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ORIGINAL RESEARCH ARTICLE

Antibacterial Potential and Time-Kill Kinetics of *Calotropis procera* Extracts

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ABSTRACT

The escalating challenge of bacterial resistance to antibiotics has prompted interest in exploring the antibacterial potential of *Calotropis procera*. To investigate the antibacterial efficacy of *C. procera*, the leaves and stems of the plant were collected and macerated in ethanol to obtain crude extracts. Using GC-MS analysis, we identified key phytochemicals in the leaf and stem extracts of the plant. *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* were selected for the study. We used the Kirby Bauer disc diffusion test to evaluate bacterial susceptibility to *C. procera* extracts, while the minimum inhibitory concentration (MIC) was determined using microdilution plates. Additionally, time-kill kinetics were assessed for each bacterium. The stem extract demonstrated superior activity, achieving complete bactericidal effects against *Pseudomonas aeruginosa* and *Escherichia coli* within 20 hours. Phytochemicals such as Lupenyl Acetate and Phytol were identified as key bioactive compounds. These findings highlight the potential of *C. procera* as a natural source for developing antimicrobial agents. Statistical analysis strongly suggests that *C. procera* extracts significantly affect the test bacteria ($p = 0.001$). In conclusion, the leaf and stem extracts of *C. procera* exhibit distinct variations in the relative abundance of bioactive compounds and further demonstrate multiple antimicrobial mechanisms. These properties of the plant highlight its potential as a valuable resource in addressing antibiotic-resistant bacterial strains.

ARTICLE HISTORY

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INTRODUCTION

Calotropis procera is a widely distributed plant species with a rich history in traditional medicine and offers a viable path for studying natural chemicals with antibacterial properties. The plant is more commonly referred to as the "Apple of Sodom," it is well-known for its numerous bioactive components, some of which have been linked to therapeutic advantages, including antibacterial activities (Dogara, 2023). Traditional medicinal plants have long been a source of therapeutic compounds, offering a rich source of bioactive compounds. Among such plants is C. procera, a plant belonging to the Asclepiadaceae family; it has garnered attention for its diverse medicinal properties. The plant is indigenous to various tropical and subtropical regions and is traditionally used to treat a range of ailments, including skin diseases, fever, and inflammation. Recent scientific investigations have begun to validate these traditional uses, particularly highlighting their potential antibacterial properties (Gorlenko *et al.,* 2020).

Antibiotic resistance has emerged as a global health crisis, posing a significant challenge in treating bacterial infections. Once highly effective, conventional antibiotics are now encountering reduced efficacy due to the development of multidrug-resistant bacterial strains. This alarming trend necessitates exploring alternative strategies to effectively combat bacterial infections (Kaushik *et al.,* 2024). Using the Time-kill kinetics technique in this study provides a dynamic assessment of *C. procera* antibacterial efficacy, offering insights beyond static methods such as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Time-kill kinetics studies measure the rate at which bacteria are killed over time. The detailed temporal profile obtained from time-kill kinetics comprehensively characterizes the antibacterial properties. This includes understanding the phase-specific actions of the bacteria in response to the agent, which supports further biochemical and molecular investigations into the mode of action. (Qadir *et al.,* 2020).

Numerous studies have documented the antibacterial properties of *C. procera*. According to Amini *et al.* (2021), the latex of *C. procera* exhibited significant inhibitory

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effects against Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus* and *Escherichia coli*. Similarly, methanolic and ethanolic extracts of the plant's leaves and stems were found to possess broad-spectrum antibacterial activity (Madhavan *et al.,* 2020). These findings suggest that *C. procera* contains potent bioactive compounds that inhibit bacterial growth. Despite these encouraging findings, there is a dearth of information on the specific mechanisms underlying the antibacterial effects of *C. procera*.

The GC-MS analysis of the plant extracts will identify bioactive phytochemicals, providing insights into their potential mechanisms of action, which can be correlated with the observed antibacterial activity of the extracts. The global escalation in antibiotic resistance poses a critical threat to public health, driving the urgent need for alternative therapeutic strategies. Among these, plant-based antimicrobials have shown significant attention due to their potential to combat resistant pathogens. While *C. procera* is extensively used in traditional medicine and has demonstrated promising antibacterial activity, the specific mechanisms underlying its bactericidal effects remain poorly understood. This gap in knowledge limits the optimization and application of *C. procera* extracts as effective antibacterial agents. This study aims to address this knowledge gap by investigating the phytochemical composition of *C. procera* and evaluating its antibacterial mechanisms against antibiotic-resistant bacteria. By assessing the active compounds and their modes of action, this research seeks to contribute to developing plant-based therapeutics in the fight against antimicrobial resistance.

MATERIALS AND METHODS

Collection and Identification of P1ant samp1es

The leaves and stems of *C. procera* were collected from the North Bank area of Makurdi, located at latitude 7°43'55.87"N and longitude 8°32'20.93"E. Botanical experts from the Biology Department of the Faculty of Sciences, Benue State University, Makurdi, verified the authenticity of the plant sample by preserving a voucher specimen (voucher No. CpN-31).

Preparation of the Extracts of *Calotropis procera*

Leaves

The leaves of *C. procera* were thoroughly washed in ethanol to remove any unwanted dust or debris. They were then air-dried for 7 days in the shade at an environmental temperature range of 30-34°C during the daytime. The dried leaves were then pulverized and ground into a fine powder using a mortar and pestle. Approximately 200 grams of this crushed leaf powder were then macerated (soaked) in 2 liters of sterile distilled water for 3 days at room temperature. The mixture was periodically stirred to ensure complete extraction of the desired compounds. Finally, the extracts were filtered using sterile Whitman filter paper and stored at 4°C until they were ready to be used for further bioassay analysis **(**Naidoo *et al.,* 2023**)**

Stems

The stems of *C. procera* were washed in ethanol to remove any unwanted dust or debris. They were then air-dried for 7 days in the shade at an environmental temperature range of 30-34°C during the daytime. After drying, the stems were pulverized and ground into a fine powder. Approximately 200 grams of this crushed stem powder were then macerated (soaked) in sterile distilled water for 3 days at room temperature. The mixture was periodically stirred to ensure complete extraction of the desired compounds from the stem material. Finally, the extracts were filtered using sterile Whitman filter paper and stored at 4°C until they were ready for further bioassay analysis (Kumar *et al.,* 2022).

Analysis of *Calotropis procera* Extract by Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analysis was performed as Madhavan et al. (2020) described. A Shimadzu Gas Chromatograph using a capillary column TR-5MS (30 m × 0.25 mm) and a film thickness of 0.25 µm was used. The carrier gas was Helium, with a flow rate of 1.2 mL. The temperature was first set at 70ºC and gradually increased to 290ºC over 16 minutes. The chromatograph was connected to a Shimadzu QP2010 Ultra MS detector 70 eV.

Identification of Constituents

The majority of the compounds were identified using Gas Chromatography by comparing their retention indices to those of authentic standards available in the laboratory or were in close agreement with references. GC-MS data were analyzed using NIST and Wiley 9 libraries, and compounds were identified based on retention indices and fragmentation patterns.

Test microorganisms

Four bacterial strains were used in this study: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. These strains were isolated from clinical samples such as wound swabs, urine, and sputum using standard microbiological techniques, including culture on selective media, Gram staining, and biochemical characterization.

Antimicrobial Bio-assay Activity using Disc Diffusion Method

The antimicrobial activity of the aqueous leaf and stem extracts from *C. procera* was evaluated against the test microorganisms using the methods described by Fatema *et al.* in their 2017 publication. This established protocol was followed to assess the antimicrobial properties of the plant extracts. Whiteman filter paper discs (6 mm diameter) were sterilized at 140°C for 30 minutes in a hot air oven before impregnating them with the aqueous leaf

or stem extracts of *C. procera*. Sterile Whiteman 6 mm filter paper discs were impregnated with 20 µL of leaf extract and dried at 50°C in an oven. The same procedure was carried out with the stem extract of *C. procera*. A sterile inoculating loop was used to pick three colonies from a 24-hour culture of each test bacterium and suspend them in a tube containing sterile distilled water. The turbidity of each tube was adjusted to 0.5 McFarland solution, corresponding to approximately $1.5x10^5$ CFU/mL. The adjusted bacterial suspension was utilized within 15 minutes of preparation. The nutrient agar plates were inoculated with the test microorganisms using a swab. The organisms were streaked across the surface of each plate in a back-and-forth motion, from one edge to the other. This repetitive streaking process was carried out to ensure that the inoculum was evenly distributed across the entire nutrient agar surface of the plate.

Application of impregnated discs

Applying impregnated filter paper discs on nutrient agar plates, sterile forceps were used. The discs were gently pressed to ensure uniform contact with the agar surface. Furthermore, each test plate contained three discs, which were placed equidistant to each other to avoid overlapping the inhibition zone. The plates were inverted and incubated for 24 hours at 37°C. The diameters of the inhibition zones were measured to assess antibacterial activity, with measurements recorded to the nearest whole millimeter. Each test was conducted thrice for reliability, and the average diameter of the inhibition zones for each extract and antibiotic was calculated. Ciprofloxacin discs served as the positive control.

Minimal Inhibitory Concentrations of the Extracts of *Calotropis* procera

The Minimum Inhibitory Concentration (MIC) of the *C. procera* extracts was determined using the microdilution plate method, as described in the 2018 publication by Stefanović. A 96-well microtiter plate was prepared by adding 100 µL of Mueller-Hinton broth (MHB) to each well. Subsequently, 100 µL of the *C. procera* leaf extract stock solution was added to the first row of the plate. The extract was then serially diluted two-fold in the MHB using a multi-channel pipette, creating a concentration gradient across the plate. Finally, 10 µL of a 24-hour bacterial culture, standardized to 1.5×10^8 CFU/mL and then diluted to 1×10^6 CFU/ml, was added to the appropriate wells. The inoculated microtiter plates were incubated for 24 hours at 37°C. The highest dilution of the leaf extract of *C. procera* that inhibited bacterial growth compared to the control tube but did not kill the organism was defined as MIC of the extract (Geraldo *et al.,* 2021).

Time kill kinetics of the extract of *Calotropis procera*

The rate of bacterial killing by the *C. procera* extracts was determined using a time-kill kinetics approach, following the method outlined by Asoso *et al.* (2018). Each test bacterium's 24-hour bacterial culture was diluted to 5×10^5 CFU/mL in freshly prepared nutrient broth. Subsequently, 1 ml of a solution containing double the previously established MIC of the *C. procera* leaf extract was introduced to the bacterial suspension in each test tube. The broth cultures were then incubated at 37°C for 24 hours. At various time intervals (0 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 20 h, 22 h, and 24 h), samples were taken from each culture and plated on a nutrient agar plate. Following an incubation period of 24 hours at 37°C, viable colonies on the plate were counted. The bacterial population was quantified regarding CFU/mL and graphed against time for each bacterial isolate.

Data Analysis

Each experiment was replicated three times. Results of the disc diffusion method of antibacterial susceptibility to the extracts of *C. procera* were expressed as means, and differences between means were statistically analyzed using one-way analysis of variance. Differences are considered significant when $p \leq 0.05$.

RESULTS

GC-MS analyses of the extracts of *C. procera* identified the presence of 23 phytochemicals in the stem and 25 phytochemicals in the leaves of *C. procera,* with various levels of relative abundance of each compound between the leaf and stem of *C. procera.*

The Kirby Bauer Disc Diffusion Test revealed the zone of inhibition produced by *C. procera* extracts against *E. coli, P. aeruginosa, S. aureus*, and *B. subtilis*. The results indicated moderate to strong antimicrobial activity, with variations observed across bacterial strains.

The stem extract exhibited lower MIC values against *E. coli* and *P. aeruginosa* than the leaf extract, suggesting higher potency against these Gram-negative bacteria. However, the leaf extract showed lower MIC values against *S. aureus* and *B. subtilis*, indicating stronger activity against these Gram-positive bacteria.

The rate of bactericidal activity of the stem extract was faster than the leaf extract, reaching 0 CFU/ml by 12 hours compared to 22 hours for the leaf extract. The leaf and stem extracts showed rapid kill rates against *P. aeruginosa*, with complete bactericidal activity observed within the first 12 hours, indicating their potent bactericidal activity against this bacterial strain. Notable differences in the rate of bactericidal activity against bacterial strains were observed, with the stem extract

generally exhibiting faster bactericidal activity sooner, particularly against *E.coli* and *S. aureus*.

The ANOVA results yielded a p-value of less than 0.001, highlighting the statistical significance of the observed differences in the zone of inhibition. This suggests that the variances in the zone of inhibition are attributable to the antibacterial activity of *C. procera* extracts rather than random variability in the data.

As shown in Figure 3, at a concentration of 100mg/mL, the stem extract of *C. procera* produced larger zones of inhibition against each bacteria compared to the leaf extract of *C. procera* at the same concentration, indicating that the test bacteria are more susceptible to the stem extract of *C. procera* ($P < 0.005$).

Figure 4 presents the minimal inhibitory concentrations (MICs) of *C. procera* extracts against the test bacteria, the stem extract of *C. procera* produced lower MIC values when compared to the leaf extract of *C. procera*. Lower MIC values indicate higher potency of the stem extract.

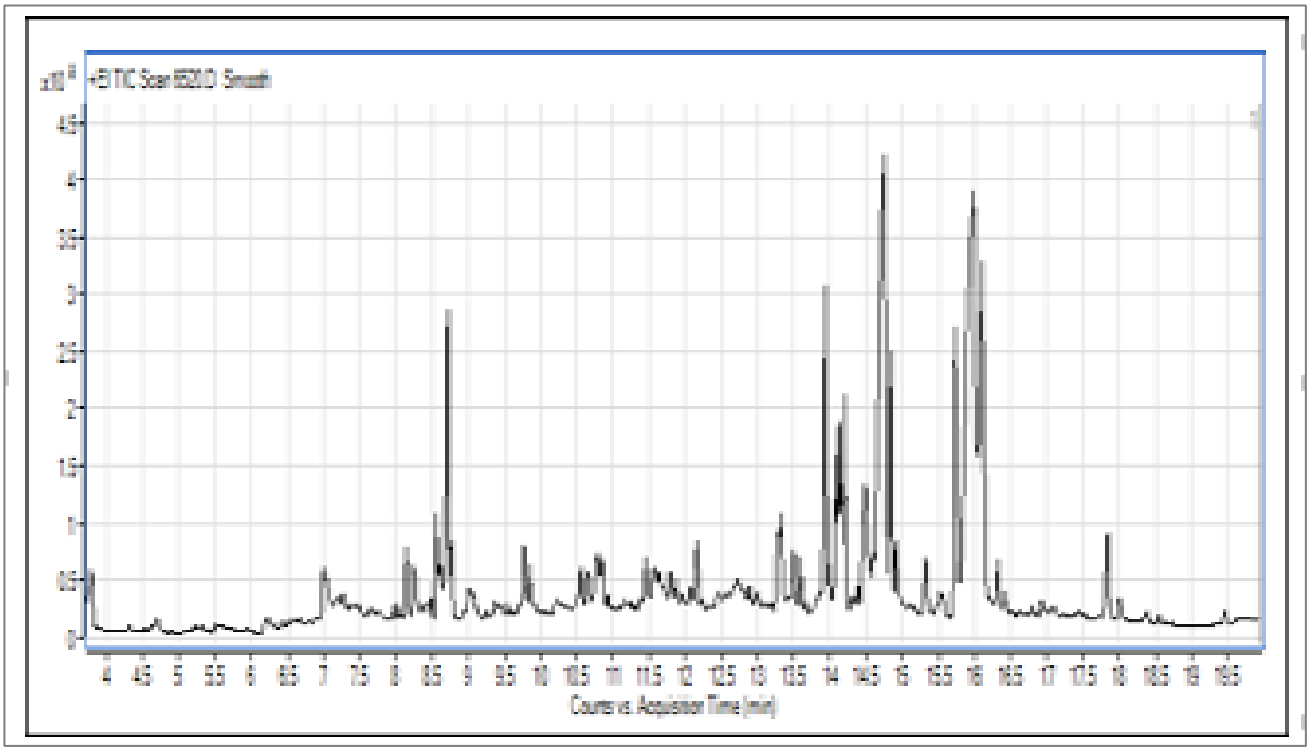

Figure 1: Chromatogram (GC-MS) of Ethanolic Leaf Extract ETCPL

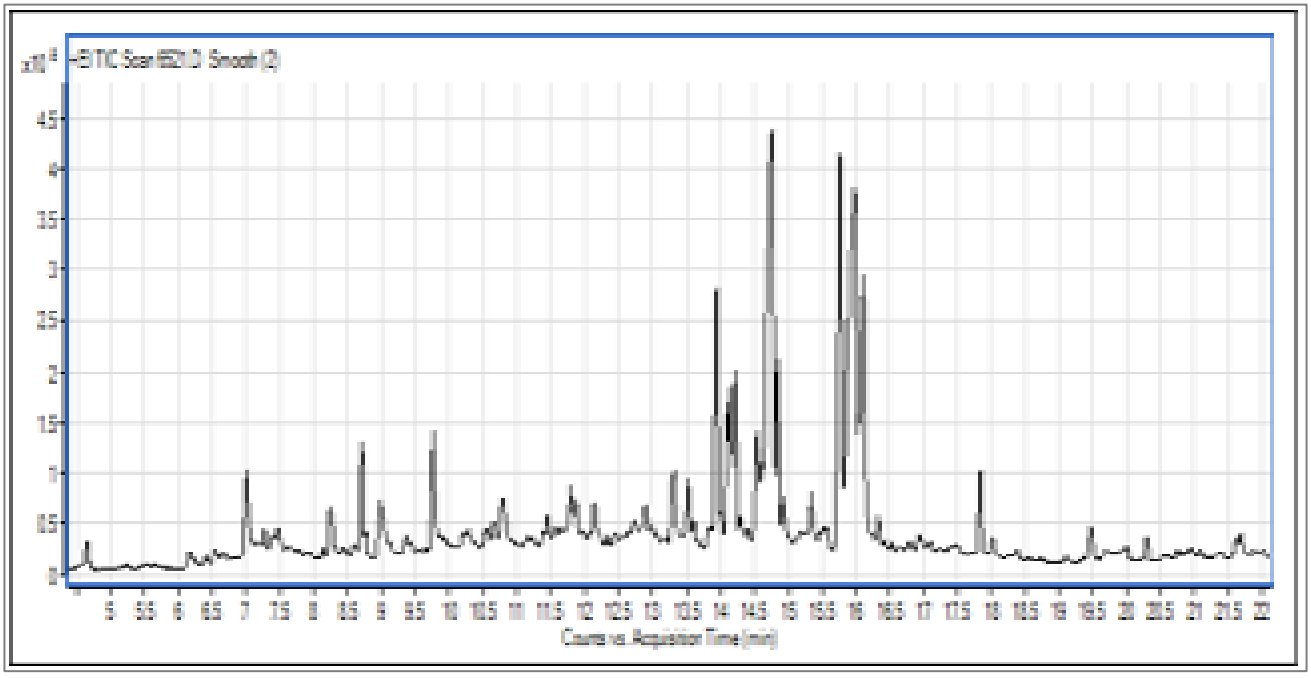

Figure 2: Chromatogram (GC-MS) Of Ethanolic Stem Extract ETCPS

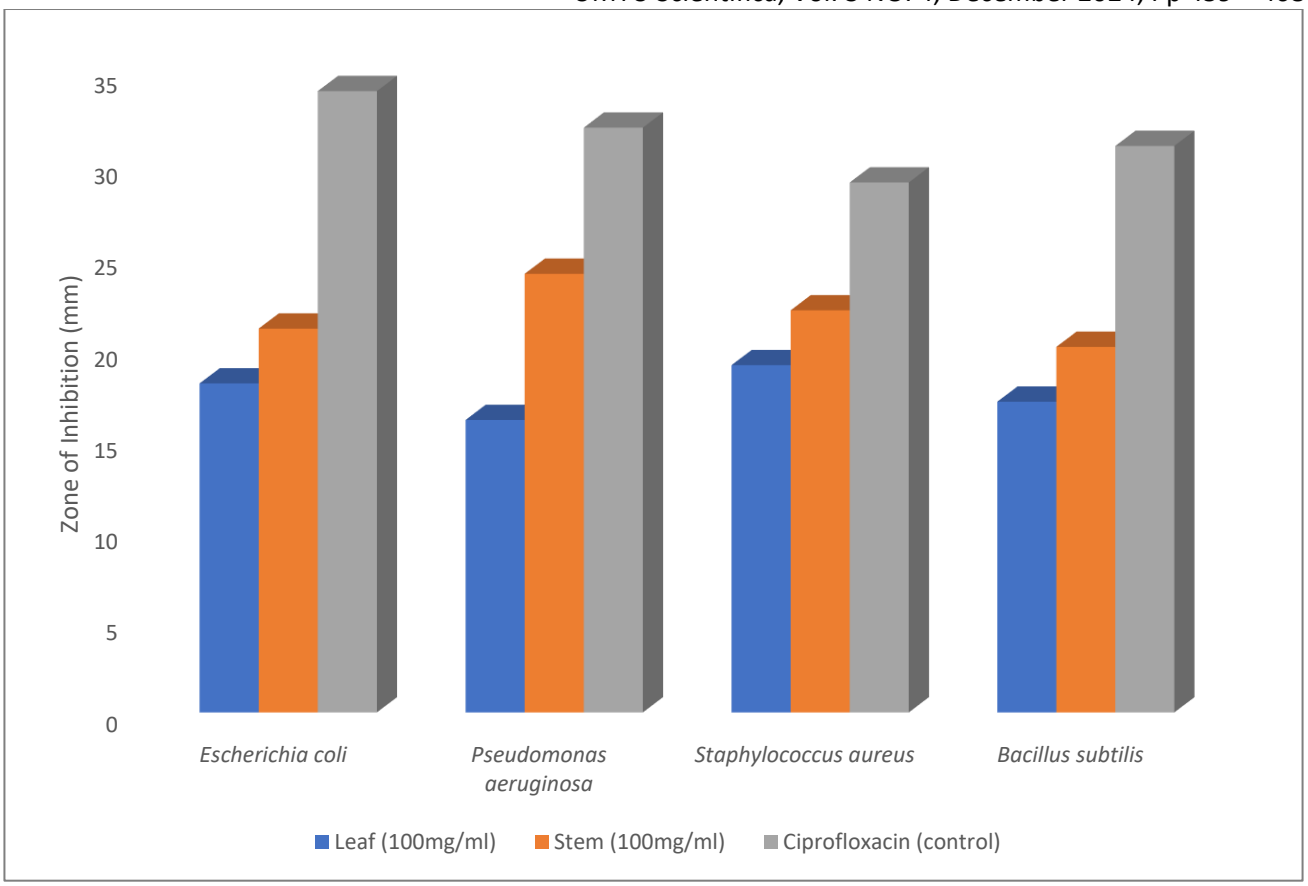

Figure 3: Antimicrobial susceptibility Test of *C. procera* Extract (Kirby Bauer Disc Diffusion Test)

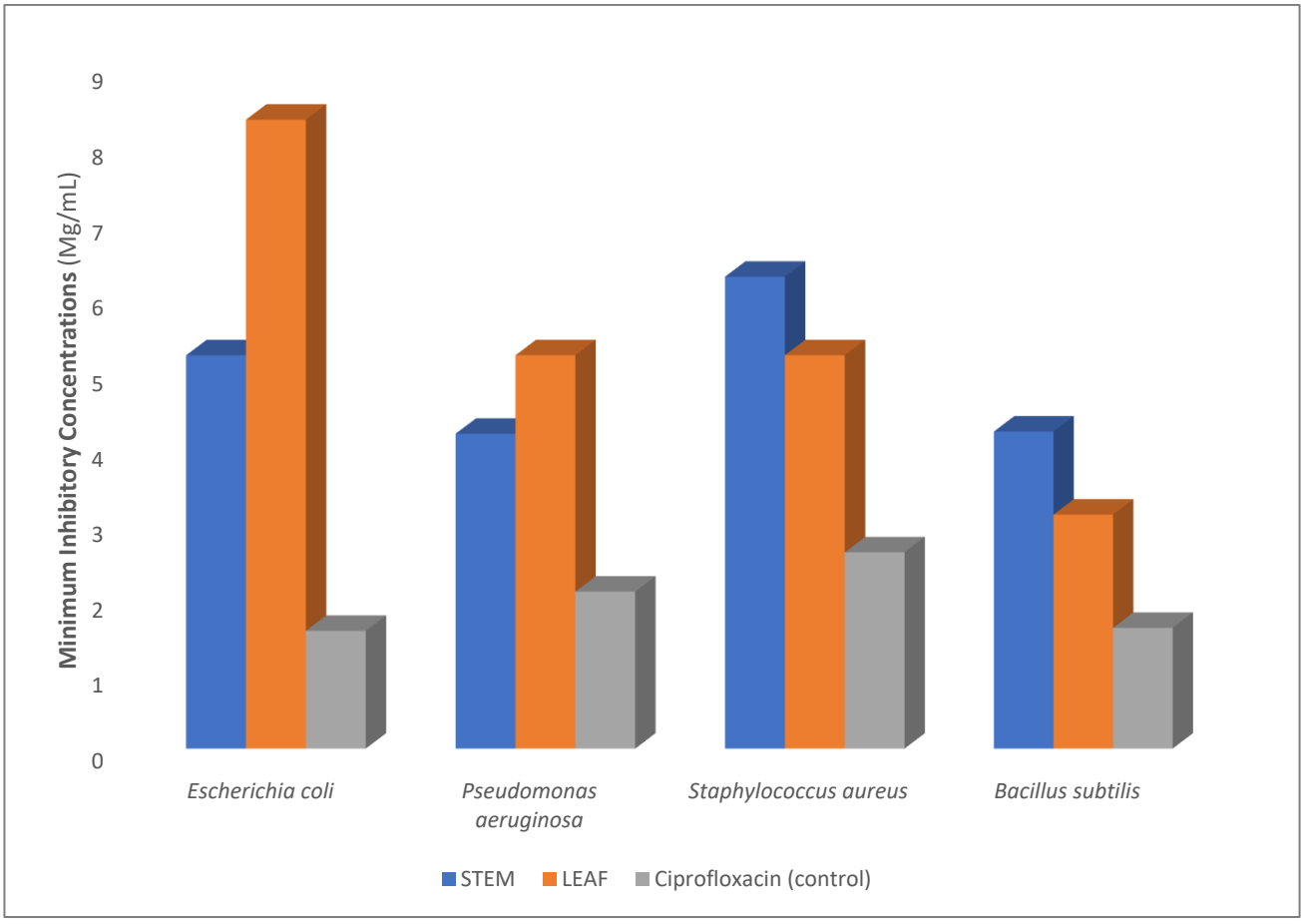

Figure 4: Minimal Inhibitory Concentrations of the Extracts of *C. procera*

DISCUSSION

The GC-MS analysis of the extracts of *C.procera* shows the presence of bioactive compounds such as Ergost-5-en-3-ol (3.beta.,24r), Lupan-3-ol acetate, Lupenyl Acetate, and Phytol. These findings align with previous studies; studies such as those of Naidoo *et al.* (2023) revealed the potent antibacterial activity of Lupan-3-ol acetate, while Saha and Bandyopadhyay (2020) and Tripathi *et al.* (2024) highlighted the roles of Phytol and Lupenyl Acetate in combating bacterial infections. Similarly, Francis *et al.* (2021) highlighted the antimicrobial properties of Ergost-5-en-3-ol. Recent studies have also shed light on these compounds' potential mechanisms of action, providing a framework to interpret their roles in the observed antimicrobial effects in this study. The relative abundance of these bioactive compounds was found to contrast between the leaf and stem extracts. It was observed that phytol, which induces oxidative stress through increased ROS production (Elkahoui *et al.,* 2024; Tripathi *et al.,* 2024), was more abundant in the stem extract. This aligns with the enhanced activity of the stem extract against *P. aeruginosa* (zone of inhibition: 24 mm), a bacterium known to be susceptible to ROS-mediated damage. A strong correlation ($r = 0.85$, $p < 0.01$) was observed between phytol abundance and antibacterial activity against *P. aeruginosa*, suggesting its significant role in disrupting bacterial cellular processes. Lupenyl acetate, present in leaf and stem extracts, is known for disrupting bacterial cell integrity (Musa *et al.*, 2024). The enhanced activity of the stem extract against *B. subtilis* (zone of inhibition: 20 mm) may be partially attributed to the higher relative abundance of this compound, supporting its role in compromising bacterial cell walls. Ergost-5-en-3-ol (3β,24R), a sterol compound, alters bacterial membrane properties (National Center for Biotechnology Information 2025, Francis *et al.* 2021). Its presence in significant amounts in both extracts correlates with the broad-spectrum activity observed, particularly against *S. aureus* (zone of inhibition: 22 mm for the stem extract and 19 mm for the leaf extract). These results highlight the compound's potential to destabilize bacterial membranes, leading to cell death. Lupan-3-ol acetate, a triterpenoid compound, modulates bacterial membrane permeability and enzyme activity (Javed *et al.,* 2021). This compound's presence supports the broad-spectrum efficacy of the extracts, particularly against *E. coli* (zone of inhibition: 21 mm for the stem extract and 18 mm for the leaf extract). The differential activity of the leaf and stem extracts can be attributed to variations in the relative abundance of these bioactive compounds. The stem extract exhibited superior antibacterial activity against most tested bacteria, likely due to its higher concentration of phytol and other potent compounds. The mechanisms by which these compounds act, including cell membrane disruption, oxidative stress induction, and enzyme inhibition, highlight their potential as multi-target antibacterial agents.

Table 1: GC-MS Analysis of the Leaf and Stem extract of *Calotropis procera*

S.No.	Name	R.Time	Area% (Leaf)	Area% (Stem)
1	Methyl 7,11,14-eicosatrienoate	7.04	1.15	1.18
2	Pthalic acid , isobutyl ester	9.22	1.2	1.24
3	Stigmasterol	9.24	1.25	1.22
4	Butyl octyl Phtalate	9.71	1.37	1.37
5	Lupan-3-ol, acetate	10.45	1.47	1.48
6	9,12,15-Octadecatrienoic acid, (Z,Z,Z)	10.51	1.54	1.54
7	Ergost-5-en-3-ol, (3.beta.,24r)	10.55	1.9	1.9
8	Hexadecanoic Acid, Ethyl Ester	11.91	1.9	1.9
9	Squalene	11.93	2.2	2.1
10	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	12.12	2.3	2.3
11	Stigmast-5-En-3-Ol, (3.Beta.)	12.34	3.45	2.42
12	Dibutyl phthalate	12.77	2.67	2.47
13	Norolean-12-Ene	13.39	2.49	2.48
14	Lupenyl Acetate	14.23	1.45	2.82
15	Hexadecanoic Acid, Methyl Ester	14.2	3.23	2.87
16	9,12,15-Octadecatrienoic acid, methyl ester,	14.73	6.45	5.72
17	LUP-20(29)-EN-3-YL ACETATE	15.52	4.55	5.73
18	Methyl Commate	15.65	8.72	7.71
19	n-Hexadecanoic acid	15.71	5.62	7.71
20	Phytol	16.1	6.76	9.77
21	Mono(2-ethylhexyl) phthalate	16.33	0.52	0.65
22	1,4,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a, 2,6,10-trimethyl,14-ethylene-14- pentadecne	17.4	1.53	1.57
23	1,2-Benzenedicarboxylic acid, bis(2- methylpropyl) ester	18.63	1.25	1.25
24	1,8-Dioxa-5-thiaoctane,8-(9-borabicyclo[3.3.1]non-9-yl)-3-(9-borabicyclo[3.3.1]non-9-yloxy)-1-phenyl	19.55	0.51	—
25	Isochiapin B	19.78	0.72	—

R= retention

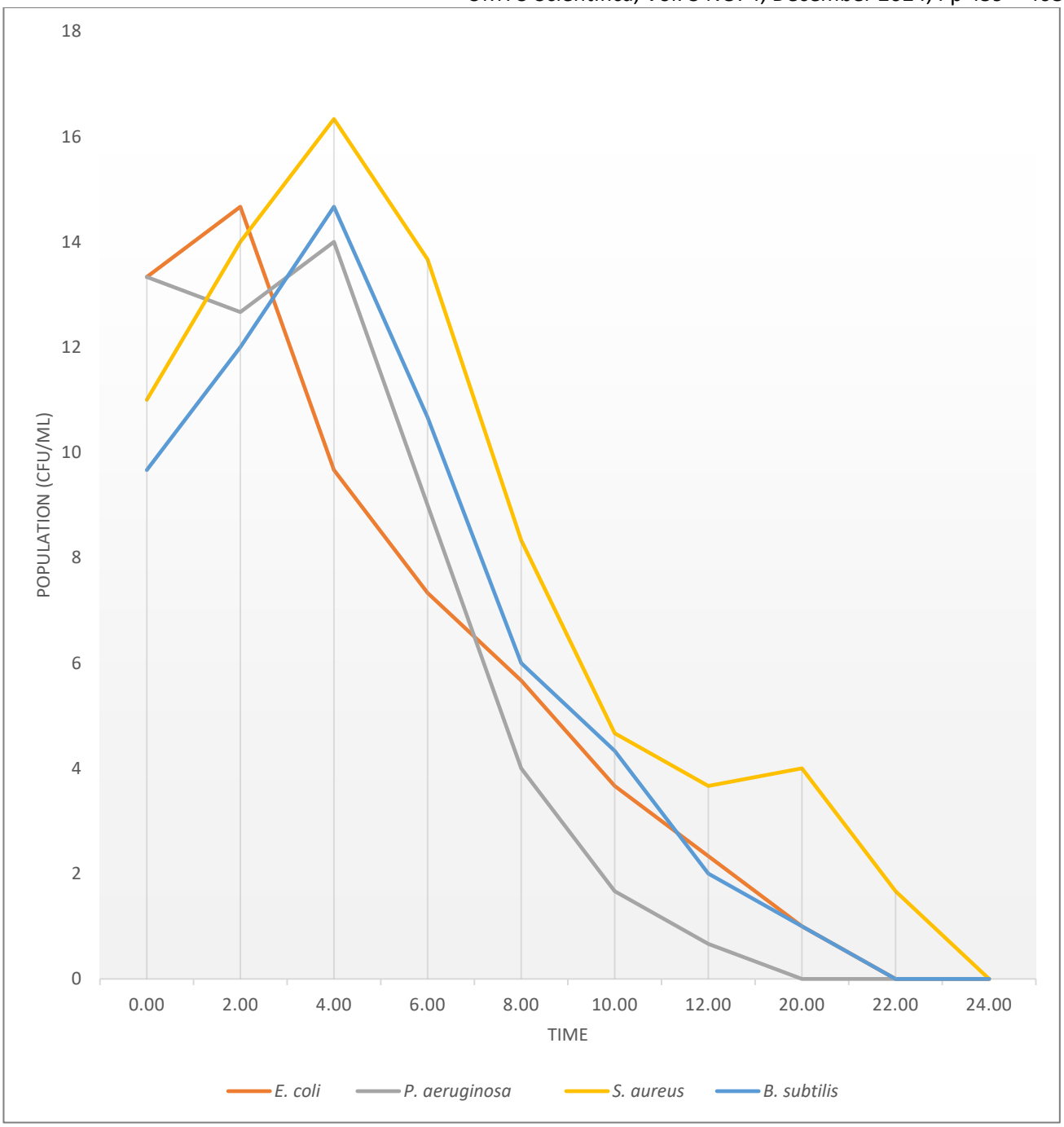

Figure 5: The rate of kill of the leaf extract of *C. procera, P. aeruginosa* that *were* completely eliminated at 20hrs, *E. coli* and *B. subtilis* that were eliminated at 22hrs and *S. aureus* that were eliminated at 24hrs

In the face of escalating antibiotic resistance, these findings highlight the potential of *C. procera* extracts as sources of novel antibacterial agents. The multi-mechanistic actions of lupenyl acetate, ergost-5-en-3-ol, lupan-3-ol acetate, and phytol reduce the likelihood of resistance development, as bacteria would need to undergo multiple simultaneous mutations to evade these effects. Combining these bioactive compounds with synthetic antibiotics presents an intriguing avenue for enhancing antibacterial efficacy. For instance, the oxidative stress-inducing properties of phytol could potentiate the effects of ROS-generating antibiotics, while membrane-disrupting compounds like lupenyl acetate and lupan-3-ol acetate could enhance antibiotic uptake. However, further studies are needed to optimize such combinations, evaluate dosing strategies, and ensure safety in vivo.

This study's findings significantly affect the global fight against antibiotic resistance. The broad-spectrum antibacterial activity exhibited by the extracts of *C. procera*, evident in the zone of inhibition, MIC, and time-kill kinetics studies, suggests their potential as alternative or adjunct therapies. For example, the leaf extract demonstrated substantial inhibition zones against *E. coli* and *S. aureus*, representing Gram-negative and Gram-positive bacteria, respectively. Meanwhile, the stem extract

showed enhanced potency against *P. aeruginosa*, a notoriously antibiotic-resistant pathogen.

These findings align with the need to develop antimicrobial agents that target resistant bacterial strains. The identified bioactive compounds, with their multiple mechanisms of action, such as membrane disruption, biofilm inhibition, oxidative stress induction, and enzyme inhibition, reduce the likelihood of bacteria developing resistance. This multidimensional approach is a vital strategy in overcoming the limitations of single-target antibiotics.

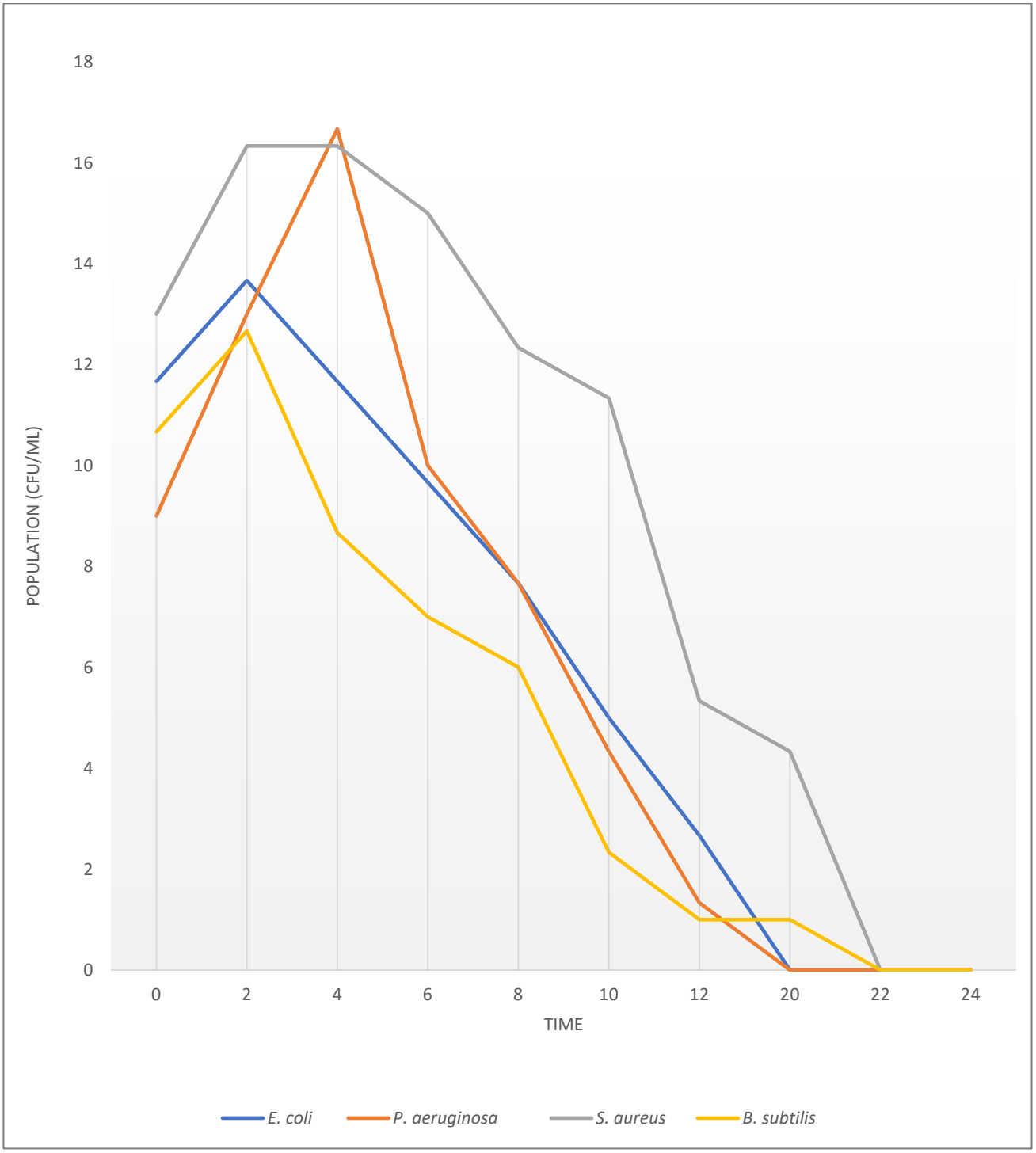

Figure 6: The rate of kill of the stem extract of *C. procera*, *P. aeruginosa* and *E. coli* that were completely eliminated at 20hrs and *B. subtilis* and *S. aureus* that were eliminated at 22hrs.

The potential for combining bioactive compounds from *C. procera* with conventional antibiotics is an exciting avenue for research. For instance, combining Lupenyl Acetate and Ergost-5-en-3-ol with antibiotics like ciprofloxacin may enhance antimicrobial efficacy and mitigate resistance development. Such combinations could act synergistically, disrupting bacterial processes more effectively than either agent alone. However, further studies are essential to optimize dosing, investigate interactions, and evaluate safety in vivo.

The current findings partially align with those of Bilal et al. (2020), who reported inhibitory effects of *C. procera* leaf extracts against *Proteus mirabilis*, *P. aeruginosa*, and *B. cereus*. In contrast, this study observed significant bactericidal activity against *E. coli* at higher extract concentrations (100 mg/ml), highlighting the concentration-dependent nature of the antibacterial effects. Additionally, the study aligns with those of Ahmad *et al.,* 2023, who demonstrated that the methanolic extract of *C. procera* exhibited significant antimicrobial activity, with inhibition zones ranging from 9.5 to 22.5 mm against tested microorganisms.

Identifying broad-spectrum bioactive compounds in *C. procera* has far-reaching implications for developing next-generation antimicrobials. The multiple mechanisms of action associated with these compounds make them particularly valuable in reducing resistance development. Furthermore, their broad-spectrum activity suggests potential applications in treating infections caused by multi-drug-resistant pathogens.

However, challenges remain. Variability in extract efficacy, issues with standardization, and the need for clinical validation must be addressed. Future research should focus on isolating individual bioactive compounds, elucidating their specific mechanisms of action, and assessing their safety and efficacy in vivo. Additionally, exploring synergistic effects with synthetic antibiotics could pave the way for novel combination therapies that enhance treatment outcomes and prolong the effectiveness of existing antibiotics.

CONCLUSION

The extracts of *C. procera* demonstrate significant antibacterial activity, particularly against *P. aeruginosa* and *E. coli*. Future studies should focus on isolating and characterizing the bioactive compounds, exploring their mechanisms of action, and assessing their safety and efficacy in animal models. The bioactive compounds identified in *C. procera* extracts exhibit significant antibacterial potential through diverse mechanisms of action. Their correlation with observed antibacterial activity highlights their individual and synergistic contributions to the efficacy of the extracts. This study provides a foundation for further exploration of these compounds as alternatives or adjuncts to conventional antibiotics in combating antibiotic-resistant bacteria.

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