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Control of Multidrug-Resistant Hospitalized Pathogenic Bacteria Using the Secondary Metabolites of *Calotropis procera* and In-silico Analysis of Bacterial Virulent Proteins

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Abstract

This study explores the multidrug-resistant pattern of hospitalized pathogens and their pharmacological impact against the secondary metabolites isolated from Calotropis procera, for its medicinal properties. Moreover, this study implies that comprehensive analysis, including isolation of multidrug-resistant hospitalized bacterial species and extraction and characterization of secondary metabolites by GC-MS from Calotropis procera, molecular docking, ADMET profiling, and, was conducted to evaluate the therapeutic potential of these compounds. The multidrug-resistant Klebsiella pneumonia, Salmonella typhi, and Pseudomonas aeruginosa were isolated and they also showed sensitivity against C. procera leaf extract. GC-MS reveals the key volatile compounds, including oleic acid, and the molecular docking with the proteasome (PDB ID: 5JXG) identified 5-Methyl-2-phenylindolizine as the most promising compound due to its high binding affinity (-6.7 kcal/mol), with 2,6-Dimethylphenol, 3,5-Dimethylaniline, and Ethyl Heptanoate showing progressively lower affinities. Interaction analysis highlighted the importance of PRO266, TRP531, GLU271, and ARG490 residues. ADMET profiling revealed that 2,6-dimethylphenol and 3,5-Dimethylaniline have favorable absorption and minimal CYP interactions, while 5-methyl-2-phenylindolizine demonstrated excellent absorption and BBB permeability. Additionally, the study found that C. procera metabolites could target furin, a proprotein convertase involved in bacterial virulence, offering a novel approach to combat multidrug-resistant bacterial infections. These findings underscore the potential of Calotropis procera metabolites as effective therapeutic agents and active against multidrug-resistant bacterial species.

Keywords: *Bacterial Multidrug Resistance, Calotropis procera, Molecular Docking, Secondary Metabolites.*

Introduction

Because of the overuse and misuse of antibiotics in both human and veterinary medicine, antimicrobial resistance (AMR) is a serious worldwide health concern. The development of resistance to antimicrobial drugs, which are intended to eradicate microorganisms such as bacteria, fungi, viruses, and parasites, is the cause of this resistance [1]. The environment is particularly concerning due to the expansion and survival of antibiotic-resistant genes (ARG) and bacteria (ARB) [2]. Multidrug-resistant (MDR) microbes have been discovered in food, soil, water, and even the air,

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indicating that this problem is not limited to hospitals but also exists in community settings. These environmental reservoirs aid in the spread of AMR, which poses a serious global health risk to the population [3]. Bacteria are becoming more and more resistant to treatment, which could undo decades of medical advancement. This makes treating bacterial infections and diseases more difficult. One of the main causes of this urgent worldwide problem is the overuse and misuse of antibiotics in a variety of fields, including clinical treatment, agriculture, animal healthcare, and the food system [4]. To combat this global issue, researchers are exploring alternative solutions, including the use of plants and their phytochemical compounds such as primary and secondary metabolites. These natural compounds have shown promise in combating AMR by inhibiting the growth of antibiotic-resistant bacteria and enhancing the efficacy of existing bacteria [5].

Metabolites are organic compounds synthesized by plants and organisms using enzyme-mediated chemical reactions within metabolic pathways. These metabolites play a crucial role in various biological processes, and they can be broadly categorized into primary and secondary metabolites [6]. Primary metabolites, such as amino acids, nucleotides, and simple carbohydrates, are essential for the basic growth, development, and reproduction of the organism. They are involved in fundamental metabolic processes and are critical for the organism's survival [7].

In contrast, secondary metabolites are not directly involved in primary metabolic functions but play significant roles in the interaction of the organism with its environment. These compounds, which include alkaloids, flavonoids, terpenoids, and phenolics, are often species-specific and are synthesized for various ecological functions such as defense mechanisms, competition, and reproduction [7,8]. Secondary metabolites can enhance an organism's ability to adapt to its environment, attract pollinators, deter herbivores, or combat pathogens. The absence of secondary metabolites does not result in immediate death but can lead to long-term impairment in survivability, fecundity, or other fitness-related traits [9].

Calotropis procera (Figure 1), commonly known as the Sodom apple, is a plant species well-known for its production of a diverse array of secondary metabolites. This plant has been traditionally used in various medicinal applications, particularly for its antimicrobial properties. The study of secondary metabolites from *Calotropis procera* is of significant interest due to their potential therapeutic applications. These metabolites are often characterized by their distinctive colors, fragrances, and flavors, which facilitate the plant's interaction with other organisms [10–12].

Recent advances in both *in-vitro* and *in-silico* techniques have allowed for a more comprehensive screening and analysis of these bioactive compounds. *In-vitro* studies provide insights into the antimicrobial efficacy of these metabolites against various pathogens, while *in-silico* studies offer a molecular-level understanding of their interactions and mechanisms of action [13–18]. By integrating these approaches, we can better understand the potential of secondary metabolites from *Calotropis procera* as antimicrobial agents and explore their applications in developing new therapeutic strategies.

This study aims to screen and characterize the secondary metabolites from *Calotropis procera* and evaluate their antimicrobial activity using a combination of *in-vitro* and *in-silico* methodologies. The *Calotropis procera* plant, renowned for its diverse medicinal properties, has yielded several bioactive compounds with potential therapeutic benefits. In particular, this research focuses on the proteasome as a key target for molecular docking studies. The proteasome plays a critical role in protein degradation and cellular

regulation, influencing various cellular processes including cell cycle control and apoptosis. Its involvement in cancer and neurodegenerative diseases makes it a significant target for drug discovery.

The secondary metabolites extracted from *Calotropis procera* show promising potential in targeting furin, a proprotein convertase critically involved in bacterial virulence and pathogenesis [19,20]. Furin activates a variety of bacterial toxins and adhesins essential for infection and survival, making it a strategic target for mitigating bacterial multidrug resistance [21–23]. The identified compounds, particularly 5-Methyl-2-phenylindolizine, demonstrated high binding affinity and favorable pharmacokinetic profiles, suggesting their efficacy in inhibiting furin. By blocking furin activity, these compounds could prevent the maturation of bacterial virulence factors, thereby reducing bacterial infectivity and resistance [24,25]. This novel approach highlights the potential of *C. procera* metabolites in developing effective therapies against multidrug-resistant bacterial infections, emphasizing the need for further in vitro and in vivo studies to confirm these findings.

Figure 1. *Calotropis procera* Plant.

Classification

Kingdom	: plantae.
Subkingdom	: Thracheobionta.
Super division	: spermatophyta.
Division	: Magnoliophyta.
Class	: Magnoilopsida
Subclass	: Asteridae
Order	: Gentianales
Family	: Asclepiadaceae
Genus	: *Calotropis*
Species	: *procera*

Materials and Methods

Isolation of Multidrug-Resistant Bacteria from Hospitalized Environment

The sterile swab was used for the collection of samples in the hospitalized environment like bed, table, floor, and utensils. After the swabbing, the swab was kept in brine heart infusion broth for 18 hrs at 37°C for enrichment. The enriched broth was used as inoculum and streak on respective selective media for isolation of pathogens. For drug-resistant analysis, the cultures were swabbed on Muller Hinton Agar plates and placed the standard antibiotic discs such as *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae* [2].

Sample Preparation

A 20 gm of *C. procera* dried leaf powder was taken for extraction using a Soxhlet apparatus. The Soxhlet apparatus, containing 250 ml of acetone solvent in a round-bottom flask, was connected to a condenser with a continuous water supply to prevent solvent evaporation. The entire setup was placed on a table and heated at 80°C in a heating mantle for 16 hours.

The crude extract was then condensed by evaporation and stored at 4°C for further analysis.

Extraction of Secondary Metabolites

The crude extract of *Calotropis procera* (from both acetone and benzene extractions) was subjected to qualitative phytochemical screening to detect the presence of secondary metabolites. Major secondary metabolites such as terpenes, flavonoids, carotenoids, alkaloids, saponins, and tannins were identified.

Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

The functional groups in the crude extracts of *Calotropis procera* leaves, fruits, and seeds were analyzed using FT-IR spectroscopy (Shimadzu IRTracer-100). The FT-IR spectrum was recorded in the range of 400 to 4000 cm^{-1}.

GC-MS Analysis

The crude extracts were filtered and subjected to GC-MS analysis to identify bioactive compounds. The analysis was performed using a gas chromatograph-mass spectrometer (GC-MS) (Shimadzu GC-MS-QP2010 PLUS) equipped with an AOC-20i auto-injector and AOC-20s auto-sampler units. The system used was a GC Clarus 500 Perkin Elmer interfaced with a mass spectrometer.

Antimicrobial Activity

This study aimed to evaluate the effectiveness of *Calotropis procera* leaf extract against MDR pathogens, which possess significant challenges in clinical settings due to their resistance to multiple antibiotics. The antibacterial activity of the leaf extract was determined using the well diffusion method on Muller Hinton agar. Nutrient broth (containing 0.3 g beef extract, 0.3 g yeast extract, 0.5 g peptone, and 0.5 g NaCl in 100 ml of double distilled water) was used to cultivate bacterial cultures of *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae.* After autoclaving and cooling the nutrient media, it was poured into Petri dishes and allowed to solidify. Overnight bacterial cultures were spread onto the solidified Muller Hinton agar plates using sterile swabs. Sterile wells were created using a gel puncture or 6 mm sterile tips under aseptic conditions. The antibiotic samples were added to the wells in aseptic conditions, mixed with DMSO solution, and the plates were incubated at 37°C for 24-48 hours. The zones of inhibition were measured in mm diameter using a transparent ruler and recorded.

Molecular Docking

Molecular docking studies were conducted using AutoDockTools to investigate the binding interactions of ligands extracted from *Calotropis procera* and identified by GC-MS profiling [26]. Ligand structures were obtained from PubChem, and the target protein structure for furin (PDB ID: 5JXG) was downloaded from the Protein Data Bank (https://www.rcsb.org/) [27–29]. The preparation of ligands and the furin protein involved adding charges, defining rotatable bonds, and removing non-essential components. Docking simulations were performed to explore the binding interactions of the plant-derived compounds with furin. The resulting binding poses were analyzed based on binding affinities and interaction patterns to determine the optimal binding configurations using PyMOL and Discovery Studio tools [30,31].

ADMET Analysis

The identified compounds were subjected to ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiling using pkCSM, an online tool for evaluating pharmacokinetic properties [32]. The chemical structures of the compounds were submitted to pkCSM to predict various pharmacokinetic parameters such as water solubility, intestinal absorption, blood-brain barrier permeability, cytochrome P450 interactions, and potential

toxicity. This step helped in filtering out compounds with unfavourable ADMET properties, ensuring that only compounds with promising drug-likeness and safety profiles were selected for further analysis.

Results

Phytochemical Screening

The powdered leaf material was subjected to solvent extraction using acetone and benzene in a Soxhlet apparatus. The qualitative phytochemical screening of these extracts revealed the presence of various secondary metabolites. The identified secondary metabolites include carbohydrates, tannins, steroids, glycosides, flavonoids, phenols, saponins, terpenoids, and coumarins. These secondary metabolites are known for their potential bioactivities, contributing to the plant's medicinal properties.

The detailed results of the phytochemical screening are presented in Table 1. The acetone extract showed the presence of carbohydrates, glycosides, terpenoids, phenols, coumarins, and steroids. In contrast, the benzene extract revealed the presence of tannins, saponins, flavonoids, terpenoids, phenols, and steroids. Notably, quinones, cardiac glycosides, and anthraquinones were absent in both extracts.

Table 1. Phytochemical Screening of *Calotropis procera* Leaf.

S.No.	Phytochemical Analysis	Acetone	Benzene
1	Carbohydrates	Present	Absent
2	Tannis	Absent	Present
3	Glycosides	Present	Absent
4	Saponins	Absent	Present
5	Flavonoids	Absent	Present
6	Quinines	Absent	Absent
7	Terpenoids	Present	Present
8	Phenols	Present	Present
9	Cardiac glycosides	Absent	Absent
10	Coumarins	Present	Absent
11	Steroids	Present	Present
12	Anthraquinones	Absent	Absent

Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

Fourier Transform Infrared Spectroscopy (FT-IR) was employed to characterize the functional groups and secondary metabolites present in the *Calotropis procera* leaf extract. The FT-IR spectra revealed several characteristic peaks at 432.08, 447.49, 470.00, 516.92, 566.60, 594.08, 686.88, 1010.70, 1242.16, 1634.54, and 2916.37 cm^{-1}. These peaks correspond to various functional groups, including nitro compounds (N-O stretch), halo compounds (C-Br stretching), benzene derivatives, and alkenes (C=C bending) (Figure 2). Additionally, peaks at 408.91, 424.34, 447.49, 470.83, 532.36, 594.08, 671.23, 902.69, 1095.57, 1219.01, 1357.89, 1419.61, 1712.79, 2144.84, and 3001.24 cm^{-1} were observed, indicating similar functional groups (Figure 3). These functional groups are associated with secondary metabolites that play a significant role in the plant's bioactivity and potential antimicrobial properties.

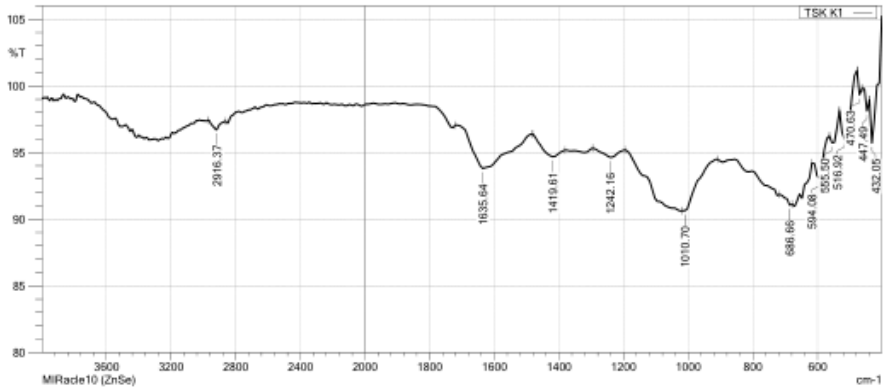

Figure 2. FT-IR Spectrum Analysis of Acetone Extraction.

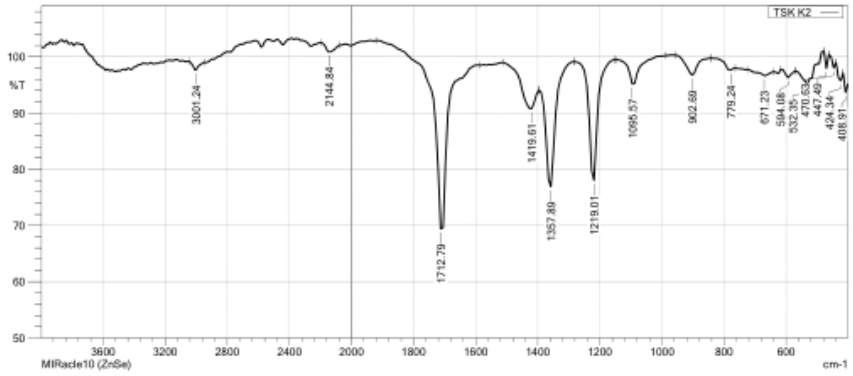

Figure 3. FT-IR Spectrum Analysis of Benzene Extraction.

GC-MS Analysis

The *Calotropis procera* extract was subjected to analysis of gas-chromatography mass spectrometer (GC-MS) analysis. The mass spectrum of GC-MS was interpreted using the National Institute of Standard and Technology (NIST) database with more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The chromatogram of fruit, leaves and seeds is represented in Figure 4. The phytochemical compounds are also given in Table 2.

Table 2. Compounds Identified from GC-MS

S.NO	Compound	PubChem ID	SMILES
1.	1,3-Dichlorobenzene	10943	C1=CC(=CC(=C1)Cl)Cl
2.	1-Methyl-2-phenylindole	77095	CN1C2=CC=CC=C2C=C1C3=CC=CC=C3
3.	2,6-dimethylphenol	11335	CC1=C(C(=CC=C1)C)O
4.	3,5-dimethylaniline	7949	CC1=CC(=CC(=C1)N)C
5.	5-Methyl-2-phenylindolizine	610180	CC1=CC=CC2=CC(=CN12)C3=CC=CC=C3
6.	Ethyl 13-methyltetradecanoate	71380066	CCOC(=O)CCCCCCCCCCCC(C)C
7.	Ethyl heptadecanoate	26397	CCCCCCCCCCCCCCCCC(=O)OCC
8.	Ethyl heptanoate	7797	CCCCCCC(=O)OCC

9.	Hexadecanoic acid, ethyl ester	12366	CCCCCCCCCCCCCCCC(=O)OCC
10.	methyl 2-chloroacetate	7295	COC(=O)CCl
11.	Naphthalene, 2-fluoro-	67583	C1=CC=C2C=C(C=CC2=C1)F
12.	Nonanoic acid, ethyl ester	31251	CCCCCCCCC(=O)OCC
13.	Phenylsilane	6327628	C1=CC=C(C=C1)[Si]
14.	Tetra ethyl silicate	6517	CCO[Si](OCC)(OCC)OCC
15.	Tetradecanoic acid, ethyl ester	31283	CCCCCCCCCCCCCC(=O)OCC

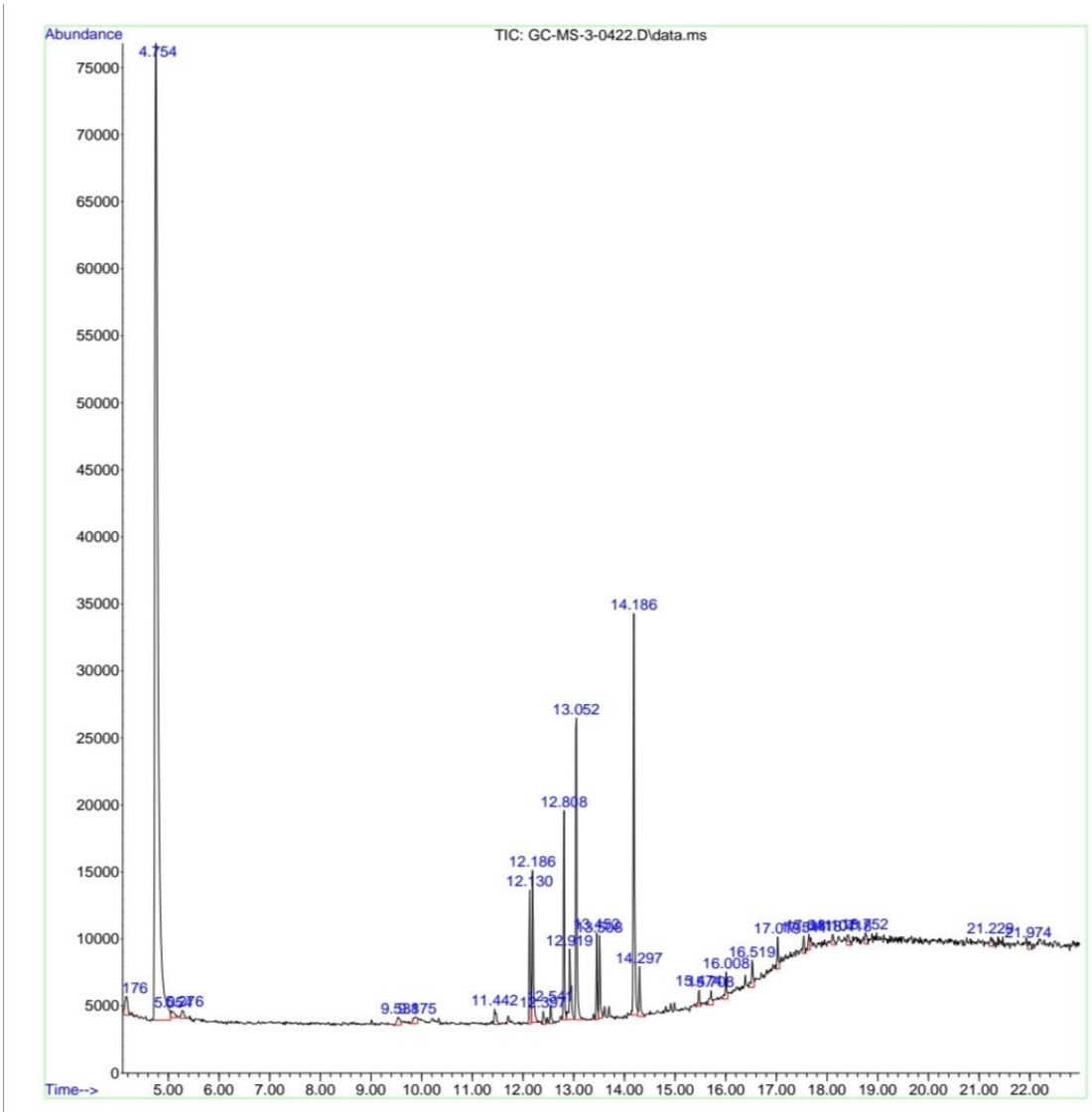

Figure 4. GC-MS Analysis of *Calotropis procera.*

Antibacterial Activity of *Calotropis procera*

Antibacterial activity of the synthesized *Calotropis procera*. Leaf extract was assessed. The testing was done against four clinically important pathogens *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia, Salmonella typhi* shows the zone of inhibition in the well diffusion method of antibacterial activity. The different patterns of the zone of inhibitions are observed in and Charted. The highest zone of inhibition was

observed for *Salmonella typhi* and *Escherichia coli* even at lower the lowest zone of inhibition was observed for *Klebsiella pneumonia* and *Pseudomonas aeruginosa*.

Multidrug-Resistant Analysis of Wound Pathogens

The antibacterial activity of five antibiotics-Oxacillin (30µg), Azithromycin (14µg), Erythromycin (15µg), Ciprofloxacin (5µg), and Norfloxacin (10µg) was evaluated against clinical isolates of *Salmonella typhi, Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. The standard strain *S. aureus* ATCC 25923 was used as a control (Table 3). *S.typhi* exhibited moderate susceptibility to all tested antibiotics, with zones of inhibition ranging from 12 mm (Erythromycin and Norfloxacin) to 14 mm (Ciprofloxacin). *S. aureus* showed moderate sensitivity to Oxacillin (15 mm) and Erythromycin (14 mm). It exhibited lower sensitivity to Azithromycin (11 mm) and Ciprofloxacin (10 mm). *E. coli* demonstrated a similar pattern, with moderate inhibition zones of 12-18 mm. Notably, it was highly susceptible to Norfloxacin (18 mm) but showed lower inhibition against Erythromycin (12 mm). *K. pneumoniae* displayed moderate resistance to most antibiotics, with zones of inhibition ranging between 11-14 mm. Ciprofloxacin (11 mm) showed the least activity. *P. aeruginosa* exhibited resistance to Ciprofloxacin (10 mm) and Norfloxacin (9 mm). It showed moderate susceptibility to Oxacillin (15 mm) and Erythromycin (14 mm). The control strain *S. aureus* ATCC 25923 was highly susceptible to all antibiotics, with inhibition zones ranging from 17 mm (Norfloxacin) to 20 mm (Azithromycin and Ciprofloxacin). *S. aureus* and *P. aeruginosa* showed notable resistance to Ciprofloxacin and Norfloxacin, reflecting the potential emergence of multidrug resistance. *K. pneumoniae* exhibited intermediate resistance to all tested antibiotics.

E. coli was resistant to Erythromycin but highly sensitive to Norfloxacin. The standard strain *S. aureus* ATCC 25923 demonstrated expected high susceptibility to all antibiotics. The observed resistance patterns emphasize the importance of continuous monitoring for antimicrobial resistance.

Table 3. Antimicrobial Resistance Pattern with Commercial Antibiotics Against Bacterial Pathogens

Bacterial Isolates	Zone of Inhibition in mm				
	Oxacillin (30µg)	**Azithromycin (14µg)**	**Erythromycin (15µg)**	**Ciprofloxacin (5µg)**	**Norfloxacin (10µg)**
Salmonella typhi	13	13	12	14	12
Staphylococcus aureus	15	11	14	10	10
Escherichia coli	13	13	12	13	18
Klebsiella pneumonia	14	12	13	11	12
Pseudomonas aeruginosa	15	11	14	10	9
***Staphylococcus aureus* ATCC 25923**	18	20	17	20	17

Antibiogram of Wound Pathogens Against Different Concentrations of *Calotropis procera*

According to the antibiogram data, *C. procera* has variable levels of antibacterial activity against a number of pathogens that cause wounds. The highest zone of inhibition was observed for *K. pneumonia* at 21mm, then *E. coli* and *S. aureus* ATCC 25923 at 20mm followed by *P. aeruginosa* at 18mm, and subsequently *S. aureus* and *S. typhii* at 16mm and 15mm at 100µg/mL concentration. This suggests that MDR pathogens *S. aureus* ATCC 25923, *E.coli*, and *K. pneumoniae* are susceptible to the effects of *C. procera* at higher concentrations (Table 4) with the control of standard oxacillin (30µg).

Table 4. Antibacterial Activity of Secondary Metabolites of *Calotropis procera* Against the Wound Pathogens

Bacterial Isolates	Zone of Inhibition in mm				
	25µg	50µg	75µg	100µg	Oxacillin (30µg)
Salmonella typhi	5±0.1	9±0.1	12±0.1	15±0.1	13±0.1
Staphylococcus aureus	3±0.1	4±0.1	9±0.1	16±0.1	15±0.1
Escherichia coli	3±0.1	4±0.1	11±0.1	20±0.1	13±0.1
Klebsiella pneumonia	4±0.1	7±0.1	13±0.1	21±0.1	14±0.1
Pseudomonas aeruginosa	5±0.1	8±0.1	14±0.1	18±0.1	15±0.1
***Staphylococcus aureus* ATCC 25923**	4±0.1	4±0.1	14±0.1	20±0.1	18±0.1

(Mean ±0.1)

ADMET Profiling

Based on the ADMET analysis, four molecules have been selected for further development due to their balanced pharmacokinetic profiles and safety characteristics. *2,6-Dimethylphenol* exhibits high absorption, moderate distribution, and minimal CYP interactions, with a safe toxicity profile. *3,5-Dimethylaniline* also shows strong absorption and distribution with minimal CYP interactions, although it has potential skin sensitization concerns. *5-Methyl-2-phenylindolizine* demonstrates excellent absorption and favorable BBB permeability, making it suitable for CNS applications, but requires monitoring for CYP enzyme interactions.

Molecular Docking Analysis

Based on the ADMET profiling of the phytochemical molecules derived from the *Calotropis procera* plant, the top 4 molecules were taken for further molecular docking analysis. Molecular docking was conducted to investigate their binding affinities and interacting residues with the furin (PDB ID: 5JXG). The results are summarized in Table 5, and the intermolecular interactions are also plotted in 2D form (Figure 5).

Table 5. Docking Score Values of Selective Molecules with Interacting Residues of Furin Enzyme Active Site.

Molecule Name	Binding Affinity *(kcal/mol)*	Interacting Residues
2,6-dimethylphenol	-5.1	GLY265, PRO266, GLY307, ASN310, ASP530, TRP531, ALA532

3,5-dimethylaniline	-4.9	PRO266, ASN310, TRP531, ALA535
5-Methyl-2-phenylindolizine	-6.7	PRO266, GLU271, SER311, ILE312, TYR313, GLN488, ARG490, TRP531, ALA532, TYR571
Ethyl heptanoate	-4.7	GLU271, ASN310, ILE312, GLN488, ARG490, TRP531, ALA532

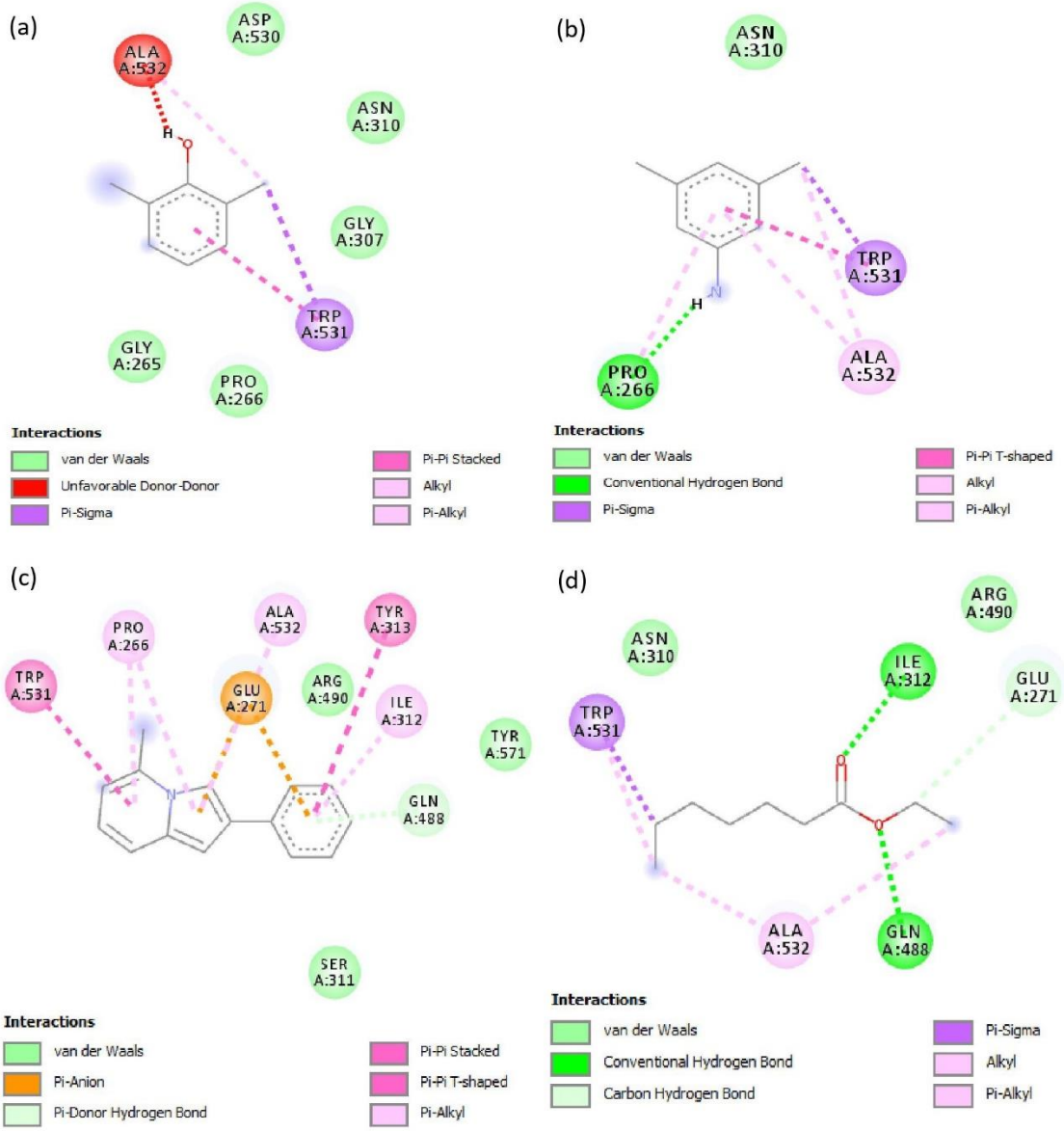

Figure 5. 2D Intermolecular Interaction Plots for Selective Molecules with the Active Site of Furin Enzyme.

The docking studies reveal varying binding affinities of the four molecules towards the proteasome, suggesting different levels of potential inhibitory activity. Among the molecules tested, 5-methyl-2-phenylindolizine exhibited the highest binding affinity with a value of -6.7 kcal/mol, indicating a strong interaction with the proteasome active site. This molecule stands out as a promising candidate for further investigation due to its substantial

binding affinity, which may translate to significant proteasome inhibition.

2,6-Dimethylphenol demonstrated a moderate binding affinity of -5.1 kcal/mol, which, while lower than that of 5-Methyl-2-phenylindolizine, still indicates a reasonable interaction with the proteasome. This molecule might serve as a lead compound for modifications aimed at enhancing its inhibitory potential.

3,5-Dimethylaniline and Ethyl heptanoate showed lower binding affinities of -4.9 kcal/mol and -4.7 kcal/mol respectively. Although these values are lower compared to the other molecules, they still suggest potential binding interactions with the proteasome, albeit weaker. These compounds may require structural optimization to improve their binding affinities and inhibitory activities.

The interaction analysis reveals that certain residues are recurrently involved in binding interactions across different molecules. For instance, PRO266 and TRP531 are common interacting residues among all four molecules. The consistent involvement of these residues suggests their crucial role in the binding interactions within the proteasome active site.

5-Methyl-2-phenylindolizine interacts with several key residues, including GLU271, SER311, ILE312, TYR313, GLN488, ARG490, TRP531, ALA532, and TYR571. The presence of multiple hydrogen bonds and hydrophobic interactions likely contributes to its high binding affinity. Specifically, the interaction with residues such as GLU271 and ARG490, which are known to play critical roles in the proteasome's enzymatic activity, suggests that 5-Methyl-2-phenylindolizine may effectively inhibit the proteasome's function.

Discussion

The qualitative phytochemical screening of these extracts revealed the presence of various secondary metabolites. The identified secondary metabolites include carbohydrates, tannins, steroids, glycosides, flavonoids, phenols, saponins, terpenoids, and coumarins. These secondary metabolites are known for their potential bioactivities, contributing to the plant's medicinal properties [33].

The FT-IR analysis thus confirmed that the functional groups identified in the plant extract were due to the presence of secondary metabolites, which are responsible for the synthesis and stabilization of bioactive compounds. This comprehensive identification of functional groups supports the hypothesis that *Calotropis procera* contains diverse secondary metabolites contributing to its medicinal properties.

Determination of metabolomic profiles by ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/ MS) and gas chromatography-mass spectrometry (GC-MS) has been recently adopted as an effective technique to understand the chemical nature of medicinal plants [28]. The identification of semi-polar chemicals has been widely applied to characterize plant secondary metabolites. GC-MS can reveal the chemical nature of volatile chemicals, which define the flavour of a plant. From the phytochemical profile obtained, it could be reported that six *C. procera* seeds had similar phytochemical profiles, but that the level of each peak varied in the previous study. GC-MS analysis showed that the major volatile compounds in *C. procera* seeds were fatty acids. It is found to be the widely used pharmacologically active chemical in China [34]. The prime advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of marker compound and mobile phase with very little time. The linear regression curve showed a good linear relationship for strychnine and brucine compounds from *C. procera.*

The highest inhibition was observed in *Calotropis procera* for the bacterial species with metal oxide nanoparticles as carriers. Based on the previous research, the biosynthesized silver nanoparticles also showed maximum antimicrobial activity. The

exact mechanism of the inhibition of the bacteria is still unknown. Still, some hypothetical mechanisms show that the inhibition is due to the ionic binding of the nanoparticles on the surface of the bacteria which creates great intensity of the proton motive force [35]. The antibacterial properties of silver compounds and silver ions have been historically recognized and applied in a wide range of applications from disinfecting medical devices and home appliances to water treatments. The individual effects of the bio-reduced antibiotics were investigated against four bacterial strains using the good diffusion method.

Ethyl Heptanoate has high absorption and favourable distribution, with minimal CYP interactions, though its potential for skin sensitization needs further assessment. These compounds show promising ADMET profiles, making them strong candidates for subsequent in vitro and in vivo studies to validate their therapeutic potential [30].

2,6-Dimethylphenol and 3,5-Dimethylaniline primarily interact with residues such as ASN310, TRP531, and ALA532. These interactions, although fewer in number, still highlight the importance of these residues in ligand binding. The presence of ASP530 in the interaction profile of 2,6-Dimethylphenol suggests potential ionic interactions, which may enhance its binding stability.

Ethyl heptanoate interacts with a similar set of residues, including GLU271, ASN310, ILE312, GLN488, ARG490, TRP531, and ALA532. The consistent involvement of GLU271 and ARG490 across multiple molecules emphasizes their significance in binding interactions and suggests that these residues could be targeted for designing more potent proteasome inhibitors.

The docking studies of secondary metabolites from *Calotropis procera* with the proteasome (PDB ID: 5JXG) highlight 5-Methyl-2-phenylindolizine as the most promising candidate due to its high binding affinity and extensive interaction with key residues. The recurring involvement of residues such as PRO266, TRP531, GLU271, and ARG490 across different molecules underscores their importance in proteasome inhibition. Further structural optimization and in-vitro studies are warranted to validate these findings and explore the therapeutic potential of these compounds as proteasome inhibitors.

Conclusion

This study investigated the antimicrobial and pharmacological properties of secondary metabolites extracted from *Calotropis procera* through molecular docking, ADMET profiling, antibacterial testing, and GC-MS characterization. Molecular docking studies with the proteasome (PDB ID: 5JXG) identified 5-Methyl-2-phenylindolizine as the most promising compound due to its high binding affinity (-6.7 kcal/mol), followed by 2,6-Dimethylphenol, 3,5-Dimethylaniline, and Ethyl Heptanoate. Interaction analysis highlighted the importance of residues PRO266, TRP531, GLU271, and ARG490 in binding interactions. ADMET profiling showed 2,6-Dimethylphenol had high absorption and minimal CYP interactions with a safe toxicity profile, while 3,5-Dimethylaniline had strong absorption and distribution but potential skin sensitization concerns. 5-Methyl-2-phenylindolizine demonstrated excellent absorption and BBB permeability but required monitoring for CYP interactions, and Ethyl Heptanoate showed high absorption and favorable distribution. Antibacterial testing revealed that *C. procera* leaf extract was most effective against *Salmonella typhi and Escherichia coli,* with lower activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Additionally, the study underscores the importance of exploring novel natural compounds from *C. procera* as potential alternatives in the fight against antimicrobial drug resistance organisms, which is a growing

global health concern. GC-MS analysis identified key volatile compounds, including oleic acid, highlighting their pharmacological potential. These findings suggest that *C. procera* possesses significant antimicrobial properties and potential therapeutic applications, warranting further in vitro and in vivo studies to validate these results.

Conflict of Interest

The authors have no conflicts of interest to declare.

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