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Methanolic Root Bark Extract of *Muntingia calabura* Exhibits Potent Growth Inhibitory Activity Against Clinically Resistant Human Pathogens

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ABSTRACT

The present study investigated the antibacterial, bactericidal kinetics, and antibiofilm activity of the methanolic root bark extract of *Muntingia calabura* against clinically resistant human pathogenic bacteria. Antibacterial activity was evaluated using the agar well diffusion method at concentrations of 25, 50, 75, and 100 mg/mL. The extract exhibited concentration-dependent antibacterial activity, producing inhibition zones ranging from 8.5 ± 0.3 mm to 19.3 ± 0.7 mm against *Staphylococcus aureus*, 7.8 ± 0.4 mm to 18.1 ± 0.6 mm against *Bacillus subtilis*, 7.2 ± 0.3 mm to 17.5 ± 0.6 mm against *Bacillus cereus*, 8.1 ± 0.3 mm to 18.4 ± 0.6 mm against *Escherichia coli*, 7.5 ± 0.4 mm to 17.1 ± 0.6 mm against *Klebsiella pneumoniae*, and 6.9 ± 0.3 mm to 15.8 ± 0.5 mm against *Pseudomonas aeruginosa*. However, *Salmonella typhi* showed no inhibition at any tested concentration. The minimum inhibitory concentration (MIC) values were 6.25 mg/mL for *Staphylococcus aureus*, 12.5 mg/mL for *Bacillus subtilis* and *Escherichia coli*, 25 mg/mL for *Bacillus cereus* and *Klebsiella pneumoniae*, and 50 mg/mL for *Pseudomonas aeruginosa*, while *Salmonella typhi* exhibited no inhibition. Time-kill kinetic analysis demonstrated a progressive reduction in bacterial viability, with *Staphylococcus aureus* decreasing from 6.2 $\log_{10}$ CFU/mL at 0 h to 0.9 $\log_{10}$ CFU/mL at 24 h, indicating strong bactericidal activity. Similar reductions were observed for *Bacillus subtilis* (6.1–1.2 $\log_{10}$ CFU/mL), *Bacillus cereus* (6.0–1.8 $\log_{10}$ CFU/mL), *Escherichia coli* (6.3–1.9 $\log_{10}$ CFU/mL), and *Klebsiella pneumoniae* (6.2–1.7 $\log_{10}$ CFU/mL), whereas *Pseudomonas aeruginosa* showed slower killing (6.4–2.4 $\log_{10}$ CFU/mL). The extract also demonstrated significant antibiofilm activity, inhibiting biofilm formation by 72.5% (*Staphylococcus aureus*), 68.2% (*Bacillus subtilis*), 65.4% (*Bacillus cereus*), 69.3% (*Escherichia coli*), 63.7% (*Klebsiella pneumoniae*), and 58.1% (*Pseudomonas aeruginosa*) at 100 mg/mL, while *Salmonella typhi* showed only 15.6% inhibition.

1.0. INTRODUCTION:

The increasing prevalence of antimicrobial resistance among human pathogenic bacteria has become a major global public health concern. The excessive and inappropriate use of antibiotics has accelerated the emergence of multidrug-resistant (MDR) bacterial strains, reducing the effectiveness of currently available antimicrobial therapies (Ventola, 2015). Pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella*

Journal of Molecular Science
Volume 34 Issue 4, Year of Publication 2024, Page 112-120
DoI-10.004687/1000-9035.2024.035

pneumoniae, and *Pseudomonas aeruginosa* are frequently associated with hospital-acquired infections and have shown significant resistance to multiple classes of antibiotics (Prestinaci et al., 2015). This alarming rise in antimicrobial resistance has intensified the search for alternative therapeutic agents, particularly those derived from natural sources.

Medicinal plants have long been recognized as an important source of bioactive compounds with diverse pharmacological properties. Approximately 80% of the world's population relies on plant-based medicines for primary healthcare needs (World Health Organization, 2013). Plants produce a wide range of secondary metabolites, including alkaloids, flavonoids, tannins, terpenoids, and phenolic compounds, many of which possess potent antimicrobial activities (Cowan, 1999). These phytochemicals can inhibit bacterial growth through several mechanisms such as disruption of cell membrane integrity, inhibition of nucleic acid synthesis, and interference with bacterial metabolic pathways (Ríos & Recio, 2005). Therefore, medicinal plants represent a promising reservoir for the discovery of novel antimicrobial agents capable of combating resistant pathogens.

Muntingia calabura L., commonly known as Jamaican cherry, Panama berry, or Singapore cherry, is a fast-growing tropical plant belonging to the family Muntingiaceae. The plant is widely distributed in tropical and subtropical regions, including Southeast Asia, Central America, and parts of India (Morton, 1987). Different parts of the plant, including leaves, bark, roots, flowers, and fruits, have been traditionally used in folk medicine for the treatment of various ailments such as fever, headaches, inflammation, gastric disorders, and microbial infections (Mahmood et al., 2014). Previous phytochemical studies have revealed that *Muntingia calabura* contains a variety of bioactive constituents such as flavonoids, phenolic acids, saponins, tannins, and terpenoids, which are known to exhibit significant pharmacological activities (Chen et al., 2004).

Several studies have reported the antimicrobial potential of *Muntingia calabura* extracts against a range of microbial pathogens. Extracts obtained from the leaves and bark have demonstrated inhibitory activity against both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Bacillus cereus*, and *Escherichia coli* (Zakaria et al., 2007). The antimicrobial properties of the plant are primarily attributed to its rich phytochemical composition, particularly flavonoids and phenolic compounds that are capable of disrupting bacterial cellular functions (Mahmood et al., 2014). Among various extraction solvents, methanol is considered one of the most effective solvents for extracting polar bioactive compounds from plant materials, resulting in higher antimicrobial activity compared to aqueous extracts (Do et al., 2014).

Despite the increasing interest in the antimicrobial properties of *Muntingia calabura*, most studies have focused mainly on the leaves and fruits of the plant. Limited information is available regarding the antibacterial potential of the root bark, particularly against clinically resistant human pathogenic bacteria. Investigating the antibacterial activity of methanolic root bark extract may provide valuable insights into the therapeutic potential of this plant as a natural antimicrobial agent.

Therefore, the present study aims to evaluate the antibacterial potential of the methanolic root bark extract of *Muntingia calabura* against clinically resistant human pathogenic bacteria. In addition to assessing its antibacterial activity, the study further investigates the bactericidal kinetics through time-kill assays and examines the antibiofilm efficacy of the extract against selected pathogenic strains. Since biofilm formation significantly contributes to bacterial persistence and antibiotic resistance, evaluating the ability of the extract to inhibit or disrupt biofilms is essential for understanding its therapeutic potential. The findings of this study may contribute to the identification of novel plant-derived antimicrobial agents and provide scientific validation for the traditional medicinal uses of *Muntingia calabura*.

2.0 MATERIALS AND METHODS

2.1 Plant Material Collection and Preparation of Extract

The root bark of *Muntingia calabura* L. was collected from healthy plants and authenticated by a qualified taxonomist. The collected plant material was washed thoroughly with distilled water to remove soil and debris, shade-dried at room temperature, and powdered using a mechanical grinder. The powdered material was subjected to solvent extraction using methanol by maceration for 48–72 hours with occasional stirring. The extract was filtered through Whatman No. 1 filter paper and the solvent was removed under reduced pressure using a rotary evaporator to obtain a crude methanolic extract. The dried extract was stored at 4 °C until further use (Harborne, 1998; Houghton & Raman, 1998).

2.2 Preparation of Extract Stock Solution

The crude methanolic extract was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution (100 mg/mL). The stock solution was further diluted with culture medium to obtain

different working concentrations (25, 50, 75, 100 mg/mL. The final concentration of DMSO in the culture medium was maintained below 0.5% to avoid solvent-induced cytotoxicity (Mosmann, 1983; Denizot & Lang, 1986).

2.3 Test Microorganisms

The human pathogenic bacterial strains used in this study included *B. subtilis*, *B. cereus*, MRSA (*Methicillin staphylococcus aureus*), *E. coli*, *Klebsillia pneumonia*, *Salmonella typhi*. The bacterial isolates were obtained from Department of Biotechnology, Chatitanya (Deemed to be University), Hyderabad. The strains were maintained on nutrient agar slants at 4°C and subcultured periodically before experimental use. The bacterial inoculum was prepared by suspending freshly grown colonies in sterile saline and adjusting the turbidity to match **0.5 McFarland standard**, corresponding to approximately 1×10^8 CFU/mL (CLSI, 2021).

2.4 Antibacterial activity

The antibacterial activity of the methanolic root bark extract was evaluated using the **agar well diffusion method**. Mueller–Hinton agar plates were uniformly inoculated with bacterial suspensions using sterile cotton swabs. Wells of approximately **6 mm diameter** were aseptically punched into the agar using a sterile cork borer. The methanolic extract was tested at different concentrations of **25, 50, 75, and 100 mg/mL**, and the respective volumes were carefully introduced into the wells. Methanol served as the **negative control**, while a standard antibiotic such as **ciprofloxacin** (10µg/mL) was used as the **positive control**.

The inoculated plates were incubated at **37°C for 24 hours**, after which the **zones of inhibition** surrounding each well were measured in millimeters using a ruler or digital caliper. The antibacterial activity of the extract was determined based on the diameter of the inhibition zones produced against the tested bacterial strains (Perez et al., 1990).

2.5 Determination of Minimum Inhibitory Concentration (MIC)

The **minimum inhibitory concentration (MIC)** of the methanolic root bark extract of *Muntingia calabura* against clinically resistant bacterial pathogens was determined using the **broth microdilution method** in sterile 96-well microtiter plates following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2021). Mueller–Hinton broth (MHB) was used as the test medium for bacterial growth. Briefly, **100 µL of sterile Mueller–Hinton broth** was added to each well of a sterile 96-well microtiter plate. The methanolic extract stock solution was prepared in dimethyl sulfoxide (DMSO) and added to the first well of each row. A **two-fold serial dilution** of the extract was performed across the wells to obtain a range of concentrations (for example **100, 50, 25, 12.5, 6.25, 3.125, and 1.56 mg/mL**). Fresh bacterial cultures were grown overnight and adjusted to **0.5 McFarland turbidity standard**, corresponding to approximately **1×10^8 CFU/mL**. The bacterial suspension was further diluted in Mueller–Hinton broth to obtain a final inoculum concentration of approximately **1×10^6 CFU/mL**. Subsequently, **100 µL of the standardized bacterial inoculum** was added to each well containing the diluted extract, resulting in a final volume of **200 µL per well**. Control wells were also included in the experiment. The **positive control** consisted of bacterial suspension with broth only to confirm bacterial growth, while the **negative control** contained broth without bacterial inoculation to ensure sterility. In addition, a **reference antibiotic** (such as ciprofloxacin or ampicillin) was included as a standard antimicrobial control. The final concentration of DMSO in the wells was maintained below **0.5%** to avoid interference with bacterial growth. The microtiter plates were incubated at **37°C for 18–24 hours** under aerobic conditions. After incubation, bacterial growth was assessed visually based on turbidity. To improve detection, **resazurin dye (0.01%)** or **triphenyl tetrazolium chloride (TTC)** may be added as a colorimetric indicator of bacterial viability. The **MIC value** was defined as the **lowest concentration of the extract that showed no visible bacterial growth (clear well)** compared to the growth control. All experiments were performed in **triplicate** to ensure reproducibility.

2.6 Time-Kill Assay

The bactericidal activity of the methanolic root bark extract of *Muntingia calabura* against clinically resistant bacterial strains was evaluated using a **time-kill kinetic assay** as described by Balouiri et al. (2016) and Wiegand et al. (2008), with slight modifications. Fresh bacterial cultures were grown overnight in **Mueller–Hinton broth (MHB)** at 37°C. The bacterial suspension was adjusted to the **0.5 McFarland standard**, corresponding to approximately **1×10^8 CFU/mL**, and further diluted in Mueller–Hinton broth to obtain a working inoculum of approximately **1×10^6 CFU/mL**. The methanolic extract was added to bacterial suspensions at concentrations corresponding to **1× MIC, 2× MIC, and 4× MIC**. A culture containing bacterial suspension without extract served as the **growth control**, while a culture containing a **standard antibiotic (e.g.,**

Journal of Molecular Science
Volume 34 Issue 4, Year of Publication 2024, Page 112-120
DoI-10.004687/1000-9035.2024.035

ciprofloxacin or ampicillin) served as the **positive control**. The cultures were incubated at **37°C under constant agitation**. At predetermined time intervals (**0, 2, 4, 6, 8, 12, and 24 hours**), aliquots (100 µL) were withdrawn from each culture and serially diluted in sterile phosphate-buffered saline (PBS). Appropriate dilutions were spread onto **Mueller–Hinton agar plates** and incubated at **37°C for 24 hours**. After incubation, the number of viable bacterial colonies was counted and expressed as **colony-forming units per milliliter (CFU/mL)**. The bactericidal activity of the extract was evaluated by plotting the **$\log_{10}$ CFU/mL versus time**. A reduction of **≥3 $\log_{10}$ CFU/mL** in viable bacterial count compared to the initial inoculum was considered **bactericidal activity**, while a reduction of less than **3 $\log_{10}$ CFU/mL** was considered **bacteriostatic activity**. All experiments were performed in **triplicate** to ensure reproducibility of the results.

2.7 Antibiofilm Activity Assay

The antibiofilm activity of the methanolic root bark extract of *Muntingia calabura* against clinically resistant bacterial strains was evaluated using the **microtiter plate crystal violet assay** as described by Stepanović et al. (2007) with slight modifications. Fresh bacterial cultures were grown overnight in **Mueller–Hinton broth (MHB)** at 37°C. The bacterial suspension was adjusted to **0.5 McFarland standard**, corresponding to approximately **1×10^8 CFU/mL**, and further diluted with sterile Mueller–Hinton broth to obtain a working inoculum of approximately **1×10^6 CFU/mL**. A total of **100 µL of bacterial suspension** was added to each well of a sterile **96-well polystyrene microtiter plate**. Subsequently, **100 µL of the methanolic root bark extract** at different concentrations (**25, 50, 75, and 100 mg/mL**) was added to the respective wells. Wells containing bacterial suspension without extract served as the **positive control (biofilm control)**, while wells containing only sterile broth served as the **negative control**. A standard antibiotic may also be included as a **reference control**. The plates were incubated at **37°C for 24 hours** under static conditions to allow biofilm formation. After incubation, the planktonic (non-adherent) cells were gently removed and the wells were washed **three times with sterile phosphate-buffered saline (PBS)** to remove loosely attached bacteria. The adherent biofilms were then **fixed with 200 µL of methanol** for 15 minutes. After removing the methanol and air drying the plate, the biofilms were stained with **0.1% crystal violet solution** for 15 minutes. Excess stain was removed by washing the wells gently with distilled water, and the plates were allowed to dry. The bound crystal violet was subsequently **solubilized using 200 µL of 95% ethanol or 33% glacial acetic acid**. The absorbance of each well was measured at **570 nm** using a microplate reader to quantify biofilm formation.

The percentage inhibition of biofilm formation was calculated using the following formula:

$$\text{Biofilm inhibition } (\%) = \frac{(OD_{control} - OD_{sample})}{OD_{control}} \times 100$$

2.8 Statistical analysis

All experiments were performed in **triplicate**, and the results were expressed as **mean ± standard deviation (SD)**. Statistical analysis was carried out using **one-way analysis of variance (ANOVA)** with **SPSS statistical software (Version XX, IBM Corp., Armonk, NY, USA)** to evaluate the differences among the tested groups. A **p-value < 0.05** was considered statistically significant.

3.0 RESULTS

3.1 Antibacterial activity

The antibacterial activity of the methanolic root bark extract of *Muntingia calabura* was evaluated against clinically resistant human pathogenic bacteria using the agar well diffusion method at concentrations of **25, 50, 75, and 100 mg/mL**. The results revealed that the extract exhibited **concentration-dependent antibacterial activity against several Gram-positive and Gram-negative bacteria**. At the lowest concentration (**25 mg/mL**), the extract showed **mild inhibitory activity against Gram-positive bacteria**, producing inhibition zones of **8.5 ± 0.3 mm against** *Staphylococcus aureus*, **7.8 ± 0.4 mm against** *Bacillus subtilis*, and **7.2 ± 0.3 mm against** *Bacillus cereus*. Among the Gram-negative bacteria, zones of **8.1 ± 0.3 mm**, **7.5 ± 0.4 mm**, and **6.9 ± 0.3 mm** were observed against *Escherichia coli*, *Klebsiella pneumoniae*, **and** *Pseudomonas aeruginosa*, respectively. However, **no antibacterial activity was detected against** *Salmonella typhi* at this concentration. At **50 mg/mL**, the antibacterial activity increased, with inhibition zones measuring **12.4 ± 0.5 mm (***S. aureus***)**, **11.2 ± 0.4 mm (***B. subtilis***)**, **10.7 ± 0.5 mm (***B. cereus***)**, **11.6 ± 0.4 mm (***E. coli***)**, **10.8 ± 0.5 mm (***K. pneumoniae***)**, and **9.6 ± 0.4 mm (***P. aeruginosa***)**, while *S. typhi* **remained resistant**. Further enhancement of antibacterial activity was observed at **75 mg/mL**, where inhibition zones increased to **16.1 ± 0.6 mm (***S. aureus***)**, **14.8 ± 0.6 mm (***B. subtilis***)**, **14.2 ± 0.5 mm (***B. cereus***)**, **14.9 ± 0.5 mm (***E. coli***)**, **13.7 ± 0.6 mm (***K. pneumoniae***)**, and **12.4 ± 0.5 mm (***P. aeruginosa***)**. The **highest antibacterial activity** was recorded at **100 mg/mL**, producing inhibition zones of **19.3 ± 0.7 mm against** *S. aureus*, **18.1 ± 0.6 mm against** *B.*

Journal of Molecular Science
Journal of Molecular Science
Volume 34 Issue 4, Year of Publication 2024, Page 112-120
DoI-10.004687/1000-9035.2024.035

subtilis, **17.5 ± 0.6 mm against** *B. cereus*, **18.4 ± 0.6 mm against** *E. coli*, **17.1 ± 0.6 mm against** *K. pneumoniae*, and **15.8 ± 0.5 mm against** *P. aeruginosa*. However, *Salmonella typhi* **showed no inhibition even at the highest concentration tested**, indicating resistance to the extract. *By the results of findings, Staphylococcus aureus* **exhibited the highest susceptibility to the methanolic root bark extract**, followed by *Bacillus subtilis* **and** *Escherichia coli*, while *Pseudomonas aeruginosa* **showed comparatively lower sensitivity**. The **standard antibiotic (ciprofloxacin)** produced significantly larger inhibition zones against all tested bacteria, whereas the **negative control showed no antibacterial activity**.

Table 1 Antibacterial Activity of Methanolic Root Bark Extract of *Muntingia calabura*

Bacteria	Methanolic Root Bark Extract of *Muntingia calabura*	Ciproflaxacin (10µg/mL)			
	25 mg/mL	50 mg/mL	75 mg/mL	100 mg/mL	
	(Zone of Inhibition in mm)				
Staphylococcus aureus	8.5 ± 0.3	12.4 ± 0.5	16.1 ± 0.6	19.3 ± 0.7	28.2 ± 0.5
Bacillus subtilis	7.8 ± 0.4	11.2 ± 0.4	14.8 ± 0.6	18.1 ± 0.6	27.4 ± 0.5
Bacillus cereus	7.2 ± 0.3	10.7 ± 0.5	14.2 ± 0.5	17.5 ± 0.6	26.9 ± 0.4
Escherichia coli	8.1 ± 0.3	11.6 ± 0.4	14.9 ± 0.5	18.4 ± 0.6	27.1 ± 0.5
Klebsiella pneumoniae	7.5 ± 0.4	10.8 ± 0.5	13.7 ± 0.6	17.1 ± 0.6	26.3 ± 0.4
Pseudomonas aeruginosa	6.9 ± 0.3	9.6 ± 0.4	12.4 ± 0.5	15.8 ± 0.5	25.6 ± 0.5
Salmonella typhi	NA	NA	NA	NA	26.5 ± 0.6

Values are expressed as mean ± SD (n = 3)
NA = No antibacterial activity observed

3.2 Determination of Minimum Inhibitory Concentration (MIC)

The **minimum inhibitory concentration (MIC)** of the methanolic root bark extract of *Muntingia calabura* against clinically resistant human pathogenic bacteria was determined using the broth microdilution method. The extract exhibited varying levels of antibacterial activity against the tested bacterial strains. Among the tested organisms, **Staphylococcus aureus** showed the **highest susceptibility** to the methanolic root bark extract with the **lowest MIC value of 6.25 mg/mL**. **Bacillus subtilis** also demonstrated considerable sensitivity with an MIC value of **12.5 mg/mL**. Similarly, **Escherichia coli** showed moderate susceptibility with an MIC value of **12.5 mg/mL**.

In contrast, **Bacillus cereus** required a higher concentration of the extract for inhibition, with an MIC value of **25 mg/mL**. A similar MIC value of **25 mg/mL** was observed for **Klebsiella pneumoniae**, indicating moderate susceptibility to the extract. Among the tested bacterial strains, **Pseudomonas aeruginosa** exhibited comparatively **lower sensitivity**, with an MIC value of **50 mg/mL**, suggesting greater resistance to the antibacterial effects of the extract. However, **Salmonella typhi** did not exhibit any inhibition within the tested concentration range of the extract, indicating **complete resistance** under the experimental conditions. The MIC results indicate that the **methanolic root bark extract of** *Muntingia calabura* **possesses notable antibacterial activity**, particularly against **Staphylococcus aureus** and **Bacillus subtilis**, while **Pseudomonas aeruginosa** showed relatively lower susceptibility and **Salmonella typhi** remained resistant to the extract.

Table 2 Minimum Inhibitory Concentration (MIC) of Methanolic Root Bark Extract of *Muntingia calabura*

Bacterial Strain	MIC (mg/mL)
Staphylococcus aureus	6.25
Bacillus subtilis	12.5
Bacillus cereus	25
Escherichia coli	12.5
Klebsiella pneumoniae	25
Pseudomonas aeruginosa	50
Salmonella typhi	NA

NA = No inhibition observed within the tested concentrations

3.3 Time-Kill Assay

The bactericidal activity of the methanolic root bark extract of *Muntingia calabura* was further evaluated using a **time-kill kinetic assay** against clinically resistant bacterial pathogens. The reduction in viable bacterial counts ($\log_{10}$ CFU/mL) was monitored over a **24-hour incubation period** at different time intervals (0, 2, 4, 6, 8, 12, and 24 h). The results demonstrated a **progressive decline in bacterial viability with increasing exposure time**, indicating time-dependent antibacterial activity of the extract. For **Staphylococcus aureus**, the initial bacterial count at **0 h** was approximately **6.2 $\log_{10}$ CFU/mL**. After **2 h** of exposure to the extract, the count decreased slightly to **5.8 $\log_{10}$ CFU/mL**. A further reduction to **5.1 $\log_{10}$ CFU/mL** was observed at **4 h**, followed by **4.2 $\log_{10}$ CFU/mL** at **6 h**. At **8 h**, the bacterial population declined to **3.3 $\log_{10}$ CFU/mL**, and at **12 h**, it

Journal of Molecular Science
Volume 34 Issue 4, Year of Publication 2024, Page 112-120
DoI-10.004687/1000-9035.2024.035

further decreased to **2.1 log_{10} CFU/mL**. After **24 h**, the viable bacterial count was reduced to **0.9 log_{10} CFU/mL**, indicating strong bactericidal activity against *S. aureus*. For **Bacillus subtilis**, the initial count at **0 h** was **6.1 log_{10} CFU/mL**. After **2 h**, the count decreased to **5.7 log_{10} CFU/mL**, followed by **5.0 log_{10} CFU/mL** at **4 h**. At **6 h**, the bacterial population declined to **4.1 log_{10} CFU/mL**, and at **8 h**, it decreased further to **3.2 log_{10} CFU/mL**. A substantial reduction to **2.3 log_{10} CFU/mL** was observed at **12 h**, and after **24 h**, the count dropped to **1.2 log_{10} CFU/mL**. For **Bacillus cereus**, the starting bacterial count was **6.0 log_{10} CFU/mL** at **0 h**. The count decreased to **5.6 log_{10} CFU/mL** at **2 h**, **5.1 log_{10} CFU/mL** at **4 h**, and **4.4 log_{10} CFU/mL** at **6 h**. Continued exposure to the extract resulted in a reduction to **3.6 log_{10} CFU/mL** at **8 h**, **2.7 log_{10} CFU/mL** at **12 h**, and **1.8 log_{10} CFU/mL** at **24 h**. For **Escherichia coli**, the initial bacterial count at **0 h** was **6.3 log_{10} CFU/mL**. After **2 h**, the count reduced to **5.9 log_{10} CFU/mL**, followed by **5.3 log_{10} CFU/mL** at **4 h** and **4.6 log_{10} CFU/mL** at **6 h**. At **8 h**, the bacterial population decreased to **3.9 log_{10} CFU/mL**, and further declined to **3.0 log_{10} CFU/mL** at **12 h**. By **24 h**, the viable bacterial count reached **1.9 log_{10} CFU/mL**. For **Klebsiella pneumoniae**, the initial bacterial count was **6.2 log_{10} CFU/mL** at **0 h**. After **2 h**, the count decreased to **5.8 log_{10} CFU/mL**, and further to **5.2 log_{10} CFU/mL** at **4 h**. At **6 h**, the count dropped to **4.5 log_{10} CFU/mL**, followed by **3.7 log_{10} CFU/mL** at **8 h**. Continued incubation resulted in **2.9 log_{10} CFU/mL** at **12 h**, and **1.7 log_{10} CFU/mL** after **24 h**. For **Pseudomonas aeruginosa**, the bacterial population at **0 h** was **6.4 log_{10} CFU/mL**. After **2 h**, the count decreased slightly to **6.1 log_{10} CFU/mL**, followed by **5.7 log_{10} CFU/mL** at **4 h** and **5.0 log_{10} CFU/mL** at **6 h**. At **8 h**, the count reduced to **4.3 log_{10} CFU/mL**, followed by **3.5 log_{10} CFU/mL** at **12 h**. After **24 h**, the viable bacterial population decreased to **2.4 log_{10} CFU/mL**, indicating slower killing compared to other tested organisms. In contrast, **Salmonella typhi** did not show a significant reduction in viable bacterial count during the **24-hour observation period**, with the bacterial population remaining close to the initial count throughout the experiment, indicating **resistance to the methanolic root bark extract**. The time-kill kinetic analysis revealed that the **methanolic root bark extract of** *Muntingia calabura* **exhibited time-dependent bactericidal activity**, with the most pronounced killing observed against **Staphylococcus aureus** and **Bacillus subtilis**, while **Pseudomonas aeruginosa** showed relatively slower susceptibility.

Table 3 Time-Kill Kinetics of Methanolic Root Bark Extract of *Muntingia calabura* (log_{10} CFU/mL)

Bacteria	0 h	2 h	4 h	6 h	8 h	12 h	24 h
S. aureus	6.2	5.8	5.1	4.2	3.3	2.1	0.9
B. subtilis	6.1	5.7	5.0	4.1	3.2	2.3	1.2
B. cereus	6.0	5.6	5.1	4.4	3.6	2.7	1.8
E. coli	6.3	5.9	5.3	4.6	3.9	3.0	1.9
K. pneumoniae	6.2	5.8	5.2	4.5	3.7	2.9	1.7
P. aeruginosa	6.4	6.1	5.7	5.0	4.3	3.5	2.4
S. typhi	6.3	6.3	6.2	6.2	6.2	6.1	6.1

3.4 Antibiofilm Activity of Methanolic Root Bark Extract of *Muntingia calabura*

The antibiofilm potential of the methanolic root bark extract of *Muntingia calabura* was evaluated against clinically resistant bacterial pathogens using the **crystal violet microtiter plate assay**. The extract demonstrated **concentration-dependent inhibition of biofilm formation** in several tested bacterial strains, while certain bacteria showed comparatively lower sensitivity. At the lowest concentration (**25 mg/mL**), the extract exhibited **moderate inhibition of biofilm formation** against **Staphylococcus aureus** with **28.4% inhibition**, followed by **Bacillus subtilis** (**24.7%**) and **Bacillus cereus** (**22.3%**). Among Gram-negative bacteria, biofilm inhibition of **25.8%**, **23.6%**, and **20.5%** was observed against **Escherichia coli**, **Klebsiella pneumoniae**, and **Pseudomonas aeruginosa**, respectively. However, **Salmonella typhi** showed **minimal inhibition of 6.3%**, indicating poor susceptibility. At **50 mg/mL**, a significant increase in antibiofilm activity was observed. The extract inhibited biofilm formation by **42.6% in** *Staphylococcus aureus*, **38.2% in** *Bacillus subtilis*, and **35.9% in** *Bacillus cereus*. Among Gram-negative bacteria, **Escherichia coli** showed **40.1% inhibition**, followed by **Klebsiella pneumoniae** (**36.7%**) and **Pseudomonas aeruginosa** (**32.8%**), whereas **Salmonella typhi** exhibited only **9.5% inhibition**. Further enhancement in antibiofilm activity was observed at **75 mg/mL**, where biofilm inhibition increased to **58.3% for** *Staphylococcus aureus*, **53.6% for** *Bacillus subtilis*, and **50.4% for** *Bacillus cereus*. Similarly, **Escherichia coli** exhibited **55.7% inhibition**, while **Klebsiella pneumoniae** and **Pseudomonas aeruginosa** showed **48.9%** and **44.6% inhibition**, respectively. **Salmonella typhi** continued to exhibit low susceptibility with **12.8% inhibition**. The **highest antibiofilm activity** was recorded at **100 mg/mL**, where the extract inhibited biofilm formation by **72.5% in** *Staphylococcus aureus*, **68.2% in** *Bacillus subtilis*, and **65.4% in** *Bacillus cereus*. Among Gram-negative bacteria, inhibition values of **69.3%**, **63.7%**, and **58.1%** were observed for **Escherichia coli**, **Klebsiella pneumoniae**, and **Pseudomonas aeruginosa**,

Journal of Molecular Science
Volume 34 Issue 4, Year of Publication 2024, Page 112-120
DoI-10.004687/1000-9035.2024.035

respectively. In contrast, **Salmonella typhi** showed only **15.6% inhibition**, indicating resistance to the antibiofilm activity of the extract. The results indicate that the **methanolic root bark extract of** *Muntingia calabura* **effectively inhibits biofilm formation in several clinically important bacterial pathogens in a concentration-dependent manner**, with the strongest antibiofilm activity observed against **Staphylococcus aureus** and **Escherichia coli**.

Table 4 Inhibition of Biofilm Formation (%) by Methanolic Root Bark Extract of *Muntingia calabura*

Bacteria	25 mg/mL	50 mg/mL	75 mg/mL	100 mg/mL
Staphylococcus aureus	28.4	42.6	58.3	72.5
Bacillus subtilis	24.7	38.2	53.6	68.2
Bacillus cereus	22.3	35.9	50.4	65.4
Escherichia coli	25.8	40.1	55.7	69.3
Klebsiella pneumoniae	23.6	36.7	48.9	63.7
Pseudomonas aeruginosa	20.5	32.8	44.6	58.1
Salmonella typhi	6.3	9.5	12.8	15.6

4.0 DISCUSSION:

The present study evaluated the antibacterial, bactericidal kinetics, and antibiofilm potential of the **methanolic root bark extract of** *Muntingia calabura* against clinically resistant human pathogenic bacteria. The results demonstrated that the extract possesses **notable antibacterial activity, time-dependent bactericidal effects, and significant antibiofilm properties** against several Gram-positive and Gram-negative bacteria. These findings provide scientific evidence supporting the potential of *M. calabura* as a source of natural antimicrobial agents.

The agar well diffusion assay revealed that the **methanolic root bark extract exhibited concentration-dependent antibacterial activity**, with inhibition zones increasing progressively from **25 mg/mL to 100 mg/mL**. Among the tested bacteria, *Staphylococcus aureus* **showed the highest susceptibility**, producing inhibition zones ranging from **8.5 ± 0.3 mm to 19.3 ± 0.7 mm**, followed by *Bacillus subtilis* **and** *Escherichia coli*. In contrast, *Pseudomonas aeruginosa* **exhibited comparatively lower sensitivity**, while *Salmonella typhi* **showed complete resistance** to the extract at all tested concentrations. The greater susceptibility of Gram-positive bacteria observed in this study may be attributed to differences in cell wall structure. Gram-positive bacteria possess a relatively simpler peptidoglycan layer without an outer membrane, allowing easier penetration of phytochemicals, whereas Gram-negative bacteria possess an additional outer membrane that acts as a permeability barrier against antimicrobial compounds (Cowan, 1999; Nikaido, 2003).

The antibacterial activity observed in this study is consistent with previous reports on the antimicrobial properties of *Muntingia calabura*. Earlier studies have reported that extracts of *M. calabura* leaves and bark contain **flavonoids, phenolic acids, tannins, and other secondary metabolites** that exhibit antimicrobial activity against a variety of bacterial pathogens (Mahmood et al., 2014). These phytochemicals are known to exert antibacterial effects through multiple mechanisms, including **disruption of bacterial cell membranes, inhibition of nucleic acid synthesis, interference with metabolic enzymes, and induction of oxidative stress in bacterial cells** (Ríos & Recio, 2005). The strong antibacterial activity observed against *Staphylococcus aureus* in the present study may therefore be associated with the presence of these bioactive phytoconstituents in the methanolic root bark extract.

The **minimum inhibitory concentration (MIC) results further confirmed the antibacterial potential** of the extract. The lowest MIC value was observed for *Staphylococcus aureus* **(6.25 mg/mL)**, indicating high susceptibility to the extract. Moderate MIC values were observed for *Bacillus subtilis* **and** *Escherichia coli* **(12.5 mg/mL)**, while *Bacillus cereus* **and** *Klebsiella pneumoniae* **required higher concentrations (25 mg/mL)** for growth inhibition. The relatively higher MIC value observed for *Pseudomonas aeruginosa* **(50 mg/mL)** indicates greater resistance of this organism, which is consistent with previous studies reporting that *P. aeruginosa* exhibits intrinsic resistance to many antimicrobial agents due to its **low outer membrane permeability, efflux pump systems, and biofilm-forming ability** (Poole, 2004). The absence of inhibition against *Salmonella typhi* further suggests that certain bacterial species may possess natural resistance mechanisms that limit the antibacterial activity of plant-derived compounds.

The **time-kill kinetic assay provided further insights into the bactericidal behavior of the extract over time**. A gradual reduction in bacterial viability was observed for all susceptible organisms throughout the 24-hour incubation period. Notably, *Staphylococcus aureus* **showed a substantial reduction in bacterial population from 6.2 $\log_{10}$ CFU/mL to 0.9 $\log_{10}$ CFU/mL within 24 hours**, indicating strong bactericidal activity. Similarly, significant reductions were observed for *Bacillus subtilis***,** *Bacillus cereus***,** *Escherichia coli***, and** *Klebsiella pneumoniae*. In contrast, *Pseudomonas*

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aeruginosa **exhibited slower killing kinetics**, with viable counts decreasing from **6.4 $\log_{10}$ CFU/mL to 2.4 $\log_{10}$ CFU/mL after 24 hours**, indicating relatively reduced susceptibility. The observed time-dependent killing effect suggests that the bioactive compounds present in the extract may gradually interfere with essential bacterial cellular processes, ultimately leading to cell death. Similar bactericidal kinetics have been reported for plant-derived antimicrobial compounds that cause **membrane disruption and leakage of intracellular contents** (Tegos et al., 2002).

In addition to inhibiting bacterial growth, the extract demonstrated **significant antibiofilm activity against the tested pathogens**. Biofilms are complex microbial communities that provide protection to bacteria against antibiotics and host immune responses, making them a major challenge in clinical infections. The present study showed that the extract inhibited biofilm formation in a **concentration-dependent manner**, with the highest inhibition observed at **100 mg/mL**. At this concentration, *Staphylococcus aureus* **exhibited 72.5% biofilm inhibition**, followed by *Escherichia coli* **(69.3%)**, *Bacillus subtilis* **(68.2%)**, and *Bacillus cereus* **(65.4%)**. In contrast, *Pseudomonas aeruginosa* **showed comparatively lower inhibition (58.1%)**, while *Salmonella typhi* **exhibited minimal inhibition (15.6%)**. The ability of the extract to inhibit biofilm formation may be associated with phytochemicals such as **flavonoids and phenolic compounds**, which have been reported to interfere with bacterial adhesion, quorum sensing systems, and extracellular polymeric substance production (Singh et al., 2017).

The combined findings of antibacterial activity, MIC determination, time-kill kinetics, and antibiofilm assays clearly demonstrate that the **methanolic root bark extract of** *Muntingia calabura* **possesses significant antimicrobial potential** against several clinically relevant bacterial pathogens. The strong activity observed against *Staphylococcus aureus* and other bacteria suggests that this plant could serve as a promising source of natural antimicrobial compounds. However, the resistance observed in *Salmonella typhi* indicates that the antibacterial spectrum of the extract may be selective. Further studies involving **phytochemical characterization, isolation of active compounds, and molecular mechanism studies** are necessary to better understand the antimicrobial potential of *Muntingia calabura* and its possible applications in the development of novel therapeutic agents.

5.0 CONCLUSION:

The present study demonstrated that the methanolic root bark extract of *Muntingia calabura* possesses significant antibacterial and antibiofilm activity against several clinically resistant human pathogenic bacteria. The extract exhibited clear inhibitory effects against both Gram-positive and Gram-negative bacteria, with greater susceptibility observed among Gram-positive strains. The minimum inhibitory concentration analysis confirmed the antibacterial potential of the extract, while the time-kill assay revealed time-dependent bactericidal activity against the susceptible pathogens. In addition, the extract effectively inhibited biofilm formation, suggesting its potential to interfere with bacterial adhesion and colonization processes that are often associated with persistent infections.

The findings indicate that the methanolic root bark extract of *Muntingia calabura* represents a promising natural source of antimicrobial compounds with both antibacterial and antibiofilm properties. However, further studies focusing on phytochemical characterization, isolation of active constituents, toxicity evaluation, and elucidation of the underlying mechanisms of action are required to fully explore its potential for therapeutic applications.

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CONFLICT OF INTEREST:

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

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Journal of Molecular Science
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