

Research Article

Phytochemical-Antibiotic Synergy: Exploring the Antibacterial Potential of *Calotropis procera* in Combination Therapy

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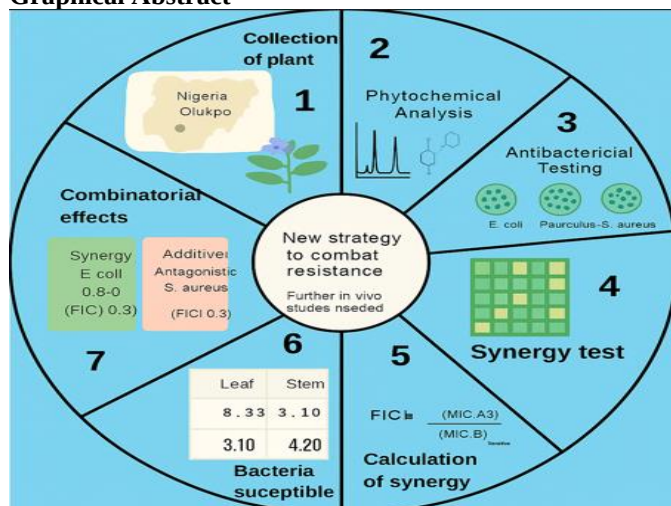


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Abstract— The rising problem of bacterial resistance necessitates alternative strategies to enhance antibiotic efficacy. This study examines the combined antimicrobial efficacy of the extracts of *Calotropis procera* and ciprofloxacin against specific bacterial pathogens. Crude extracts were prepared from the leaves and stems of *C. procera* collected in Makurdi, Nigeria. Using GC-MS analysis, we identified notable phytochemicals in the extracts from the plant's leaves and stems. The minimum inhibitory concentrations (MICs) for *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* were measured using a microdilution method. The checkerboard assay was utilized to evaluate the synergistic effect of *C. procera* extracts when combined with ciprofloxacin. The Fractional Inhibitory Concentration Index (FICI) was calculated using the formula: $FICI = (MIC \text{ of extract in combination} / MIC \text{ of extract alone}) + (MIC \text{ of ciprofloxacin in combination} / MIC \text{ of ciprofloxacin alone})$. The leaf extract showed an MIC of 3.10 µg/ml for *B. subtilis* and 8.33 µg/ml for *E. coli*, whereas the stem extract had an MIC of 4.20 µg/ml for *B. subtilis*. When combined with ciprofloxacin, the FICI values indicated synergistic interactions against *E. coli* (FICI = 0.4 for leaf and 0.3 for stem extract) and against *S. aureus* (FICI = 0.3 for the stem extract). In contrast, the interactions against *P. aeruginosa* and *B. subtilis* ranged from additive to antagonistic. The findings indicate that extracts from *C. procera* significantly enhance the antibacterial efficacy of ciprofloxacin, which may enable reduced doses of the antibiotic and offer a new approach to address antibiotic resistance. Nonetheless, additional in vivo research is necessary to confirm these promising results and to clarify the mechanisms involved in their action.

Keywords— *Calotropis procera*, synergy, antimicrobial, antibacterial, antibiotic, phytochemical

Graphical Abstract-



- **Research on plant-based antibacterial properties:** *Calotropis procera* was examined for its phytochemical content, and evaluated for antibiotic activity against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* as shown in 1,2 and 3
- **Evaluation of synergy:** The combinatorial effect of *C. procera* extract in conjunction with antibiotics, was assessed using the Fractional Inhibitory Concentration Index (FICI) as shown 4, 5 and 6
- **Potential in addressing resistance:** The findings indicated synergistic, additive, and antagonistic interactions, showing the plant's potential as a component of innovative approaches to tackle antimicrobial resistance as shown in 7

1. Introduction

The increasing incidence of antibiotic-resistant bacteria presents a significant challenge to global health, compromising the efficacy of standard antimicrobial treatments and jeopardizing the progress made in medicine over the past several decades. The inappropriate use and excessive prescription of antibiotics have quickened the development of resistant strains, leading to a decrease in the effectiveness of even our strongest medications [1]. In light of this crisis, scientists have started to explore traditional medicinal plants as sources of bioactive compounds that could potentially restore or enhance the activity of antibiotics. One such plant, *Calotropis procera*, a perennial shrub commonly found in tropical and subtropical regions, has a long history of use in ethnomedicine due to its diverse pharmacological benefits, including substantial antimicrobial properties [2]. Phytochemical studies of *C. procera* have revealed a diverse array of secondary metabolites, like alkaloids, flavonoids, tannins, and terpenoids, all known for various antimicrobial, anti-inflammatory, and antioxidant effects [3].

Although the antibacterial capabilities of *C. procera* extracts against specific pathogens have been documented, there is limited information regarding the interactions between these phytochemicals and standard antibiotics that may produce synergistic effects. Initial findings indicate that combining plant extracts with antibiotics can enhance bacterial inhibition and enable lower antibiotic dosages, thus reducing side effects and slowing the development of resistance [2, 3]. Nonetheless, significant knowledge gaps exist: particularly, there is a dearth of information on the systematic investigation into how leaf and stem extracts of *C. procera* differ in their ability to enhance the effectiveness of ciprofloxacin, a commonly used fluoroquinolone antibiotic, against important clinical bacteria.

1.1 Objective of the Study

This study aims to explore the synergistic properties of *C. procera* extracts when used with ciprofloxacin against four key bacterial pathogens: *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*.

1.2 Specific Objective of the Study

Specifically, we;

1. Performed GC-MS analysis of the plant extracts (leaf and stem),
2. Evaluated the combined effectiveness of leaf and stem extracts in improving antibiotic activity,
3. Identified the fractional inhibitory concentrations of the antibiotics and plant extract in order to assess their synergistic effect.

By pursuing these objectives, our research intends to promote the idea of combining phytochemicals and antibiotics as a feasible strategy to revitalize existing antibiotics, ultimately leading to more effective, cost-efficient, and sustainable methods for addressing multidrug-resistant infections.

2. Related Work

The growing issue of antibiotic resistance has prompted researchers to investigate creative methods for enhancing the efficacy of existing antimicrobial medications. One significant strategy is the combination of plant-derived phytochemicals with traditional antibiotics, which has shown significant potential. Phytochemicals are secondary metabolites produced by plants and include an extensive variety of compounds like alkaloids, flavonoids, tannins, terpenoids, phenolic acids, and glycosides [4]. Their ability to inhibit or kill bacteria cells from different mechanisms, such as disrupting cell wall integrity, blocking nucleic acid synthesis, interfering with energy metabolism, and modulating efflux pumps [5]. By utilizing these diverse mechanisms along with antibiotic effects, combination therapies seek to produce synergistic outcomes that can lower minimum inhibitory concentrations (MICs), postpone the development of resistance, and lessen the side effects resulting from high-dose antibiotic use.

Initial research into the synergy between plants and antibiotics established a conceptual framework by systematically assessing fractional inhibitory concentration indices (FICIs). The FICI serves as the most prevalent quantitative measure for assessing the interactions, whether synergistic, additive, indifferent, or antagonistic, between two antimicrobial substances used together [6]. This index is calculated from checkerboard dilution tests where different concentrations of agent A (such as an antibiotic) and agent B (like a plant extract or another antibiotic) are assessed in every possible combination against a target microorganism. The possible interaction of the combination of antimicrobials includes: synergism ($FICI \leq 0.5$), additivity ($0.5 < FICI \leq 1$), indifference ($1 < FICI \leq 4$), and antagonism ($FICI > 4$) [2,7]. According to [8], bacterial isolates that were treated with combinations at the fractional inhibitory concentration index (FICI) exhibited noticeably decreased bacterial growth and protein content (5-62%) compared to those treated solely with extracts or cefixime. These results paved the way for future studies aimed at broadening the application of this concept to various phytochemical groups and antibiotic types. Plant-based compounds can exhibit either direct antibacterial effects or indirect antibacterial effects by serving as agents that modify antibiotic resistance, thereby enhancing the effectiveness of antibiotics when utilized in combination [9]. Conducting a comprehensive evaluation of bioactive chemicals derived from plants as agents that modify resistance, including those that can work cooperatively with antibiotics, is a promising strategy to combat bacterial resistance.

Further mechanistic analyses indicated that the polyphenolic compounds, such as lawsone and ellagitannins, functioned to inhibit DNA gyrase and disrupt efflux pump activity, thereby reducing the concentration of antibiotics necessary to achieve bactericidal effects when they are used in concert.[8]. Additionally, time-kill assays demonstrated that combination treatments resulted in a ≥ 3 -log₁₀ decrease in bacterial counts within four hours, in contrast to eight to twelve hours

required for antibiotics alone, underscoring the potential for rapid elimination of bacteria [2].

According to [7] The supplementary application of biofilm-modulating plant compounds like zingerone can change the typically resistant structure of *Pseudomonas aeruginosa* biofilms into a more accessible form, thus enhancing and even increasing the effectiveness of traditional antibiotics such as ciprofloxacin. Essentially, addressing biofilm stability with natural substances offers a promising approach to battling antibiotic penetration obstacles and reducing resistant infections linked to biofilms. In a combined treatment approach, every bioactive compound contributes to a synergistic or additive effect, enhancing therapeutic effectiveness while lowering dosage and toxicity. Furthermore, one of the benefits of synergism in combination therapy is the reduction or postponement of the development of resistance by the pathogenic organism towards the individual elements included in the combination [6].

Although these investigations highlight the extensive potential of synergy between phytochemicals and antibiotics, there is still a pressing need to assess particular plant species that have not been thoroughly studied but possess a wealth of traditional uses and unique phytochemical characteristics. One such candidate is *Calotropis procera*, popularly referred to as the "apple of Sodom." Found throughout tropical Africa, Asia, and the Arabian Peninsula, *C. procera* has been utilized in traditional medicine for a long time, notably for addressing skin infections, wounds, and gastrointestinal ailments [4]. Phytochemical evaluations indicate that both the leaves and stems contain a variety of compounds, including cardenolides (such as calotropin and uscharin), flavonoids (like rutin and quercetin derivatives), tannins, alkaloids (notably calotropine), and terpenoids (including lupeol) all of which contribute to antibacterial, antifungal, and anti-inflammatory properties [5].

Detailed analyses of *C. procera* leaf extract show considerable amounts of tannins, flavonoids, alkaloids, and terpenoids through qualitative screening and chromatographic techniques [10]. In-vitro studies on antimicrobial activity indicated a dose-dependent inhibition of *S. aureus* and *E. coli*, with inhibition zones reaching up to 19.8 mm and MIC values between 25 and 100 mg/mL [2]. These results implied that phenolic compounds, especially tannins and flavonoids, could damage bacterial cell walls and chelate vital metal ions, whereas alkaloids might disrupt nucleic acid synthesis [11]. However, the comparatively high MICs highlighted the necessity for combination strategies to enhance these effects for practical clinical use [12].

The use of multiple categories of drug components is a common practice in medicine. A specific approach does not always enhance its pharmacological effect; combinations with two or more drugs can result in additive, synergistic, or antagonistic outcomes. Evidence indicates that if a drug combination demonstrates superior pharmacological effects compared to monotherapy in clinical studies, it can be inferred that it exhibits a synergistic effect [6].

In summary, these studies reveal three key points. Firstly, plant extracts and their phytochemicals often possess inherent antibacterial properties through various mechanisms, but they typically require high concentrations to achieve therapeutically relevant outcomes when used individually. Secondly, combining these extracts with antibiotics can significantly reduce the effective doses of both the extract and the medication, facilitating faster bacterial elimination while alleviating toxicity concerns. Lastly, although many plant species and antibiotic classes have demonstrated synergy, *C. procera* has not been sufficiently studied in this regard, despite its significant phytochemical composition and traditional medicinal importance.

In light of this context, the current study aims to address the existing gaps in knowledge by methodically assessing the synergistic effects of *C. procera* leaf and stem extracts in conjunction with ciprofloxacin. Through standardized checkerboard assays, time-kill kinetics, and FICI assessment, we will measure the levels of interaction among four pathogens of clinical relevance: *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*. This research intends to evaluate both leaf and stem extracts, identify crucial phytochemical fractions, and link their composition with the observed synergistic effects, thereby validating *C. procera* as an antibiotic enhancer and providing mechanistic insights that could guide the informed development of phytochemical-antibiotic therapies. Ultimately, these results may lead to the creation of innovative, cost-effective, and sustainable strategies to tackle the growing global challenge of antibiotic resistance.

3. Experimental Procedure

Calotropis procera plant parts were collected from the North Bank area of Makurdi close to the Benue River at coordinates approximately 7°44'N, 8°30'E. Experts in botany from the Department of Biological Sciences at Benue State University in Makurdi confirmed the plant's identification by keeping a voucher specimen (voucher No. CpN-37).

3.1. Extraction Process for *Calotropis Procera* (Leaves and Stems)

To get rid of any extra dust or debris, *C. procera* leaves and stems were carefully washed with distilled water respectively. And were further allowed to air dry for seven days in the shade, with daily temperatures between 30 and 34°C. After that, the dried leaves and stem were crushed and powdered respectively using a mortar and pestle to a fine powder. 200 grams of this crushed leaf and powder were macerated, in two liters of sterile ethanol, for three days at room temperature, to guarantee full extraction of the required phytochemicals, the mixture was agitated frequently. The extracts were then kept at 4°C until they were prepared for use in further bioassay analysis after being filtered through sterile Whitman filter paper [2].

3.2 Gas Chromatography–Mass Spectrometric Characterization of *Calotropis procera* Extract

The GC-MS analysis was conducted following the protocol of [2]. A Shimadzu Gas Chromatograph equipped with a TR-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness) was utilized, with helium as the carrier gas at a flow rate of 1.2 mL/min. The oven temperature was initially set at 70°C and ramped to 290°C over 16 minutes. The system was interfaced with a Shimadzu QP2010 Ultra MS detector operating at 70 eV.

3.3. Determination of Chemical Components

Most compounds were identified via Gas Chromatography by comparing their retention indices with those of authenticated laboratory standards or reference values. The GC-MS data were subsequently analyzed using the NIST and Wiley 9 libraries, with compound identification based on both retention indices and fragmentation patterns [2].

3.4. Test Bacteria

Four bacterial species were selected from Benue State University's Microbiology Laboratory in Makurdi: *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus*. In accordance with CLSI rules, confirmatory identification was carried out. Cellular morphology was established by Gram staining; *P. aeruginosa* was identified as a Gram-negative rod based on its motility, oxidase positivity, and characteristic pigment on cetrimide agar; *B. subtilis* was confirmed as a Gram-positive rod with endospore formation, positive catalase, and motility; *S. aureus* was identified as a Gram-positive cocci through its yellow colonies on Mannitol Salt Agar, as well as positive results in both catalase and coagulase tests; meanwhile, *E. coli* was recognized as a Gram-negative rod due to its lactose fermentation on MacConkey agar, along with positive indole and methyl red tests, and negative results in both Voges-Proskauer and citrate tests [2].

3.5. Minimal Inhibitory Concentrations of *Calotropis procera* Extracts

The Minimum Inhibitory Concentration (MIC) of *C. procera* extract was assessed using the microdilution plate technique as outlined by Andrew et al. (2024). A 96-well microtiter plate was set up by adding 100 µl of Mueller-Hinton broth (MHB) to all wells. A stock solution (100 µl) of the *C. procera* leaf extract was placed into the first row of the plate. Employing a multichannel pipette, the *C. procera* extract was diluted serially by twofold in MHB. Each well received a 24-hour culture (10 µl) of the respective bacterium, which was standardized to 1.5×10^8 and diluted to 1×10^6 CFU/ml. The infected microtiter plates were then incubated at 37 degrees Celsius for 24 hours. The highest dilution of the *C. procera* leaf extract that inhibited bacterial growth compared to the control tube but did not eliminate the organism was identified as the MIC of the extract [2].

3.6. Fractional Inhibitory Concentration Index of *Calotropis procera* Extract

The combined effect of each extract (leaf or stem) of *C. procera* and antibiotics was evaluated by the checkerboard

method. The combination of antimicrobials used for this study included

- leaf extract of *C. procera* + ciprofloxacin
- stem extract of *C. procera* + ciprofloxacin

The experiments were carried out using 96-well microtiter plates arranged in a 6-by-6 configuration. A volume of 100 µl of MHB was introduced into each well of the microtiter plate. Subsequently, 50 µl of each dilution of *C. procera*'s leaf or stem extract was added horizontally across the 6 rows, while 50 µl of each ciprofloxacin dilution was vertically introduced into the 6 columns. Each well of the microtiter plates could accommodate 200 µl. Additionally, each well received 10 µl of a bacterial solution at 106 CFU/ml concentration. The microtiter plates were then incubated at 37 °C for 24 hours. The combination that exhibited no growth was deemed the combined MIC. Each test included controls for sterility and growth. The in vitro interactions between the *C. procera* extracts and ciprofloxacin were assessed and quantified by calculating the fractional inhibitory concentration index (FICI) using the following formula:

$$FICI = \frac{MICa \text{ in Combination}}{MICa} + \frac{MICb \text{ in Combination}}{MICb}$$

MICa represents the MIC for *C. procera* extracts, while MICb denotes the MIC for ciprofloxacin. The results of the FICI were evaluated as follows: $FICI \leq 0.5$ signifies synergy (where the overall effect is greater than the individual effects of the drugs combined). $FICI > 0.5-1$ denotes additivity, meaning the total effect equals the individual contributions of the agents. $FICI > 1-4$ indicates indifference (the combined effect is equivalent to that of either single agent). $FICI > 4$ suggests antagonism, where the combined effect of the agents is weaker than the effects of each agent alone or their sum [13].

4. Results and Discussion

The findings of this research indicate that the extracts from the leaves and stems of *C. procera* demonstrate a wide range of antibiotic efficacy against the tested bacteria. The details of these results are presented in the tables below:

Table 1: Analysis of Leaf and Stem Extracts of *Calotropis procera* Using Gas Chromatography–Mass Spectrometry

S/N	NAME	RETENTION TIME (MIN)	AREA% (LEAF)	AREA% (STEM)
1	Norolean-12-Ene	63.39	1.25	1.22
2	Lupenyl Acetate	64.23	3.5	4.1
3	Hexadecanoic Acid, Methyl Ester	64.2	5.55	4.2
4	9,12,15-Octadecatrienoic acid, Methyl Ester	64.73	13.32	10.5
5	LUP-20(29)-EN-3-YL ACETATE	65.52	17.94	15
6	Methyl Commate	65.65	2	1.8
7	n-Hexadecanoic Acid	65.71	4.2	3.5
8	Phytol	66.1	13.32	10.2
9	Mono(2-ethylhexyl) Phthalate	66.33	0.75	0.8
10	2,6,10-Trimethyl,14-	67.4	1.8	1.7

	ethylene-14-pentadecane			
11	1,2-Benzenedicarboxylic Acid, Bis(2-methylpropyl) Ester	68.63	1.5	1.4
12	1,8-Dioxo-5-thiaoctane,8-(9-borabicyclo[3.3.1]non-9-yl)-3-(9-borabicyclo[3.3.1]non-9-yloxy)-1-phenyl	69.55	0.8	0.75
13	Isochiapin B	69.78	0.9	0.85
14	Calotropin (cardenolide)	70.12	2.5	3
15	Uscharin (cardenolide)	70.45	1.5	1.8
16	Calactin (cardenolide)	70.8	1.2	1.3
17	Quercetin (flavonoid)	71.3	2.2	2
18	Kaempferol (flavonoid)	71.5	2	1.9
19	Methyl 7,11,14-Eicosatrienoate	57.04	1.05	1
20	Pthalic Acid, Isobutyl Ester	59.22	1.1	1.08
21	Stigmasterol	59.24	1.5	1.4
22	Butyl Octyl Phthalate	59.71	1.2	1.18
23	Lupan-3-ol, Acetate	60.45	1.4	1.38
24	9,12,15-Octadecatrienoic acid, (Z,Z,Z)	60.51	1.6	1.58
25	Ergost-5-en-3-ol, (3.beta.,24r)	60.55	2	1.9
26	Hexadecanoic Acid, Ethyl Ester	61.91	2	1.9
27	Squalene	61.93	2.5	2.4
28	9,12-Octadecadienoic acid (Z,Z)-, Methyl Ester	62.12	2.4	2.3
29	Stigmast-5-En-3-Ol, (3.Beta.)	62.34	3.8	3
30	Dibutyl Phthalate	62.77	3	2.8

moderate concentrations. These polar compounds are known to influence enzyme activity, inhibit efflux pumps, and disrupt quorum sensing, potentially working in conjunction with the lipophilic components to boost antibacterial effectiveness. While the extracts from leaves and stems contain the same twenty major compounds, the leaf generally has slightly higher levels of unsaturated fatty acid esters and phytol, whereas the stem tends to contain marginally more cardenolides. Such organ-specific differences in relative concentrations likely account for the variations in MIC values and the synergistic interactions with ciprofloxacin.

Table 2: Fractional Inhibitory Concentration of the Extracts of *Calotropis procera*

Combination	Bacteria	MICa	MICb	MIC (a+b)	MIC (a+b)/MICa	MIC (a+b)/MICb	FIC Index
Leaf + ciprofloxacin	<i>E. coli</i>	8.33	1.56	0.52	0.06	0.33	0.40
Stem + ciprofloxacin	<i>E. coli</i>	5.21	1.56	0.39	0.07	0.25	0.30
Leaf + ciprofloxacin	<i>P. aeruginosa</i>	5.21	2.08	1.04	0.19	0.50	0.70
Stem + ciprofloxacin	<i>P. aeruginosa</i>	4.17	2.08	0.78	0.18	0.38	0.60
Leaf + ciprofloxacin	<i>S. aureus</i>	5.21	2.60	2.34	0.45	0.9	1.40
Stem + ciprofloxacin	<i>S. aureus</i>	6.25	2.60	0.65	0.10	0.25	0.30
Leaf + ciprofloxacin	<i>B. subtilis</i>	3.10	1.60	4.17	1.34	2.6	4.00
Stem + ciprofloxacin	<i>B. subtilis</i>	4.20	1.60	0.78	0.19	0.49	0.70

a=plant extract, b=ciprofloxacin.

The GC-MS profiles of *C. procera* extracts from leaves and stems (Table 1) demonstrate a largely comparable phytochemical composition, with differences in quantity that likely account for their varying antibacterial efficacies. The primary components in both plant parts are triterpenoid esters and unsaturated fatty acid methyl esters: LUP-20(29)-en-3-yl acetate represents 17.9% of the leaf extract and 15.0% of the stem extract, while 9,12,15-octadecatrienoic acid methyl ester and phytol each make up about 10–13% of both extracts. These highly lipophilic substances are recognized for their ability to compromise membrane integrity, which may significantly contribute to their antibacterial effectiveness. Intermediate compounds encompass hexadecanoic acid methyl ester (5.6% in leaves vs. 4.2% in stems) and lupenyl acetate (3.5% in leaves vs. 4.1% in stems), with their fatty acid and triterpenoid structures indicating similar mechanisms targeting membranes. Sterols, including stigmasterol, squalene, and ergost-5-en-3-ol,

appear at approximately 2–3%, further supporting the notion of membrane-affecting properties. Significantly, cardenolides such as calotropin (2.5% in leaves, 3.0% in stems), uscharin (1.5% vs. 1.8%), and calactin (1.2% vs. 1.3%) alongside the flavonoids quercetin (2.2% vs. 2.0%) and kaempferol (2.0% vs. 1.9%) are detected in both extracts at fairly low to

Table 2 illustrates the interaction between *C. procera* extracts and ciprofloxacin against various bacterial pathogens, with the effects differing based on the bacterial species and the specific plant extract used (leaf and stem). Both the leaf and the stem extracts combined with ciprofloxacin exhibit synergistic activity against *E. coli*, as indicated by FIC indices of 0.4 and 0.3, respectively. In the case of *P. aeruginosa*, both combinations yield additive effects (FIC indices of 0.7 and 0.6), suggesting that the combined effect is approximately the sum of the individual activities without significant enhancement. Notably, the interaction against *S. aureus* varies depending on the extract: while the leaf extract combination shows indifference (FIC index of 1.4), the stem extract combination demonstrates synergy (FIC index of 0.3). This divergence implies that the phytochemical composition of the stem extract may better potentiate ciprofloxacin's activity against this Gram-positive bacterium.

Conversely, when combined with ciprofloxacin against *B. subtilis*, the leaf extract exhibits antagonistic activity (FIC index of 4.0), whereas the stem extract combination results in an additive effect (FIC index of 0.7). These discrepancies highlight the influence of extract-specific constituents on antibacterial efficacy and highlight the complexity of interactions when natural products are used in combination with conventional antibiotics.

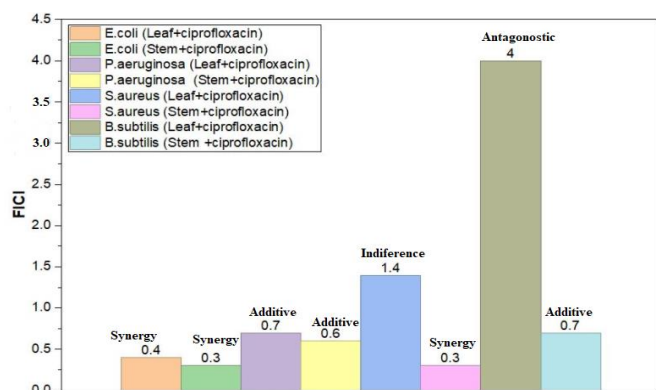


Figure 1. FIC indices of the extracts from *C. procera* (FIC Indices ≤ 0.5 indicate synergy, $> 0.5-1$ indicates additivity, $> 1-4$ indicates indifference, and > 4 indicates antagonism).

The bar chart (Figure 1) shows how combining ciprofloxacin with *C. procera* leaf or stem extracts affected four bacteria: *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis*. The results varied widely depending on the bacteria and plant part used. For *E. coli*, both extracts worked strongly with ciprofloxacin, with low FICI values (0.4 and 0.3), meaning the combinations were much more effective than either treatment alone. The stem extract also showed strong synergy against *S. aureus* (FICI 0.3). Additive effects, which still improved activity though less dramatically, were seen for *S. aureus* treated with the leaf extract and for *B. subtilis* with both extracts (FICI values between 0.6 and 0.7). However, against *P. aeruginosa*, the leaf extract did not improve the antibiotic's action (FICI 1.4), and the stem extract actually interfered, showing antagonism (FICI 4.0). This suggests that in some cases, plant extracts can reduce antibiotic effectiveness, possibly by blocking or disrupting its activity.

The current study validates that extracts from *C. procera* show significant antibacterial effects, with their effectiveness depending on the specific microbial target and the particular plant part utilized. GC-MS analysis (Table 1) revealed the presence of cardenolides including calotropin, uscharin, and calactin as well as the flavonoids quercetin and kaempferol as key phytochemical constituents. These bioactive compounds are closely associated with the antimicrobial properties of *C. procera*.

Interestingly, extracts derived from the stem exhibited lower minimum inhibitory concentrations (MICs) against Gram-negative bacteria (*E. coli* and *P. aeruginosa*), while leaf extracts showed greater potency against Gram-positive bacteria (*S. aureus* and *B. subtilis*). This variation in activity likely stems from both quantitative and qualitative differences in phytochemical composition across the different plant parts, as documented by [3]. According to [4], the higher concentration of cardenolides found in the stem may enhance the disruption of the Gram-negative outer membrane, whereas the flavonoid-rich extracts from leaves may more effectively hinder the synthesis of peptidoglycan in Gram-positive cell walls.

The checkerboard assay results highlighted significant synergistic effects particularly between the stem extract and

ciprofloxacin against *E. coli* and *S. aureus* with fractional inhibitory concentration indices (FICIs) ≤ 0.5 . This synergy could be attributed to multiple factors: (i) improved cell membrane permeability that allows for greater antibiotic accumulation inside the bacterial cell; (ii) inhibition of efflux pump mechanisms that typically contribute to resistance against fluoroquinolones; and (iii) potential interference from flavonoid components with bacterial DNA gyrase or topoisomerase IV, enhancing the action of ciprofloxacin. In contrast, some combinations against *P. aeruginosa* and *B. subtilis* demonstrated additive or antagonistic effects, highlighting the intricate nature of the interaction between phytochemicals and antibiotics and suggesting that specific membrane characteristics or inherent resistance traits can influence synergy [13] [14].

These results obtained from this study align with the wider literature describing the synergistic potential between plant extracts and antibiotics. According to [5], [15], the ethanol extract from the rind of *Punica granatum* demonstrated a remarkable synergistic effect when combined with ciprofloxacin, leading to a 34-fold reduction in MIC and restoring sensitivity in resistant strains of *Klebsiella pneumoniae*. The antibacterial and modulatory properties of ethanol extracts derived from the leaves and bark of *Azadirachta indica* were examined in conjunction with aminoglycosides and carbapenems against *S. aureus*, *E. coli*, and *P. aeruginosa*. The combination of the ethanol extract from the bark and amikacin exhibited a synergistic interaction against the *P. aeruginosa* PA 24 strain. The ethanol bark extract combined with imipenem, amikacin, or gentamicin showed a synergistic effect on *E. coli*, while imipenem demonstrated a synergistic interaction with *S. aureus* strains [9] [16]. The above studies add to the increasing evidence of the synergistic effect of phyto-extracts and antibiotics. Furthermore, this study broadens these concepts to *C. procera*, providing new evidence that combinations of cardenolides and flavonoids can synergize with fluoroquinolones.

Nonetheless, the in-vitro design of this research and the restricted selection of bacterial strains introduce limitations. Future in-vivo studies, such as those using murine infection models, will be necessary to verify pharmacokinetic compatibility, toxicity profiles, and therapeutic indices of the extract-ciprofloxacin combinations. Additionally, mechanistic investigations utilizing efflux pump mutants, membrane permeability assessments, and studies on enzymatic inhibition will clarify the specific molecular interactions that contribute to synergy or antagonism.

In conclusion, this research highlights the dual antibacterial effects and potentiating capability of *C. procera* extracts, showing that particular combinations with ciprofloxacin result in notable synergistic effects against significant pathogens. The MIC values, FICI calculations, and strain-specific reactions lay a solid groundwork for further preclinical and mechanistic research. Ultimately, these findings could guide the strategic design of phytochemical-

antibiotic therapies aimed at addressing multidrug resistance and refining existing antimicrobial treatment protocols.

5. Conclusion and Future Scope

This research shows that *C. procera* extracts contain strong antibacterial activity that is variable depending on both the bacterial strain and the plant part used, underlining the importance of phytochemical variability in antimicrobial activity. The stem extracts from *C. procera* demonstrated particularly low minimum inhibitory concentrations against Gram-negative bacteria such as *E. coli* and *P. aeruginosa*, while the leaf extracts showed greater effectiveness against Gram-positive species like *S. aureus* and *B. subtilis*. GC-MS analysis detected the presence of biologically active cardenolides and flavonoids, such as Calotropin, Uscharin, Calactin, Quercetin, and Kaempferol, and are most likely responsible for differential effects. Checkerboard assays also detected remarkable synergy between stem extract and ciprofloxacin against *E. coli* and *S. aureus*, suggesting potential synergy via increased membrane permeability or efflux inhibition to enhance antibiotic activity. The additive and antagonistic effects in interactions with *P. aeruginosa* and *B. subtilis* highlight the intricate nature of phytochemical and antibiotic interactions, underscoring the importance of carefully choosing combination therapies. They are important in the context of emerging antibiotic resistance, because they justify the traditional utilization of *C. procera* and render plausible the exploration of phytomedicine as an adjunct to existing antibiotics. The clear demonstration of synergistic and non-synergistic effects makes focused screening of plant-antibiotic mixtures rather than mass use crucial. By the discovery of bioactive components with potentiating activity, this study initiates the potential to isolate or structurally alter lead compounds towards drug development. Nonetheless, reliance on in vitro assays and an array of restricted molecular strains poses a fundamental limitation, as cellular complexity, pharmacokinetics, and host interactions cannot be sufficiently managed outside of a living system. To achieve these encouraging in vitro data clinically, further studies are warranted, including tests of toxicity, resistance development analysis, and formulation optimization for clinical administration.

Subsequent studies should be directed toward demonstrating antibacterial efficacy and safety by appropriate in vivo models to establish pharmacodynamic and pharmacokinetic profiles under conditions of physiology. Detailed mechanistic studies at the molecular level will be needed to establish how individual cardenolides and flavonoids interact with bacterial targets and antibiotics. Expansion of the panel of pathogens to be studied, such as clinical isolates with multiple drug resistance, will increase generalizability. Finally, resistance emergence studies long-term and overall cytotoxicity evaluation are needed to facilitate the assessment of combination therapies based on *C. procera* as an antidote against resistant bacterial infections.

Author's Statements

All authors have reviewed and given their approval for the final version of the manuscript and acknowledge their accountability for every aspect of the work, assuring that any inquiries concerning the accuracy or integrity of any portion of the work will be properly investigated and addressed.

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Data Availability- All data produced or evaluated in this study are contained within this published article and its supplementary files. Any additional questions can be addressed to the corresponding author.

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the goal of contributing new perspectives on the development of plant-based antimicrobial solutions.

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