituted in 1 mL of 0.85% sodium chloride solution. A staining solution containing SYTO 9 (3.34 mM, 1.5 µL) and PI (30 mM, 1.5 µL) was added to each sample and incubated at 37°C in darkness for 15–30 min. Twenty microliters of stained cells were mounted on a slide with a coverslip. Imaging was conducted using a confocal fluorescence microscope equipped with FITC (excitation 465–495 nm) and TRITC (excitation 532–554 nm) filters, following the manufacturer's protocol in the LIVE/DEAD BacLight bacterial viability and counting kit (Thermo Fisher, L34856). SYTO 9, a green-fluorescent nucleic acid stain, penetrates intact bacterial membranes, marking viable cells. In contrast, propidium iodide, which fluoresces red, only enters bacteria with compromised membranes, indicating non-viable cells.

*The Congo Red method*: The Congo Red Agar (CRA) assay was followed to qualitatively evaluate slime formation, as a presumptive test for biofilm formation, where slime-producing strains form black colonies, while non-producers remain red (Freeman *et al.* 1989 , Cotter *et al.* 2009). *Pseudomonas* strains were grown on BHI agar supplemented with 0.08% Congo Red and 8% glucose at 37°C for 24–48 h, with autoclaved Congo Red added to the medium cooled to 55°C.

*Evaluation of antibiofilm activity*

*Phenotypic biofilm formation assay*: The ability of bacterial isolates to form biofilms was phenotypically quantified employing the microtitre plate method in 96-well sterile flat-bottomed microtitre plates with a lid (Nunc), where a 20 µL aliquot of bacterial inoculum ($10^7$ CFU/mL) and 180 µL of TSB were added to each well in triplicate and incubated at 37°C for 24 h as per (Stepanović *et al.* 2000). After incubation, the contents of the wells were removed and rinsed twice using distilled water to clear non-adherent (planktonic) cells. To fix the biofilm, 200 µL of 99% methanol was added to each well and incubated for 15 min. After removing the methanol, the plates were left to air-dry at ambient temperature for one hour. To stain the biofilm biomass, each well was treated with 200 µL of 1% crystal violet solution and incubated for 20 min. Excess stain was rinsed off under running tap water. To solubilize the bound crystal violet, 200 µL of 33% glacial acetic acid was added to each well and absorbance was recorded at 570 nm using a microplate ELISA reader. The positive control consisted of a known biofilm-producing strain, while wells with only 200 µL of TSB acted as negative controls. The optical density cut-off (ODc) was determined by adding three times the standard deviation to the mean OD of the negative control (ODc = mean OD of negative control + 3×SD). Based on the measured OD values, strains were grouped into four categories: non-biofilm formers (OD ≤ ODc), weak (OD > ODc to ≤ 2×ODc), moderate (OD > 2×ODc to ≤ 4×ODc) and strong biofilm formers (OD > 4×ODc).

*Crystal violet antibiofilm microtiter plate assay*: The antibiofilm activity of MPE was evaluated by assessing its ability to inhibit biofilm formation when administered prior to biofilm development. The assay was performed following the method described by Saeloh and Visutthi (2021). In brief, bacterial isolates ($10^7$ CFU/mL) were incubated in tryptic soy broth (TSB) in a 96-well microplate at 37 °C for 24 h, with 2MIC, MIC, 1/2 MIC and 1/4 MIC of propolis (treated) and without propolis (untreated). The biofilm was fixed by treating with absolute methanol for 20 min, followed by air drying. Subsequently, 200 µL of 0.1% crystal violet was added to each well and allowed to stain for 15 min. After rinsing with water, the plate was air-dried. The stained biofilm was then solubilized using 200 µL of 33% (v/v) glacial acetic acid and the optical density was measured at 595 nm using a microplate reader. Biofilm inhibition was calculated by evaluating the optical density (OD) of treated samples with that of the untreated control using the formula: [(OD of control − OD of treated sample)/OD of control] × 100, as described by (Prabhakar *et al.* 2024).

*Statistical analysis*: Bioassays were conducted with three independent experiments, each performed in triplicates. Results are presented as mean ± standard deviation (SD).

## RESULTS AND DISCUSSION

*Prevalence rate of Pseudomonas*: Out of 175 environmental bacterial isolates obtained from milk samples of subclinical and clinical mastitis cases in buffaloes (n = 472 animals) from organized and unorganized herds, 41 isolates were identified as *Pseudomonas spp*. *Pseudomonas spp.* accounted for 23.4% of the total environmental isolates, as the second most prevalent environmental

Table 1. Antimicrobial Susceptibility profile of *Pseudomonas* isolates (n=41).

| Antibiotic sensitivity pattern of *Pseudomonas*, (n=41) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Class | Antimicrobials | S | % | I | % | R | % |
| Penicillin with beta-lactamase inhibitor | AMS | 31 | 74 | 8 | 19 | 3 | 6 |
| Aminoglycoside | GEN | 38 | 91 | 3 | 9 | 0 | 0 |
| Cephalosporin | CFS | 27 | 66 | 7 | 17 | 7 | 17 |
| | CTR | 10 | 23 | 16 | 38 | 16 | 38 |
| Fluoroquinolone | EX | 13 | 32 | 27 | 66 | 1 | 2 |

AMS (30/15 µg): amoxycillin/sulbactam, GEN (10µg): gentamicin, CFS (75/30µg): cefoperazone/sulbactam, CTR (30µg): ceftriaxone, EX (10µg): enrofloxacin

mastitis pathogen after *Escherichia coli* (24.6%). These findings are consistent with a previous report highlighting *Pseudomonas* as an emerging environmental pathogen in bovine mastitis, particularly in herds exposed to poor environmental sanitation (Schauer *et al.* 2021, Mallick *et al.* 2025). All 41 isolates showed yellow-colored colonies on MacConkey agar and were oxidase positive.

*Resistance profile of Pseudomonas*: The results of antimicrobial susceptibility testing of 41 *Pseudomonas* strains to 5 antibiotics from four antimicrobial classes are summarized in Table 1.

The highest susceptibility was observed for gentamicin (GEN) at 91%, followed by amoxicillin/sulbactam (AMS) with 74% and cefoperazone/sulbactam (CFS) with 66% susceptibility. Gentamicin showed the highest susceptibility against *Pseudomonas*, indicating their potential as effective treatment which is in agreement with the previous reports (Park *et al.* 2014, Yadav *et al.* 2020, Sekhri *et al.* 2021, Kumari 2024). The low resistance rates to gentamicin among *Pseudomonas* isolates in this study could be attributed to short-term, limited usage and low preference for its use in mastitis treatment (Yadav *et al.* 2023).

*Molecular detection of P. aeruginosa*: Genus-level identification of *Pseudomonas* spp. was performed by targeting the outer membrane lipoprotein gene oprI, which yielded the expected 249 bp amplicon in all six clinical isolates (PS_01 to PS_06) (Fig. 1). Species-specific confirmation of *P. aeruginosa* was carried out using primers targeting the *oprL* gene, resulting in a 504 bp amplicon. Out

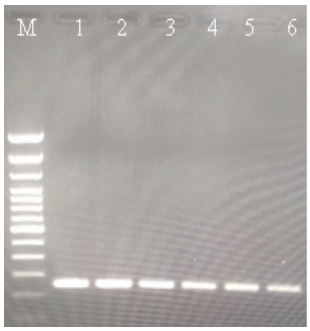


Fig. 1. Amplification of outer membrane lipoprotein genes (*oprI*) of *Pseudomonas*, M: 100 bp DNA marker, lane 1-6: the amplified gene segments of PS_01 to PS_06, 249 bp from representative *Pseudomonas* isolates.

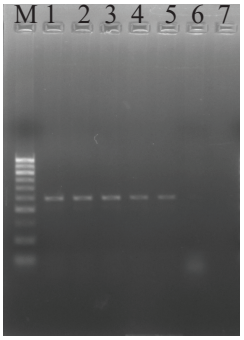


Fig. 2. PCR amplification of the *Pseudomonas aeruginosa* -specific *opr L* gene (504 bp). M: 100 bp DNA marker, lanes 1–5: positive amplification from clinical isolates PS_01 to PS_05, lane 6: no amplification in PS_06, lane 7: negative control (no template DNA).

of the six isolates, five (PS_01 to PS_05) showed positive amplification for the *oprL* gene, confirming their identity as *P. aeruginosa*, while PS_06 showed no amplification (Fig. 2).

*Extended-spectrum beta-lactamase (ESBL) detection*: All six isolates showed inhibition zone ≤27 mm for CTX whereas five isolates with ≤ 22 mm for CAZ, therefore all isolates were screened positive for ESBL. All these were further confirmed as ESBL producers by combination disk ESBL phenotype confirmatory test (Table 2, Fig. 3). The detection of ESBL producing *Pseudomonas* strains in mastitis milk supports earlier observations of β-lactam resistance in *Pseudomonas* spp. from dairy environments (Salem *et al.* 2023, Kumari 2024).

*Molecular detection of ESBL*: ESBL genes (*blaTEM*, *blaSHV* and *blaCTX-M* ) tested in this study demonstrated

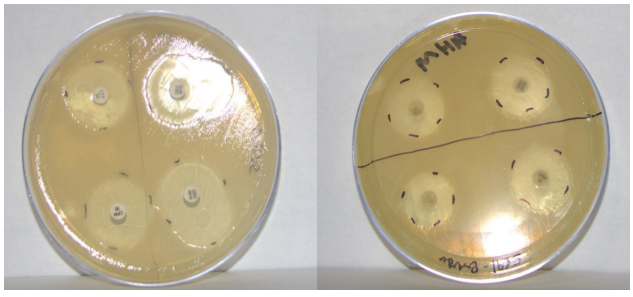

Fig. 3. Combined disk synergistic test (CDT)* for phenotypic confirmation of ESBL producer *Pseudomonas* (n=6).

Table 2. Combined disk synergistic test (CDT)* for phenotypic confirmation of ESBL producer *Pseudomonas* (n=6)

| Antibiotic | Required inhibition zone for screening test | Positive isolates by screening test | ESBL confirmation by CDT* |
|---|---|---|---|
| CAZ | ≤22mm | 5 | 4 |
| CTX | ≤27mm | 6 | 6 |

*With CEC (cefotaxime with clavulanic acid)/ CTX (cefotaxime, 30 µg) and CAC (ceftazidime with clavulanic acid)/ CAZ (Ceftazidime, 30 µg)

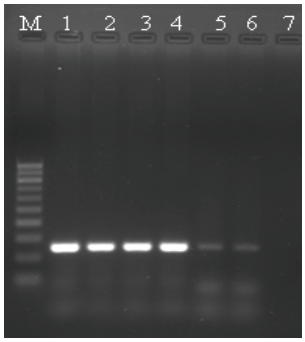


Fig. 4. PCR amplification of *blaTEM* gene showing positive bands in all six isolates, M: 100 bp DNA marker , lane 1-6: the amplified gene segments of PS_01 to PS_06, 296 bp of *blaTEM* gene, lane 7 negative control.

that all 6 isolates were positive for *blaTEM* gene (Fig. 4), while no amplification was observed for *blaSHV* and *blaCTX-M* genes.

*Antibacterial activity*: The antibacterial potential of different concentrations of crude MPE in the concentration range of 250 mg/mL to 0.49 mg/mL was evaluated using broth microdilution assays. The MPE showed MIC ranging from 7.81 mg/mL to 15.62 mg/mL toward the tested strains

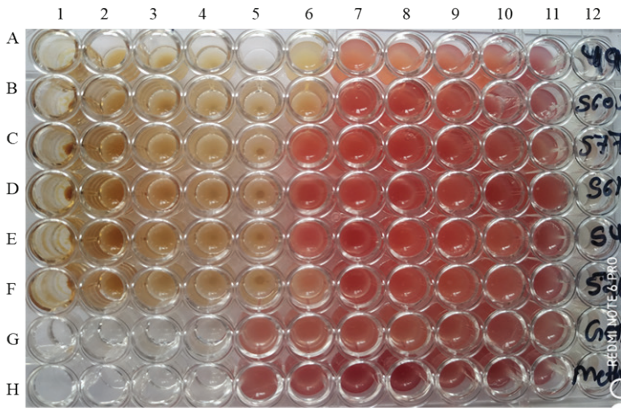


Fig. 5. Assay showing detection of MIC for MPE against *Pseudomonas* isolates in a 96 well plate. A1-10 to F1-10: MPE against different *Pseudomonas* strains, G1-G10: Gentamicin, H1-H10: methanol control, A11-H11: bacterial culture without MPE, A12-H12: broth control. MIC was determined as the lowest propolis concentration capable of inhibiting bacterial growth, indicated by the absence of a color change in the medium from yellow to red.

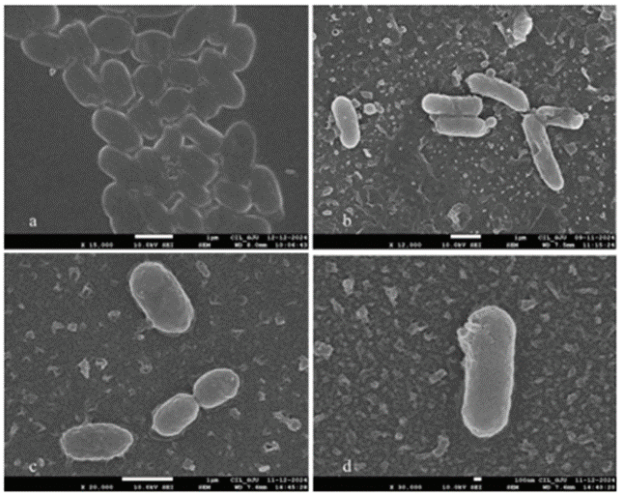


Fig. 6. Representative scanning electron micrographs of *Pseudomonas* for morphological changes: membrane rupture, surface irregularities (wrinkled and irregular), and cell deformation in the presence of MIC of MPE: a & b) control; c & d) MIC 15.62 mg/mL.

(Fig. 5) Kazemi *et al.* 2024 reported MIC values of 25–50 mg/mL for Iranian propolis against *P. aeruginosa*, whereas De Marco *et al.* 2017 observed a much lower MIC of 0.125 mg/mL for Brazilian propolis against the same pathogen. These findings highlight the variability in propolis efficacy, which may be attributed to differences in its chemical composition, geographical origin, and the specific bacterial strains tested, which significantly influence its antibacterial activity.

Membrane integrity evaluation

*SEM analysis*: As depicted in Fig. 6, morphological changes in bacteria treated with MPE (at MIC) showed wrinkled, irregular and fractured surface of bacterial cells, indicating structural damage. These results further validated the above experiment on antibacterial activity of propolis. These findings corroborate that propolis exerts its antibacterial activity by damaging the structural integrity of the cell, resulting in the leakage of essential ions and macromolecules critical for cell viability (Wang *et al.* 2021).

*SYTO 9 /propidium iodide flow cytometric analysis*: Treatment of *Pseudomonas* with Indian propolis at MIC (15.3 µg/mL) significantly increased late apoptotic cells (97.59%) compared to control (48.39%), as assessed by SYTO 9/PI flow cytometry. (Fig. 7). These findings are consistent with the above experiment assessing cellular structural integrity, supporting the apoptosis-mediated antibacterial action of Indian propolis. Similar flow cytometric analyses have previously confirmed that propolis can induce apoptosis-like pathways in eukaryotic systems, highlighting the potential of propolis as an apoptosis-inducing agent (Begnini *et al.* 2014, Elnakady *et al.* 2017, Silva *et al.* 2017).

*Fluorescence microscopy*: *Pseudomonas* cells in the logarithmic growth phase were stained with the LIVE/

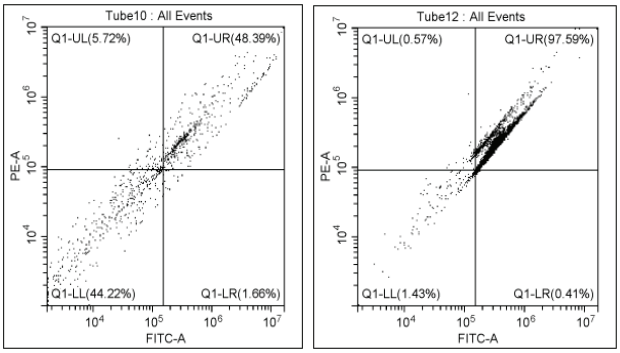


Fig. 7. Apoptosis induction by Indian propolis at MIC value at 15.3 µg/mL in *Pseudomonas* bacterial culture after overnight treatment was determined by flow cytometry. (a) Dot plot of control showing the percentages in each panel: late apoptotic (48.39%), upper right quadrant and necrotic (5.72%), upper left quadrant bacterial cells, (b) Treatment with propolis MIC 15.3 µg/mL indicating late apoptotic (97.59%), upper right quadrant and necrotic (0.57%), upper left quadrant bacterial cells.

DEAD BacLight Bacterial Viability Kit in the presence and absence of MPE for fluorescence microscopy analysis. In the untreated control, most cells showed predominant green fluorescence, indicating intact cell membranes (Fig. 8a). In contrast, treatment with MPE at its minimum inhibitory concentration (15.3 µg/mL) led to an increase in red-fluorescing cells (Fig. 8e, 8f), indicating membrane damage and loss of viability. These observations are consistent with SEM and SYTO 9/PI flow cytometry results, further supporting that MPE induces membrane damage as a mechanism of antibacterial action. Similar membrane permeabilizing effect of propolis have been

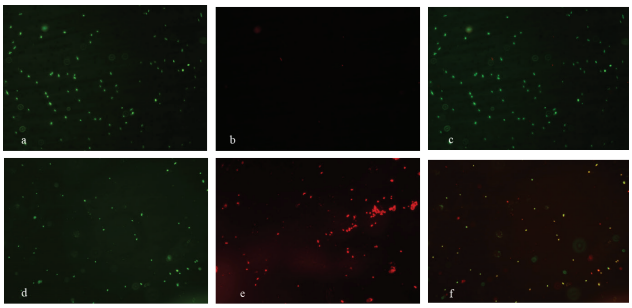

Fig.8. Fluorescence images of *Pseudomonas* with LIVE/DEAD BacLight Bacterial Viability and Counting Kit (a) Control sample without propolis, showing live bacteria with intact membranes fluorescing green. (b) Control sample exhibiting red fluorescence due to propidium iodide (PI) uptake. (c) Merged image of live and dead *Pseudomonas* cells in the control sample. (d) Propolis-treated bacterial suspension, showing a reduced number of live cells. (e) Propolis-treated bacteria with compromised membranes fluorescing red due to PI uptake. (f) Merged image of live and dead *Pseudomonas* cells following propolis treatment. Fluorescence microscopy was performed using FITC/TRITC filter sets. SYTO 9, a green-fluorescent nucleic acid stain, penetrates intact membranes, marking viable cells, whereas PI, a red-fluorescent stain, selectively enters membrane-compromised cells, indicating loss of viability.

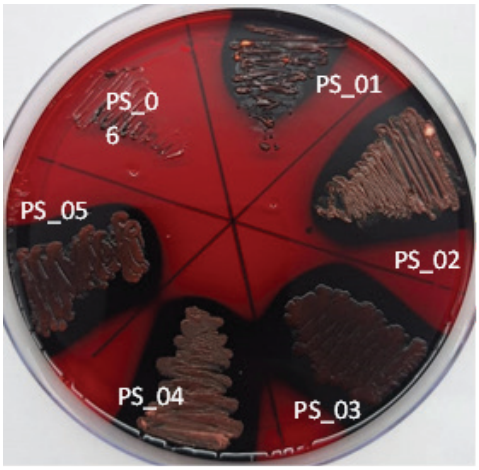


Fig.9. Black colonies on CRA method.

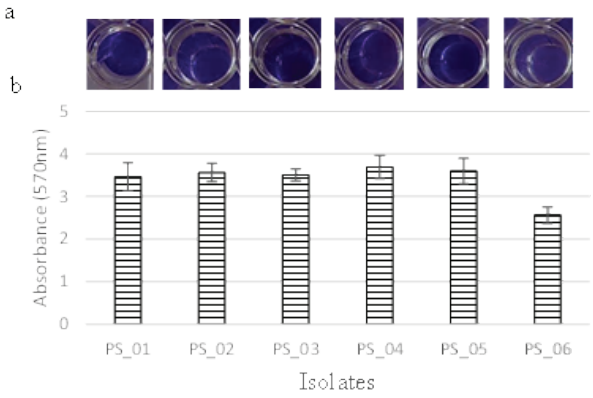


Fig. 10. Biofilm biomass phenotype of six *Pseudomonas* isolates a) crystal violet-stained biofilms on 96 well microtiter plate and b) the corresponding quantification of biofilm. Error bars represent standard deviation.

previously reported by (Veloz *et al.* 2019, Grecka *et al.* 2020) confirming the ability of propolis components to disrupt bacterial membrane integrity.

*The Congo Red method*: All 6 isolates were strong biofilm producers by CRA method (Fig. 9).

*Bacterial biofilm biomass quantification by crystal violet staining*: As shown in Fig. 10, all Pseudomonas isolates exhibited high biofilm biomass, as quantified by crystal violet absorbance at 570 nm, with values ranging from 2.56 to 3.69, indicating that all tested isolates were strong biofilm producers.

*Antibiofilm activity*: Biofilm inhibition was assessed by considering the untreated control as representing 100% biofilm formation. When MPE was added at 2MIC and MIC, the values of biofilm inhibition were 72.5±0.39% and 71.2±0.95%, respectively across six clinical *Pseudomonas* isolates (Fig. 11). A dose-dependent decline was observed at ½ MIC and ¼ MIC resulted in biofilm inhibition of 68.07±0.8% and 21.42±2.29, respectively. Compared to the untreated control, all treatment concentrations showed

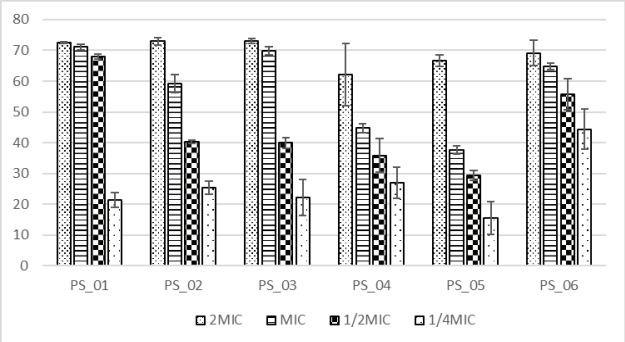


Fig. 11. Dose-dependent antibiofilm activity of methanolic propolis extract (MPE) against clinical *Pseudomonas* isolates (PS_01 to PS_06) at concentrations of 2 MIC, MIC, ½ MIC, and ¼ MIC. Maximum biofilm inhibition was observed at 2MIC, with a gradual decline at lower concentrations.

statistically significant biofilm inhibition ($p< 0.001$).

In conclusion, the current study indicates that MPE exhibit antibacterial properties, presenting a potential alternative antimicrobial and antibiofilm agent against *Pseudomonas* mastitis infection in buffaloes. Furthermore, it is suggested to carry out the identification of the bioactive compounds found in propolis fractions, aiming for their potential use as antibacterial agents.

## ACKNOWLEDGEMENTS

The authors deeply appreciate the invaluable participatory support provided by the farmers under the Farmer FIRST Program. We also express our gratitude to the Director of ICAR-CIRB, Hisar, for funding this project and supplying the essential facilities needed for this study.

## REFERENCES

Anand U, Jacobo-Herrera N, Altemimi A and Lakhssassi N. 2019. A comprehensive review on medicinal plants as antimicrobial therapeutics: Potential avenues of biocompatible drug discovery. *Metabolites* **9**(11): 258.

Aslam B, Wang W, Arshad M I, Khurshid M, Muzammil S, Rasool M H, Nisar M A, Alvi R F, Aslam M A, Qamar M U *et al.* 2018. Antibiotic resistance: A rundown of a global crisis. *Infection and Drug Resistance* **11**: 1645–58.

Begnini K R, Moura de Leon P M, Thurow H, Schultze E, Campos V F, Martins Rodrigues F, Borsuk S, Dellagostin O A, Savegnago L, Roesch-Ely M *et al.* 2014. Brazilian red propolis induces apoptosis-like cell death and decreases migration potential in bladder cancer cells. *Evidence-Based Complementary and Alternative Medicine* **2014**(1): 639856.

Berney M, Hammes F, Bosshard F, Weilenmann H U and Egli T. 2007. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. *Applied and Environmental Microbiology* **73**(10): 3283–90.

CLSI. 2012. Performance standards for antimicrobial susceptibility testing; twenty-second informational supplement CLSI document M100

Cotter J J, O'Gara J P, Mack D and Casey E. 2009. Oxygen-mediated regulation of biofilm development is controlled by the alternative sigma factor σB in *Staphylococcus epidermidis*. *Applied and Environmental Microbiology* **75**(1): 261–64.

da Silva L M, de Souza P, Jaouni S K A, Harakeh S, Golbabapour S and de Andrade S F. 2018. Propolis and its potential to treat gastrointestinal disorders. *Evidence-Based Complementary and Alternative Medicine* **2018**: 2035820.

Daly M, Power E, Bjorkroth J, Sheehan P, O'Connell A, Colgan M, Korkeala H and Fanning S. 1999. Molecular analysis of *Pseudomonas aeruginosa*: Epidemiological investigation of mastitis outbreaks in Irish dairy herds. *Applied and Environmental Microbiology* **65**(6): 2723–29.

De Marco S, Piccioni M, Pagiotti R and Pietrella D. 2017. Antibiofilm and antioxidant activity of propolis and bud poplar resins versus *Pseudomonas aeruginosa*. *Evidence-Based Complementary and Alternative Medicine* **2017**(1): 5163575.

De Vos D, Lim Jr A, Pirnay J-P, Struelens M, Vandenvelde C, Duinslaeger L, Vanderkelen A and Cornelis P. 1997. Direct detection and identification of *Pseudomonas aeruginosa* in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane lipoprotein genes, *oprI* and *oprL*. *Journal of Clinical Microbiology* **35**(6): 1295–99.

Doosti A, Pourabbas M, Arshi A, Chehelgerdi M and Kabiri H. 2015. TEM and SHV genes in *Klebsiella pneumoniae* isolated from cockroaches and their antimicrobial resistance pattern. *Osong Public Health And Research Perspectives* **6**(1): 3-8.

El-Sakhawy M. 2023. Propolis harvesting and extraction. *Egyptian Journal of Chemistry* **66**(1): 313–21.

Elnakady Y, Rushdi A, Franke R, Abutaha N, Ebaid H, Baabbad M, Omar M and Al-Ghamdi A. 2017. Characteristics, chemical compositions and biological activities of propolis from Al-Bahah, Saudi Arabia. *Scientific Reports* **7**: 41453.

Erskine R J, Unflat J G, Eberhart R J, Hutchinson L J, Hicks C R and Spencer S B. 1987. *Pseudomonas* mastitis: Difficulties in detection and elimination from contaminated wash-water systems. *Journal of the American Veterinary Medical Association* **191**(7): 811–15.

Freeman D, Falkiner F and Keane C. 1989. New method for detecting slime production by coagulase negative staphylococci. *Journal of Clinical Pathology* **42**(8): 872–74.

Grecka K, Xiong Z R, Chen H, Pełka K, Worobo R W and Szweda P. 2020. Effect of ethanol extracts of propolis (EEPs) against staphylococcal biofilm-microscopic studies. *Pathogens* **9**(8): 646.

Kazemi S, Shahandeh Z, Alizadeh M J, Nikoueian Shirvan B, Eslamdoust Z, Moulana Z and Asgharpour F. 2024. Propolis nanoemulsion enhance the antimicrobial effect on multidrug-resistant strains of *Pseudomonas aeruginosa* in combination with ciprofloxacin. *Natural Product Communications* **19**(9): 1934578X241284006.

Kirk J H and Bartlett P C. 1984. Nonclinical *Pseudomonas aeruginosa* mastitis in a dairy herd. *Journal of the American Veterinary Medical Association* **184**(6): 671–73.

Kumari G D. 2024. Identification of extended spectrum beta lactamases producing *Pseudomonas aeruginosa* from subclinical mastitis milk samples in an organized dairy farm of SVVU, Andhra Pradesh. *International Journal of Bio-resource and Stress Management* **15**(3): 1–7.

Mallick T T, Rahman M M, Siddique N, Shuvo K H, Arafat K Y, Homa S F, Akter S, Karim M R, Chandra Das Z and Hoque M N. 2025. Molecular and genomic investigation

unveils *Pseudomonas putida* as an emerging multidrug-resistant pathogen linked to bovine clinical mastitis. *Microbial Pathogenesis* **203**: 107461.

Pantha S, Parajuli H, Arjyal C, Karki S T and Shrestha D. 2024. Phenotypic characterization of ESBL-producing urinary isolates of *E. coli* and *Klebsiella* spp. in a tertiary care children's hospital in Nepal. *Tropical Medicine and Health* **52**(1): 20.

Park H R, Hong M K, Hwang S Y, Park Y K, Kwon K H, Yoon J W, Shin S, Kim J H and Park Y H. 2014. Characterisation of *Pseudomonas aeruginosa* related to bovine mastitis. *Acta Veterinaria Hungarica* **62**(1): 1–12.

Pitout J D, Hossain A and Hanson N D. 2004. Phenotypic and molecular detection of CTX-M-β-lactamases produced by *Escherichia coli* and *Klebsiella* spp. *Journal of Clinical Microbiology* **42**(12): 5715–21.

Prabhakar U P S, Shanmugam P, Boonyuen S, Chandrasekar L P, Pothu R, Boddula R, Radwan A B and Al-Qahtani N. 2024. Non-covalent functionalization of surfactant-assisted graphene oxide with silver nanocomposites for highly efficient photocatalysis and anti-biofilm applications. *Materials Science for Energy Technologies* **7**: 205–15.

Quinn P J. 1994. *Clinical veterinary microbiology*: Wolfe.

Rivera-Yañez N, Rivera-Yañez C R, Pozo-Molina G, Méndez-Catalá C F, Reyes-Reali J, Mendoza-Ramos M I, Méndez-Cruz A R and Nieto-Yañez O. 2021. Effects of propolis on infectious diseases of medical relevance. *Biology (Basel)* **10**(5).

Rozloznik M, Lochmanova A, Chmelar D, Hajek M, Korytkova K and Cisarikova M. 2020. Experimental use of flow cytometry to detect bacteria viability after hyperbaric oxygen exposure: Work in progress report. *Diving Hyperbaric Medicine* **50**(2): 152–56.

Salem M, Awad A and Younis G. 2023. Antibiotic susceptibility and molecular detection of virulent pseudomonas aeruginosa isolated from bovine mastitis milk in Egypt. *Journal of Advanced Veterinary Research* **13**(4): 664–71.

Schauer B, Wald R, Urbantke V, Loncaric I and Baumgartner M. 2021. Tracing mastitis pathogens- Epidemiological investigations of a *Pseudomonas aeruginosa* mastitis outbreak in an Austrian dairy herd. *Animals* **11**(2): 279.

Sekhri I, Chandra M, Kaur G, Narang D, Gupta D and Arora A. 2021. Prevalence of *Pseudomonas aeruginosa* and other microorganisms from mastitis milk and their antimicrobial resistance pattern. *Indian Journal of Animal Research* **55**(6): 716–21.

Silva L, Herrera Y, Bartolomeu A, Gorgulho C and Sforcin J. 2017. Mechanisms involved in the cytotoxic action of Brazilian propolis and caffeic acid against HEp-2 cells and modulation of P-glycoprotein activity. *Journal of Pharmacy and Pharmacology* **69**.

Stepanović S, Vuković D, Dakić I, Savić B and Švabić-Vlahović M. 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods* **40**(2): 175–79.

Šuran J, Cepanec I, Mašek T, Radić B, Radić S, Tlak Gajger I and Vlainić J. 2021. Propolis extract and its bioactive compounds-from traditional to modern extraction technologies. *Molecules* **26**(10): 2930.

Saeloh D and Visutthi M. 2021. Efficacy of Thai plant extracts for antibacterial and anti-biofilm activities against pathogenic bacteria. *Antibiotics (Basel)* **10**(12): 1470. doi: 10.3390/antibiotics10121470

Veloz J J, Alvear M and Salazar L A. 2019. Evaluation of alternative methods to assess the biological properties of propolis on metabolic activity and biofilm formation in *Streptococcus* mutans. *Evidence-Based Complementary and Alternative Medicine* **2019**(1): 1524195.

Wang F, Liu H, Li J, Zhang W, Jiang B and Xuan H. 2021. Australian propolis ethanol extract exerts antibacterial activity against methicillin-resistant *Staphylococcus aureus* by mechanisms of disrupting cell structure, reversing resistance, and resisting biofilm. *Brazilian Journal of Microbiology* **52**(4): 1651–64.

Wayne P. 2011. Clinical and laboratory standards institute. performance standards for antimicrobial susceptibility testing.

Yadav R, Chhabra R, Shrinet G and Singh M. 2020. Isolation of *Pseudomonas aeruginosa* from bovine mastitic milk sample along with antibiogram study. *Journal of Animal Research* **10**(2): 269–73.

Yadav S, Devi P, Boora A, Kumar P, Lohchab R K and Kumar A. 2023. Prevalence, extended-spectrum β-lactamase and biofilm production ability of *Escherichia coli* isolated from buffalo mastitis. *The Indian Journal of Animal Sciences* **93**(12): 1145–49.