# Phytochemical and Antibacterial Evaluation of *Pterygota Alata* Leaf Extracts Against CSOM Bacteria

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| Article's Information | Abstract |
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| <br><br> | The increasing rates of multidrug-resistant (MDR) organisms among patients with chronic suppurative otitis media (CSOM) have caused treatment procedures to become more complex, raising healthcare costs and significantly increasing the time of recovery. Since medicinal plants are the natural repositories for bio-active and secondary metabolites, they are an excellent alternative to antibiotics to combat MDR bacterial strains. This study aims to assess the antibacterial activity and the presence of phytochemicals in the aqueous and methanol leaf extracts of the Indian deciduous tree *Pterygota alata* against 5 MDR strains of CSOM-causing bacteria: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae*. Fresh *P. alata* leaves were harvested, dried, and extracted with methanol and water. Qualitative and quantitative assessments demonstrated the detection of primary and secondary metabolites. The antibacterial activities were determined using the agar well diffusion method, including minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), dose-response, and time-kill studies against CSOM bacterial isolates. Most phytochemicals, except proteins, were present in moderate to high concentrations in aqueous and methanol extracts. The methanol extracts had higher levels of phenolics (85.6 mg/g), flavonoids (42.3 mg/g), tannins (40.8 mg/g), and saponins (36.9 mg/g). Steroids were minimal in both extracts, while alkaloids were moderately present. All extracts showed significant antibacterial activity, particularly the methanol extracts against *S. aureus* and *P. mirabilis* (ANOVA, $p \leq 0.05$). The effect was concentration-dependent, with rapid bactericidal action observed at higher concentrations. It was further confirmed with one-way ANOVA followed post-hoc Tukey test, there were significant differences among different treatments ($p \leq 0.05$), supporting the reliability of the results. Therefore, it can be concluded from this study that methanolic leaf extracts of *P. alata* can be used as an alternative or complementary antibacterial agent against MDR CSOM infections. |

## 1. Introduction

Chronic suppurative otitis media (CSOM) is a prolonged middle ear infection that is generally characterized by tympanic membrane perforation and otorrhea and usually leads to deafness and intracranial infection if not treated. (1) In the last two decades, there has been a substantial rise in CSOM cases, primarily in children and in countries

with limited medical facilities. Moreover, the emergence of multidrug-resistant (MDR) bacterial strains has also complicated and escalated the CSOM infections. It has significantly hampered conventional antibiotic therapy, which has led to an increase in the recovery period as well as the healthcare costs (2). As the current options for antibiotics are continuously losing their effectiveness, herbal medicines have become a reliable choice to counter this onslaught of MDR bacteria. The traditional Indian medicinal system, namely Ayurveda, is primarily based on plant-derived medicines, and it was the only option for treating infectious diseases in ancient times (3). Likewise, herbal medicines were popular in traditional Chinese treatment regimens and Middle Eastern countries under the name Unani. For ages, medicinal plants have been known to be the reservoir of a wide range of chemically complex bio-active and secondary metabolites having significant therapeutic values. Hence, it makes them a potent choice for an alternative or complementary therapeutic agent to treat MDR bacteria. The mechanisms employed by bacteria to counter or break down antibiotics are not potent enough to break plant-derived complex bioactive compounds (4). In this perspective, *Pterygota alata* is one such medicinal plant that has not been much explored as a potential option of herbal drug to address MDR CSOM infections. *P. alata* (vernacular name: Buddha Coconut) is a deciduous tree widely distributed in India and other Southeast Asia countries. Its leaves are supported by short stalks, typically 3 to 10 cm long, and tend to cluster at the ends of branches. The leaf blades are broad, ranging from ovate to heart-shaped, measuring approximately 10 to 25 cm in length and 7 to 15 cm in width. They have smooth surfaces with gently undulating edges and tapering or pointed tips (5). Ayurveda and other traditional medicine systems have long used its bark, seeds, and leaves to treat various diseases, including inflammation, wounds, and microbial infections (5-8). A few phytochemical and antimicrobial studies related to *P. alata* have documented the presence of several secondary metabolites and their antibacterial potency against both Gram-positive and Gram-negative bacteria (7). These results further substantiate the hypothesis that the pharmacological properties of *P. alata* should be scientifically investigated and clinically validated for its use as an antibacterial agent. Researchers and scientists around the world have been supporting the use of antimicrobial medicinal plants as antimicrobial agents. However, the lack of scientific validation and clinical trials has restricted

their full-scale commercial use against MDR bacteria. Integrating *P. alata* as an alternative and complementary herbal drug also requires both *in vitro* and *in vivo* studies for its scientific validation (9, 10). There are no research studies, which this plant has been exclusively tested against CSOM-causing bacteria. Therefore, this study investigates the antibacterial efficacy against the common bacterial strains causing CSOM. Various methods were used to assess its antimicrobial effects. This included agar diffusion, tests to determine the lowest concentrations needed to stop or kill the bacteria, dose-response evaluations, and time-kill studies. Additionally, the extracts were examined to identify and measure the presence of key secondary metabolites using qualitative and quantitative approaches.

## 2. Materials and Methods
### 2.1. Collection and processing of plant material
Fresh leaves of *P. alata* were collected from their natural habitat, locally from Bhubaneswar, Odisha, India, soon after the monsoon season during September–October 2023, and were officially authenticated with the help of a plant taxonomist. The leaves were washed with distilled water to remove dirt and debris and were air-dried at room temperature. The dried leaves were converted to fine powder using an electric grinder (11).

### 2.2. Preparation and concentration of aqueous and methanol *P. alata* leaf extracts
The dried leaf powder of *P. alata* was extracted by soaking 10 g of powder in 100 mL of methanol or distilled water (1:10 w/v) in conical flasks and shaking for 48 h at 150 rpm on an orbital shaker (Remi CM-101, Mumbai, India). The mixtures were filtered through muslin cloth and Whatman No. 1 filter paper (GE Healthcare, India). All filtrates were concentrated using a Borosil Rotary Evaporator (India) at 40°C under reduced pressure, freeze-dried using a Yorco Lyophilizer (India), and stored in amber glass vials (Borosil, India) at 4°C until use. Extraction yield was calculated as:

$$\text{Extraction Yield (\%)} = (\text{Weight of dried extracts} \div \text{Weight of plant material}) \times 100.$$

From 10 g of leaf powder, the methanol extracts yielded 1.82 g (18.2%), while the aqueous extracts yielded 0.95 g (9.5%). All solvents and analytical-grade chemicals used were procured from HiMedia Laboratories (Mumbai, India) and SRL Chemicals (Mumbai, India). Absorbance for quantitative phytochemical assays was measured using a

Systronics UV–Visible Spectrophotometer (Model 117, Ahmedabad, India). The extracts were not sterilized because 0.22 µm filtration reduced activity; they were prepared aseptically, and extracts-only controls showed no contamination.

## 2.3. Phytochemical analysis of extracts

Phytochemical screening of the methanol and aqueous extracts of *P. alata* was carried out using standard colorimetric assays, including Wagner's test (alkaloids), alkaline reagent test (flavonoids), ferric chloride test (phenols and tannins), froth test (saponins), Salkowski test (terpenoids), Keller–Killiani test (glycosides), Liebermann–Burchard test (steroids), Molisch's test (carbohydrates), and the Biuret test (proteins). All chemicals and reagents used in these assays were obtained from Sigma-Aldrich (India). Quantitative estimation of major phytochemical groups was performed using established spectrophotometric methods, with absorbance measured on a Systronics UV–Visible spectrophotometer at 415 nm (flavonoids), 765 nm (phenolics), 760 nm (tannins), 544 nm (saponins), and 490 nm (carbohydrates) (6, 11).

## 2.4. Isolation and identification of bacterial strains

Ear swabs from 500 CSOM patients were analyzed using microbiological, biochemical, and molecular methods to identify major bacterial pathogens, with antibiotic susceptibility tested by the Kirby-Bauer method and Vitek 2. The predominant isolates included *Pseudomonas aeruginosa* – 149 cases, *Staphylococcus aureus* – 75 cases, *Escherichia coli* – 72 cases, *Proteus mirabilis* – 65 cases, and *Klebsiella pneumoniae* – 62 cases.

One representative strain from each species was selected for testing against *P. alata* leaf extracts (12). A single isolate per species was used because this study served as a preliminary antibacterial screening. From each isolated species, the clinical isolate resistant to the largest number of antibiotics was selected for the antibacterial screening test.

## 2.5. Antimicrobial studies method for *P. alata,* the aqueous and methanol leaf extracts

The agar well diffusion method was used to determine the antibacterial efficacy (zone of inhibition) of the aqueous and methanol leaf extracts against clinical isolates of five significant bacteria responsible for CSOM infections. Based on the results of the phytochemical analysis and zone of inhibition from the agar-well diffusion method against all five bacteria, the best from the two leaf extracts were further subjected to the determination of minimum inhibitory concentration (MIC) and

minimum bactericidal concentration (MBC) values, dose-response curve determination and the time-kill assay (6, 11). In the agar well diffusion assay, wells of 6 mm diameter were made on Mueller–Hinton (MH) agar plates, and 50 µL of each extract concentration was carefully dispensed into each well. Plates were incubated at 37°C for 24 hours before measuring the zones of inhibition

## 2.6. Determination of MIC and MBC values

MIC was determined by the microbroth dilution method following CLSI M07-A11 guidelines. Each well contained 100 µL of Mueller–Hinton Broth (MHB) and 100 µL of the extracts (total volume 200 µL). After incubation at 37°C for 24 h, the MIC was recorded as the lowest concentration without visible growth. For MBC, 10 µL from wells at and above the MIC were sub-cultured onto MH agar, and the lowest concentration showing ≥99.9% bacterial killing was taken as the MBC (6, 11, 13, 14).

## 2.7. Dose-response curve protocol for methanol leaf extracts.

The dose-response assay evaluated the antibacterial efficacy of *P. alata* methanol leaf extracts at varying concentrations. Serial dilutions of the extracts were prepared at 25, 50, 100, 150, and 200 mg/mL using 1% v/v DMSO as the solvent, depending on solubility. MH agar plates were inoculated with a standardized bacterial suspension equivalent to 0.5 McFarland turbidity (approximately $10^6$ CFU/mL). Subsequently, 50 µL of each methanol extract concentration was added to agar wells. Control wells included a Ciprofloxacin (10µg/ml) served as the positive control, and its MIC was measured alongside the plant extracts using identical CLSI-standard microbroth dilution conditions for direct comparison. The plates were incubated at 37°C for 24 hours, after which the diameter of the zone of inhibition around each well was measured in millimetres using a calliper (15). High concentrations were used for exploratory *in vitro* screening of crude extracts; future work will involve fractionation to isolate active principles and cytotoxicity, and formulation studies to determine clinically relevant dosing

## 2.8. Time-kill assay

The time–kill assay was performed to evaluate the bactericidal effect of *P. alata* methanol leaf extracts. Bacterial cultures of the five clinical isolates were grown to the logarithmic phase and adjusted to 0.5 McFarland ($\approx 10^6$ CFU/mL). The assay was conducted in 50 mL Erlenmeyer flasks, each containing a 10 mL reaction volume of MH Broth

with the extracts added at 1× and 2× MIC. Controls included a growth control (no extracts), ciprofloxacin (10μg/ml) as the positive control, and a solvent control (1% v/v DMSO). Flasks were incubated at 37°C on an orbital shaker (Remi CM-101, India) set at 120 rpm to ensure uniform mixing. At 0, 2, 4, 6, 8, 12, and 24 hours, 100 μL samples were withdrawn, serially diluted, and plated on MH agar. After overnight incubation, CFU/mL values were counted and plotted as time–kill curves with $\log_{10}$ CFU/mL versus time (16).

## 2.9. Statistical Analysis
All experiments were performed in triplicate, and data are expressed as mean ± standard deviation (SD). Statistical analyses were conducted using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). One-way ANOVA followed by Tukey's post-hoc test was employed to statistically compare the zone of inhibition, MIC/MBC values, and the time kill assay data. A threshold of $p \leq 0.05$ was considered statistically significant. In all tables, different superscript letters denote statistically significant differences, while identical letters indicate no significant difference between means. Exact p-values for all ANOVA tests ranged from $p = 0.0004$ to $p = 0.018$, indicating statistically significant differences across treatments ($p \leq 0.05$).

## 3. Results
### 3.1. Phytochemical analysis of *P. alata* methanol and aqueous leaf extracts
Preliminary phytochemical screening was performed to detect the presence of various secondary metabolites in methanol and aqueous extracts of *P. alata* leaves (Table 1). Further, quantitative assays were carried out to estimate the concentration of key bioactive groups in both extracts (Table 2). The methanol extracts of *P. alata* exhibited a higher content of total phenolics, flavonoids, alkaloids, tannins and saponins than the aqueous extracts, suggesting a better antibacterial potential. The presence of a broad spectrum of phytochemicals indicates that *P. alata* possesses significant therapeutic potential due to the synergistic effect of its diverse bioactive components.

### 3.2. Antibacterial activity of *P. alata* crude extracts (zone of inhibition, MIC & MBC)
The methanol extracts of *P. alata* demonstrated stronger antibacterial activity than the aqueous extracts, as evident from the larger zones of inhibition ranging from 18–20 mm, compared to 12–17 mm for the aqueous extracts (Table 3, Figures 1 & 2). For the methanol extracts, MIC values ranged from 0.62 mg/mL for *S. aureus* to 2.50 mg/mL for *P. aeruginosa*, with corresponding MBC values of 1.25–5.00 mg/mL. The aqueous extracts showed higher MICs, ranging from 3.50 mg/mL for *S. aureus* to 6.25 mg/mL for *P. aeruginosa*, and MBCs of 7.50–12.50 mg/mL. These results indicate that the methanol extracts of *P. alata* demonstrate stronger inhibitory and bactericidal activity against all tested strains, including *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *P. mirabilis*, and *S. aureus*. For comparison, ciprofloxacin exhibited MIC and MBC values of 0.25–1.00 μg/mL and 0.50–2.00 μg/mL, respectively (Table 4). All experiments, including phytochemical and antibacterial assays, were performed in triplicate (n = 3), and results are presented as mean ± standard deviation.

Table 1. Qualitative phytochemical screening

| Phytochemical | Methanol extracts | Aqueous Extracts | Observation / Test Used |
|---|---|---|---|
| Alkaloids | +++ | ++ | Wagner's test: reddish-brown precipitate. |
| Flavonoids | ++ | ++ | Alkaline reagent test; yellow colour → colourless with acid; λ = 415 nm. |
| Tannins | ++ | ++ | $FeCl_3$ test; blue-black precipitate; λ = 760 nm. |
| Saponins | ++ | + | Froth test; stable persistent froth; λ = 544 nm. |
| Terpenoids | ++ | ++ | Salkowski test; reddish-brown interface. |
| Phenols | ++ | +++ | $FeCl_3$ / Folin–Ciocalteu; bluish-black colour; λ = 765 nm. |
| Glycosides | ++ | ++ | Keller–Killiani test; brown ring at the interface. |
| Steroids | + | + | Liebermann–Burchard-Test; green ring. |
| Carbohydrates | ++ | ++ | Molisch's test; violet ring; λ = 490 nm. |
| Proteins & Amino Acids | – | – | Biuret test; no colour change; λ = 540 nm. |
| *Note: +++ = Strongly present, ++ = Moderately present, + = Weakly present, − = Absent* | | | |

Table 2. Quantitative estimation of phytochemicals (mg/g dry extracts)

| Phytochemical | Methods used | Methanol extracts (mg/g) | Aqueous Extracts (mg/g) |
|---|---|---|---|
| Total Phenolics | Folin-Ciocalteu Method | 85.6 ± 2.1 | 72.4 ± 1.9 |
| Total Flavonoids | Aluminium Chloride Colourimetric Assay | 42.3 ± 1.8 | 30.6 ± 1.5 |
| Tannins | Folin-Denis Method | 40.8 ± 2.3 | 31.5 ± 2.0 |
| Saponins | Vanillin-Sulfuric Acid Method | 36.9 ± 1.8 | 24.7 ± 1.3 |
| Alkaloids | Bromocresol Green | 15.2 ± 0.9 | 10.4 ± 1.0 |
| Note: Values are expressed as mean ± SD from three replicates. | | | |

Table 3. Zone of Inhibition of the aqueous and methanol leaf extracts.

| Bacteria | Positive Control | Methanol extracts | Aqueous Extracts | Negative Control |
|---|---|---|---|---|
| *E. coli* | 22.0 ± 0.4[a] | 18.0 ± 0.5[b] | 15.0 ± 0.3[c] | 0.0 ± 0.0[d] |
| *P. aeruginosa* | 21.0 ± 0.3[a] | 19.0 ± 0.4[b] | 17.0 ± 0.5[c] | 0.0 ± 0.0[d] |
| *K. pneumoniae* | 21.0 ± 0.4[a] | 19.0 ± 0.5[b] | 12.0 ± 0.4[c] | 0.0 ± 0.0[d] |
| *P. mirabilis* | 20.0 ± 0.5[b] | 19.0 ± 0.3[a] | 16.0 ± 0.4[c] | 0.0 ± 0.0[d] |
| *S. aureus* | 21.0 ± 0.3[b] | 20.0 ± 0.2[a] | 14.0 ± 0.3[c] | 0.0 ± 0.0[d] |
| Note: Values represent mean ± SD (n = 3). Different superscript letters within each row indicate statistically significant differences among treatments (ANOVA, Tukey; $p \leq 0.05$). | | | | |
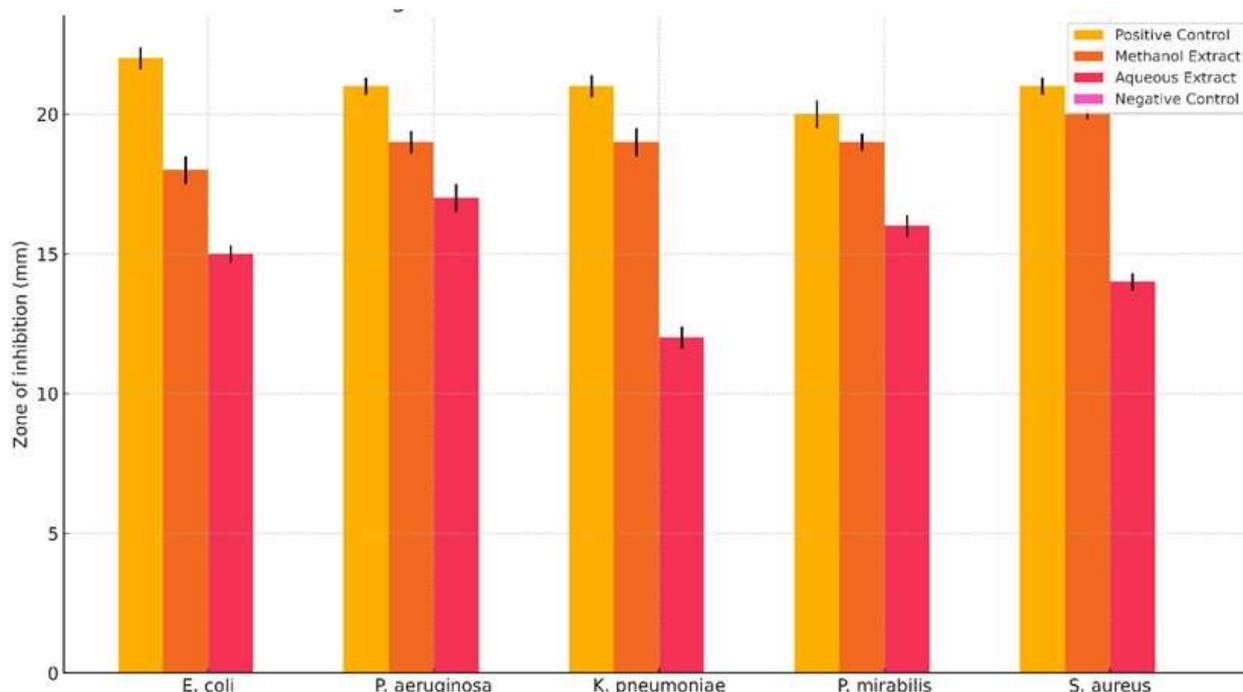


Figure 1. Zone of inhibition (mean ± SD, n = 3) produced by *P. alata* methanol and aqueous extracts against CSOM-causing bacteria. Different superscript letters within each row indicate statistically significant differences (ANOVA, Tukey; $p \leq 0.05$).
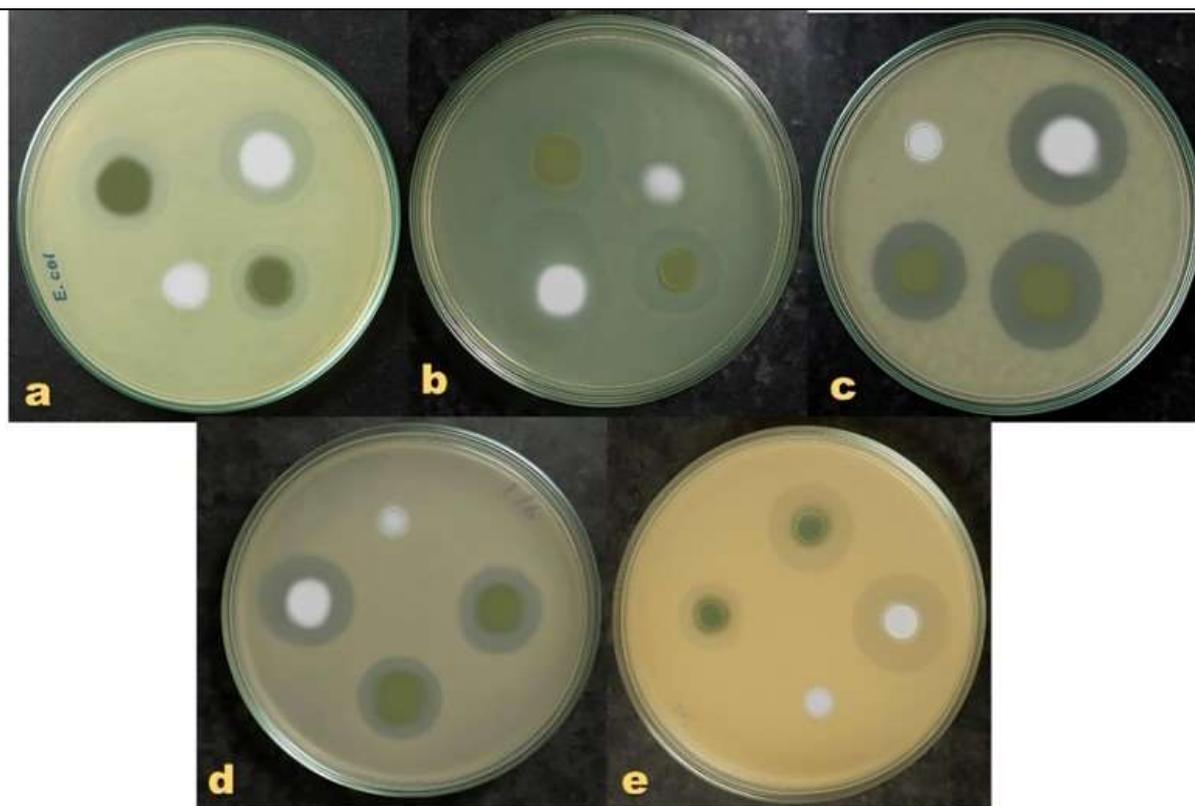
Figure 2. Zone of inhibition (mm) at various concentrations of methanol extracts in Dose-Response Study against CSOM-causing bacteria (a. *E. coli*; b. *P. aeruginosa*; c. *K. pneumoniae*; d. *P. mirabilis* e. *S. aureus*

Table 4. MIC and MBC of Methanol and Aqueous Extracts with Ciprofloxacin Control

| Bacterial isolate | Methanol MIC | Methanol MBC | Aqueous MIC | Aqueous MBC | Ciprofloxacin MIC | Ciprofloxacin MBC |
|---|---|---|---|---|---|---|
| *E. coli* | 1.25 ± 0.05[b] | 2.50 ± 0.10[b] | 5.00 ± 0.20[c] | 10.00 ± 0.25[c] | 0.25[a] | 0.50[a] |
| *P. aeruginosa* | 2.50 ± 0.10[b] | 5.00 ± 0.15[b] | 6.25 ± 0.20[c] | 12.50 ± 0.30[c] | 1.00[a] | 2.00[a] |
| *K. pneumoniae* | 1.00 ± 0.04[b] | 2.00 ± 0.08[b] | 4.00 ± 0.15[c] | 8.00 ± 0.20[c] | 0.50[a] | 1.00[a] |
| *P. mirabilis* | 0.75 ± 0.03[b] | 1.50 ± 0.05[b] | 3.75 ± 0.12[c] | 7.50 ± 0.18[c] | 0.25[a] | 0.50[a] |
| *S. aureus* | 0.62 ± 0.02[b] | 1.25 ± 0.04[b] | 3.50 ± 0.10[c] | 6.25 ± 0.15[c] | 0.25[a] | 0.50[a] |
| Note: Values represent mean ± SD (n = 3). Superscript letters within each row denote significant differences between extracts and the ciprofloxacin control (ANOVA, Tukey; $p \leq 0.05$). | | | | | | |

Table 5: Zone of inhibition (mm) at various concentrations of methanol extracts.

| Bacteria | 25 mg/mL | 50 mg/mL | 100 mg/mL | 150 mg/mL | 200 mg/mL | Positive Control |
|---|---|---|---|---|---|---|
| *E. coli* | 8.2 ± 0.5[e] | 12.3 ± 0.4[d] | 15.6 ± 0.6[c] | 17.2 ± 0.3[b] | 18.9 ± 0.2[a] | 22.0 ± 0.4[a] |
| *P. aeruginosa* | 7.8 ± 0.6[e] | 11.1 ± 0.5[d] | 14.4 ± 0.5[c] | 16.3 ± 0.4[b] | 17.6 ± 0.3[a] | 21.0 ± 0.3[a] |
| *K. pneumoniae* | 6.5 ± 0.4[e] | 10.0 ± 0.3[d] | 13.2 ± 0.5[c] | 15.0 ± 0.3[b] | 16.7 ± 0.2[a] | 21.0 ± 0.4[a] |
| *P. mirabilis* | 9.0 ± 0.5[e] | 13.1 ± 0.4[d] | 16.0 ± 0.6[c] | 18.4 ± 0.3[b] | 19.1 ± 0.2[a] | 20.0 ± 0.5[a] |
| *S. aureus* | 9.5 ± 0.4[e] | 13.8 ± 0.4[d] | 17.0 ± 0.5[c] | 19.2 ± 0.3[b] | 19.5± 0.2[a] | 21.0 ± 0.3[a] |
| *Values represent mean ± SD (n = 3). Different superscript letters within each row indicate significant differences among concentrations and the positive control (ANOVA, Tukey; $p \leq 0.05$).* | | | | | | |
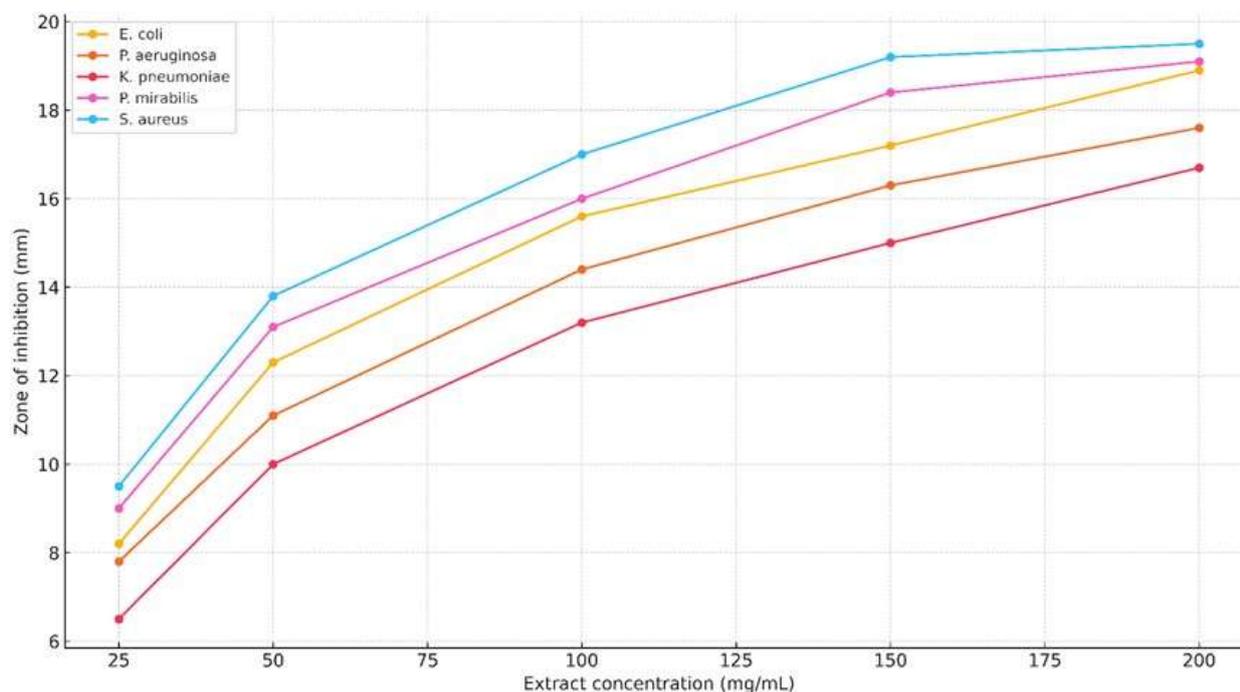
Figure 3. Dose–response curves of methanol extract of *P. alata* at 25–200 mg/mL against CSOM-associated bacteria (mean ± SD, n = 3). Antibacterial activity increases in a concentration-dependent manner. Different superscript letters in Table 5 indicate significant differences (p ≤ 0.05).

Table 6. $Log_{10}$ CFU/mL Over Time (Extracts at 2× MIC)

| Bacteria | 0 h | 2 h | 4 h | 6 h | 8 h | 12 h | 24 h | Effect |
|---|---|---|---|---|---|---|---|---|
| E. coli | 6.00[a] | 5.20[b] | 4.60[b] | 3.20[c] | 2.00[c] | 1.40[b] | 0.90[b] | BC |
| P. aeruginosa | 6.00[a] | 5.40[a] | 5.00[a] | 4.10[b] | 3.00[b] | 2.50[a] | 1.80[a] | BC |
| K. pneumoniae | 6.00[a] | 5.30[a] | 5.00[a] | 4.20[b] | 3.50[a] | 2.60[a] | 2.00[a] | BL |
| P. mirabilis | 6.00[a] | 4.80[c] | 3.60[c] | 2.40[d] | 1.20[d] | 0.80[c] | 0.40[c] | BC |
| S. aureus | 6.00[a] | 4.50[c] | 3.00[d] | 1.60[e] | 0.80[e] | 0.30[d] | <1.0[d] | RK |
| Note: BC = Bactericidal (≥3 $log_{10}$ reduction/24 h); BL = Borderline bactericidal (1–3 $log_{10}$ reduction/24 h); RK = Rapid kill (≥3 $log_{10}$ reduction in <12 h). Values represent mean ± SD (n = 3). Different superscript letters within each time point indicate statistically significant differences in bacterial counts among the tested strains (ANOVA; p ≤ 0.05). | | | | | | | | |
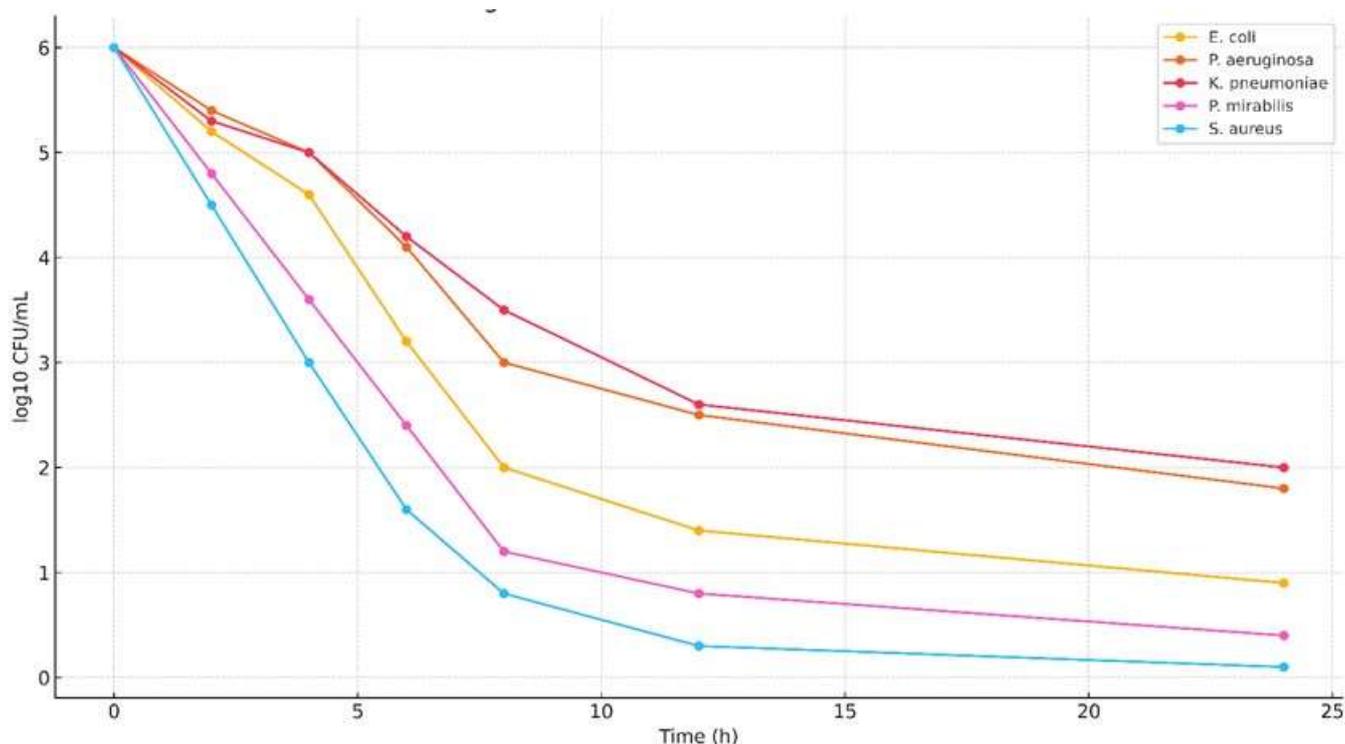
Figure 4. Time–kill kinetics of *P. alata* methanol leaf extract at 2× MIC against five CSOM pathogens. Values represent mean log10 CFU/mL (n = 3). Points approaching 0 log10 indicate counts below the detection limit (≤1 CFU/mL). BC = bactericidal (≥3 log10 reduction/24 h); BL = borderline bactericidal (1–3 log10 reduction/24 h); RK = rapid kill (≥3 log10 reduction in <12 h).

### 3.3. Antibacterial activity of *P. alata* methanol extracts (dose-response study)

The antibacterial activity of *P. alata* methanol leaf extracts was evaluated against five clinically relevant bacterial strains using the agar well diffusion method at various concentrations ranging from 25 mg/mL to 200 mg/mL (Table 5, Figure 3). A significant increase in antibacterial activity was observed with increasing concentrations of the extracts. The highest zone of inhibition at 200 mg/mL was recorded for *S. aureus* (19.5 ± 0.2 mm), whereas for ciprofloxacin, 20.0 ± 0.3 mm was recorded. It is to be noted that the values obtained for plant extracts cannot be compared to values obtained for a pure antibiotic. *P. mirabilis* also showed potent inhibition (20.1 ± 0.2 mm), followed by *E. coli*. Although responsive*, K. pneumoniae and P. aeruginosa* showed comparatively lower inhibition zones, with maximum activity reaching 16.7 mm and 17.6 mm, respectively, at 200 mg/mL. The dose-response curve suggested a concentration-dependent increase in antimicrobial efficacy, indicating the potential of *P. alata* as a source of bio-active compounds with bactericidal properties.

### 3.4. Time-kill assay of *P. alata* methanol extracts

The death curve for all five bacteria begins with the same initial concentration of $\log_{10}$ 6 CFU/mL, as shown in Table 6, allowing for a valid comparison of the extracts' bactericidal effects. The time-kill assay confirmed that *P. alata* methanol extracts exhibit time-dependent bactericidal activity at 2× MIC. *S. aureus* showed the most rapid killing, reaching complete eradication within 24 hours. *P. mirabilis* also demonstrated strong bactericidal activity, with a ≥3 $\log_{10}$ CFU/mL reduction by 12 hours. *E. coli* and *P. aeruginosa* showed gradual but consistent reductions, achieving bactericidal levels by 24 hours. In contrast, *K. pneumoniae* displayed a slower decline, remaining near the bacteriostatic–bactericidal threshold, indicating moderate sensitivity (Table 6, Figure 4). Statistical analysis using ANOVA followed by Tukey's post-hoc test revealed significant differences among treatments across all assays ($p \leq 0.05$). Zone-of-inhibition values showed clear variation between the methanol extracts, aqueous extracts, positive control, and negative control (Tables 3 and 5). Similarly, MIC and MBC values differed significantly between both extracts and ciprofloxacin, indicating distinct

antibacterial potency profiles (Table 4). Time–kill assays also demonstrated significant reductions in viable bacterial counts over time, with meaningful differences among species (Table 6). These statistically significant comparisons are denoted by different superscript letters in the respective tables.

## 4. Discussion
This study highlights the significant antibacterial activity of *P. alata* leaf extracts, with the methanol extracts showing greater potency than the aqueous extracts against bacterial strains isolated from patients with CSOM. Phytochemical analysis supported the superior efficacy of the methanol extracts, particularly against Gram-positive bacteria. Inhibition zones increased in a concentration-dependent manner, reaching 19.5 mm for *S. aureus* at 200 mg/mL. Time-kill kinetics demonstrated that at twice the minimum inhibitory concentration, the methanol extracts achieved complete eradication of *S. aureus* within 24 hours. Additionally, potential activity was observed against *P. mirabilis* and *E. coli*, while *K. pneumoniae* exhibited moderate sensitivity. This trend is in agreement with recent findings that methanolic extracts often yield a higher concentration of phenolic and flavonoid compounds responsible for antimicrobial and antioxidant effects (17, 18). The higher antibacterial activity and phytochemical yield obtained from the methanol extracts may be due to the solvent's intermediary polarity and strong extraction capability. Methanol can solubilize a wide spectrum of bio-active compounds such as phenolics, flavonoids, tannins, and alkaloids, which are only partially soluble in water. Since many of these secondary metabolites are semi-polar or non-polar, methanol serves as a more efficient solvent for extraction. The MIC and MBC findings revealed that *S. aureus* was the most sensitive organism, indicating strong inhibitory and bactericidal potential. In contrast, *P. aeruginosa* required higher concentrations, reflecting the structural resistance of Gram-negative bacteria, whose outer membrane restricts entry of plant-derived molecules (18). Such differences confirm that cell wall architecture significantly influences susceptibility to phytochemicals. Methanolic and ethanolic extracts have shown activity against both Gram-positive and Gram-negative pathogens, including biofilm-forming *MDR S. aureus*, along with notable antioxidant properties (6; 17). Egyptian studies have further identified glycosides, sterols, and alkaloids as major antibacterial constituents in this plant (19). The present study corroborates these findings and extends them by linking antibacterial efficacy with the extracts' phytochemical profile. The methanol extracts were rich in phenolics, flavonoids, tannins, saponins, and alkaloids, all known to damage bacterial membranes, inhibit nucleic acid synthesis, or interfere with energy metabolism (20, 21). The synergistic action of these compounds likely accounts for the concentration-dependent inhibition and rapid killing observed. Such rapid effects suggest interference with vital cellular pathways such as oxidative phosphorylation or membrane integrity. Similar time-dependent killing has been reported for polyphenol-rich extracts against resistant pathogens (22, 23). Hence, these results highlight *P. alata* as a promising source of plant-derived antibacterial agents effective against *MDR* bacteria (24).

The primary novelty of this study is that it focuses on CSOM-causing bacteria isolates rather than general antimicrobial screening. Previous research on *P. alata* primarily highlighted its anti-inflammatory and antioxidant properties, but this study exhibited its antibacterial activity on MDR bacteria. Additionally, the phytochemical analysis reveals the presence of complex secondary metabolites, which are cumulatively responsible for this plant's antibacterial properties. Moreover, the dose-response study and the time-kill assay provided excellent data, which, on further research, can reveal more pharmacological properties of this plant. The statistical analysis highlighted the difference in the antibacterial activity, MIC and MBC values along with the dose-response and time kill assay, that the methanolic extracts have superior antimicrobial activity in comparison to the aqueous extracts. The findings clearly suggested that appropriate concentrations of *P. alata* will have significant antibacterial activity. Despite the promising results that validates the antimicrobial properties of *P. alata*, the study has a few limitations. Firstly, the study only evaluates the antimicrobial properties of the crude methanol and aqueous leaf extract and qualitatively identifies the phytochemicals present. Further studies are required to find out the exact bio-active compound to formulate the crude extract for a possible antimicrobial drug. Secondly, the cytotoxicity and pharmacokinetics assessment are necessary for the clinical application of this crude extract.

In this study, ciprofloxacin was used as the positive control against the crude extract; however, plant extracts cannot be directly compared with a pure antibiotic, as their concentration, molecular structure, mode of action, and bioavailability characteristics differ fundamentally. Therefore,

more detailed research should be done on bioactivity-guided fractionation, compound identification, host toxicity, along with animal and human trials, before the large-scale commercial use of this plant as a potential antibacterial drug.

## 5. Conclusion

In conclusion, the result of the current study strongly advocates the use of *P. alata* as a natural alternative or complementary herbal antibacterial drug against MDR CSOM infections. The presence of several complex phytochemicals further strengthens its candidacy for an effective phytopharmaceutical drug. However, further *in vivo* studies related to its pharmacokinetics and host-toxicity, including animal and human trials, must be pursued before its commercial use. The integration of traditional herbal medicine into the contemporary treatment regime can significantly improve the healthcare quality and reduce expenses related to MDR infections. These natural remedies can significantly help to reduce the dependency on antibiotic therapy and counter the threat of global antimicrobial resistance.

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