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EXPLORING THE POTENTIAL OF 4-(2-HYDROXYPROPAN-2-YL)CYCLOHEX-1-EN-1-OL FROM *CALOTROPIS PROCERA* AS A NEW ANTIBACTERIAL AGENT

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ABSTRACT

This study investigates the antibacterial efficacy of the novel compound 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol compared to the standard antibiotic ampicillin. The *Calotropis procera* root extract was prepared with using different solvents and thin layer chromatography (TLC) was used for isolation and 1H NMR, 13C NMR, Mass spectral studies are used for characterization of isolated compound for subsequent antibacterial testing. Using standard agar diffusion methods, the compound was tested for antibacterial activity against *Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa,* and *Escherichia coli*. The zone of inhibition values for the novel compound were 4 mm against *Staphylococcus aureus*, 4 mm against *Escherichia coli,* and 5 mm against *Pseudomonas aeruginosa,* indicating superior antibacterial activity compared to ampicillin. Given the rising antibiotic resistance, the effectiveness of 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol against these pathogens underscores its potential as a therapeutic candidate. Further studies are necessary to elucidate its mechanism of action, pharmacokinetics, and *in vivo* efficacy, which will be crucial for evaluating its suitability in clinical applications. Overall, this research contributes to the ongoing search for new antibacterial agents capable of combating resistant bacterial strains, emphasizing the need for continued exploration of novel compounds in the fight against infectious diseases.

Keywords:
Antibacterial, 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol, *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* Antibiotic resistance

1. INTRODUCTION

Antimicrobial resistance (AMR) has become a pressing global health issue, primarily fueled by the misuse of antibacterial medications. This misuse has contributed to a notable increase in bacterial infections resistant to existing treatments (Ameen et al., 2022). The World Health Organization (WHO) estimates that approximately 700,000 people die each year from infections that do not respond to antibiotics. Alarmingly, recent statistics show that in 2022, AMR was associated with about 5.25 million deaths, with 1.85 million directly linked to resistant infections (ARC, 2022). Projections indicate that by 2050, deaths from bacterial infections could exceed 10 million annually, largely due to AMR. To address this growing crisis, it is essential to develop new antimicrobial agents that can work alongside existing antibiotics to maintain their efficacy. Moreover, exploring novel strategies to disarm bacterial populations, either by sensitizing them to current antibiotics or using alternative approaches, is vital for alleviating the burden of antimicrobial resistance.

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Calotropis procera, commonly referred to as giant milkweed or Sodom apple, is a widely recognized medicinal plant that belongs to the Apocynaceae family and is distributed across various regions of the world. This perennial shrub or small tree is native to North Africa, the Middle East, and parts of Asia, where it has been traditionally utilized in folk medicine to treat a multitude of ailments and health conditions (Gujjeti et al., 2014; Mamidala et al., 2013). The plant is particularly noted for its rich array of phytochemical compounds, including cardiac glycosides, triterpenoids, flavonoids, and alkaloids, which contribute significantly to its impressive pharmacological properties (Lunavath et al., 2013; Gujjeti et al., 2013). These diverse constituents not only enhance the therapeutic potential of *C. procera* but also make it a compelling subject for further scientific research and exploration in the context of antimicrobial activity.

In a previous study (Burgula et al., 2024), we gathered extensive information on 23 medicinal plants from traditional healers across various mandals in Khammam district, Telangana, India, with the aim of documenting and preserving valuable traditional knowledge. To scientifically validate this knowledge, a comprehensive literature review was conducted on these 23 plants, revealing that the antibacterial properties of *C. procera* roots have not yet been thoroughly investigated in depth. Consequently, this particular study focuses specifically on *C. procera*, as its root extract may harbor unique phytochemicals with unexplored antimicrobial potential that could contribute to addressing the growing issue of antibiotic resistance. The primary objective of this research is to isolate and characterize a novel compound from the root extract of *C. procera* and evaluate its antibacterial activity against a range of pathogenic bacteria, thereby contributing to the search for new antimicrobial agents.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material:

The roots of *Calotropis procera,* a flowering plant belonging to the Apocynaceae family, were gathered from the forest region of Thallada mandal, located in Khammam District, Telangana, India. The plant specimen was meticulously identified and authenticated by Dr. R. Sneha Rajendran, a taxonomist affiliated with the Department of Botany at Government Degree College (W), Khammam. Thallada mandal is recognized for its rich and diverse forest ecosystem, which provides an ideal habitat for a variety of plant species, including *C. procera*. The involvement of a qualified taxonomist in the identification process guarantees the accuracy and reliability of the plant material utilized in this study, which is crucial for the validity and reproducibility of the research outcomes.

2.2 Preparation of Crude Extract:

The roots of *C. procera* were harvested from the forest area of Thallada mandal in Khammam District, Telangana, India. After collection, the roots were cut into medium-sized pieces, thoroughly washed with tap water, and then dried. The dried roots were ground into a powder using a grinder and sieved to achieve a particle size of ≤ 2.00 mm. For extraction, a solvent mixture of hexane and ethyl acetate in an 85:15 [v/v] ratio was utilized. A total of 200 g of the dried sample powder was combined with 1 L of the extraction solvent and subjected to an orbital shaker for 24 hours at 200 rpm at room temperature. Following the extraction, the mixture was filtered through Whatman filter paper 1, and the resulting filtrate was evaporated under reduced pressure using a rotary evaporator. The concentrated extract was stored at -4°C for subsequent analysis. The percent yield of the crude extract was calculated using the

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formula:
Yield (%) = (Weight of solvent-free extract (g) / Dried extract weight) × 100.

2.3 Isolation of Novel Compound:

The crude extract derived from the *C. procera* root underwent preparative thin layer chromatography (TLC) to isolate the desired compound. Initially, the crude sample was dissolved in ethanol, and 5 µL of the solution was spotted onto a Merck silica gel 60 F254 glass plate. The TLC was conducted using a solvent system consisting of toluene and acetic acid in a 7:3 ratio.

After allowing the TLC plate to dry briefly, it was visualized under UV light at both 254 nm and 365 nm wavelengths. Subsequently, the plate was sprayed with a stable solution of anisaldehyde-sulfuric reagent (ANS: anisaldehyde/acetic acid/methanol/sulfuric acid–0.5:10:85:5) until fully saturated. The plate was then heated at 110°C until the spots were fully developed and visible under standard light. The retention factor (Rf) value of the compound spots was calculated based on their positions on the TLC plate. The band corresponding to the target compound was carefully scraped off the TLC plate using a spatula.

To extract the compound from the silica gel powder, the powder was dissolved in the same mobile phase used for TLC separation. The mixture was centrifuged at 1000 rpm for 10 minutes, and the supernatant was collected and dried. The purity of the dried sample was re-evaluated using TLC to confirm that the compound of interest had been successfully isolated from the crude extract. This preparative TLC method facilitated the isolation and purification of the target compound from the complex crude extract of *C. procera* roots, representing a critical step in the overall analysis and characterization of the compound.

2.4 Characterization of Compound:

The isolated compound obtained from the preparative thin layer chromatography of the *C. procera* root extract was further characterized using a variety of analytical techniques, including 1H NMR, 13C NMR, IR, and LC-MS. These comprehensive analytical methods were employed to elucidate the structural and chemical properties of the isolated compound from the *C. procera* root extract, providing valuable insights into its identity and purity.

2.5 Antibacterial Activity

The study evaluated the antibacterial activity of various compounds against a wide range of bacterial strains, including the Gram-positive *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pneumoniae* (ATCC 33400), as well as the Gram-negative *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). These bacterial strains were sourced from the American Type Culture Collection (ATCC), a reputable provider of well-characterized and standardized microbial cultures, ensuring the reliability and reproducibility of the study's findings. The selection of these specific bacterial strains enabled a thorough assessment of the antibacterial properties of the tested compounds, yielding valuable insights into their potential therapeutic applications.

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The antibacterial activity of the isolated compound and standard drug was assessed using the Agar well-diffusion method. Four different concentrations (25, 50, 75, and 100 µl) of the compounds were tested against several bacterial pathogens, including *Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa,* and *Escherichia coli.* The plates were incubated at 37°C for 18-24 hours, and at the conclusion of the experiment, the diameter of the inhibition zone (measured in millimeters) was recorded. Additionally, the activity index was calculated. Measurements were taken in three fixed directions, and the average values were documented to ensure the accuracy of the results.

3. RESULTS AND DISCUSSION

3.1 Identification of Novel Compound :

The yield from the crude extract of C. procera roots was determined to be 2.548 grams. Following preparative TLC, the purified sample obtained weighed 1350 µg, which was utilized for characterization. The isolated compound was characterized using 1H NMR, 13C NMR, and LC-MS data.

1H NMR: δ 1.22-1.32 (6H, 1.27 (s), 1.27 (s)), 1.45-1.66 (3H, 1.53 (dddd, J = 10.1, 10.0, 3.9, 2.1 Hz), 1.57 (dddd, J = 12.9, 7.0, 6.1, 2.9 Hz)), 2.07 (2H, ddd, J = 13.7, 7.0, 6.7 Hz), 2.43 (2H, ddd, J = 13.9, 6.9, 3.0 Hz), 5.24 (1H, dd, J = 8.5, 5.0 Hz). 13C NMR: δ 24.5-24.6 (2C, 24.6 (s), 24.6 (s)), 28.4 (1C, s), 32.4 (1C, s), 34.4 (1C, s), 43.4 (1C, s), 72.7 (1C, s), 121.8 (1C, s), 167.9 (1C, s). Chemical Formula: C9H16O2. Molecular Weight: 156.23. m/z: 156.12 (100.0%), 157.12 (9.7%).

The 1H NMR spectrum of the compound reveals several key signals that support its structural identity as 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol (Figure-1). The region δ 1.22-1.32 shows two singlets at 1.27 ppm, indicating the presence of two equivalent methyl groups, likely from the isopropyl group. The signals between δ 1.45 and 1.66 are complex, with a quartet-like pattern suggesting coupling interactions typical of a cyclohexene structure. Notably, the peaks at δ 2.07 and 2.43 correspond to protons on carbons adjacent to a double bond, reinforcing the presence of a cyclohexene framework. The signal at δ 5.24 indicates the presence of a double bond, characteristic of an alkene.

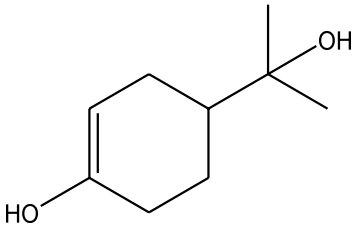

Figure-1. 2D structure of Isolated Novel Compound,4-(2-Hydroxypropan-2-yl)cyclohex-1-en-1-ol

The 13C NMR spectrum further elucidates the structure of the compound. The signals at δ 24.5-24.6 and 28.4 ppm suggest a saturated carbon environment typical of cycloalkanes, consistent with the cyclohexene backbone. The peak at δ 121.8 ppm confirms the presence of a double bond, indicating the alkene functionality. Additionally, the signal at δ 167.9 ppm is indicative of a carbonyl or related

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functional group, which supports the presence of the hydroxyl functionality in the compound. This combination of signals aligns well with the proposed structure, confirming the presence of both the isopropyl and cyclohexene moieties.

The molecular formula $C_9H_{16}O_2$ and the molecular weight of 156.23 further substantiate the identification of the compound as 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol. The significant m/z values observed at 156.12 and 157.12 in mass spectrometry confirm the molecular weight and suggest the presence of isotopic variants, reinforcing the structural integrity of the compound. Overall, the combination of NMR data and molecular formula supports the conclusion that this compound is novel and structurally unique.

3.2 Antibacterial Activity

The antibacterial activity of the isolated novel compound, 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol was evaluated against four different bacterial strains, namely *Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae*, and *Pseudomonas aeruginosa*. The results, as shown in Table-1, demonstrated a clear correlation between the concentration of 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol and the zone of inhibition observed.

Table-1. Anti-Bacterial Activity of isolated novel compound, 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol.

S No	Strain	Concentration (µg) / Zone of Inhibition (mm)			
		25	50	75	100
1	*Staphylococcus aureus*	2	3.5	3.5	4
2	*E coli*	2	2.5	3	4
3	*Streptococcus pneumonia*	2	2	2.5	3
4	*Pseudomonas aeruginosa*	3	4	4.5	5

The antibacterial activity of the isolated novel compound, 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol, was assessed against four bacterial strains: *Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae,* and *Pseudomonas aeruginosa*. The findings, presented in Table-1 and Figure-2, revealed a clear relationship between the concentration of 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol and the observed zone of inhibition.

For *Staphylococcus aureus,* the novel compound 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol exhibited a zone of inhibition ranging from 2 mm at 25 µg to 4 mm at 100 µg, indicating stronger antibacterial activity compared to ampicillin, which showed no inhibitory effect at 25 µg and 50 µg, and a minimal zone of inhibition of 0.5 mm at 75 µg and 2 mm at 100 µg.

In the case of *Escherichia coli,* the novel compound demonstrated a substantial zone of inhibition, from 2 mm at 25 µg to 4 mm at 100 µg, suggesting more potent antibacterial activity than ampicillin, which had no inhibition at 25 µg and 50 µg, with a small zone of inhibition of 0.5 mm at 75 µg and 1 mm at 100 µg.

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For *Streptococcus pneumoniae*, the novel compound showed a zone of inhibition ranging from 2 mm at 25 µg to 3 mm at 100 µg, indicating comparable or slightly stronger antibacterial activity relative to ampicillin, which had no inhibition at 25 µg, and zones of inhibition of 0.5 mm at 50 µg, 1 mm at 75 µg, and 2 mm at 100 µg.

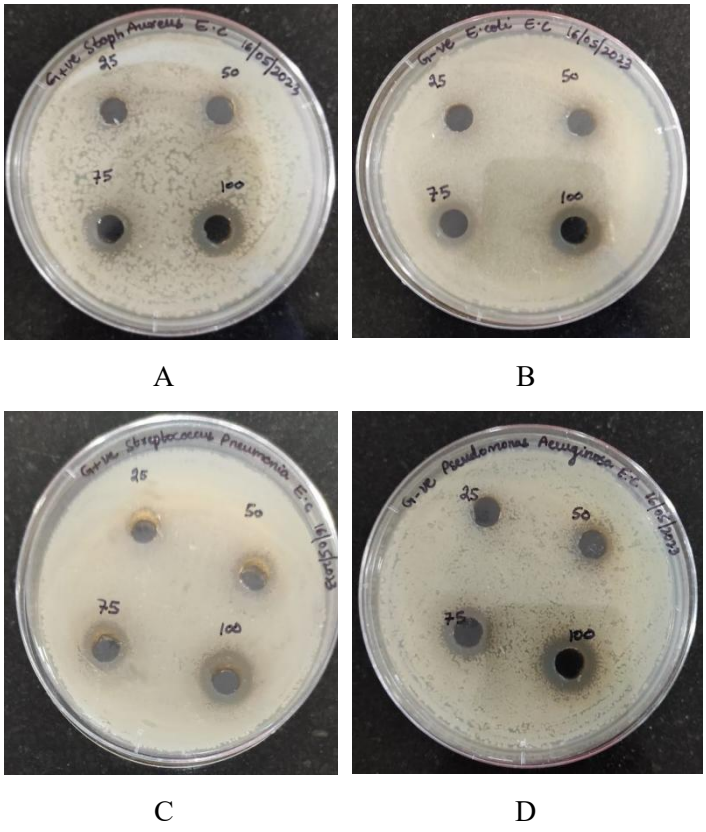

Figure-2: Anti-Bacterial Activity of isolated compound, 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol against (A) *Staphylococcus aureus* (B) *E. coli* (C) *Streptococcus pneumonia* (D) *Pseudomonas aeruginosa*

Regarding *Pseudomonas aeruginosa*, the novel compound exhibited a larger zone of inhibition, from 3 mm at 25 µg to 5 mm at 100 µg, suggesting more effective antibacterial activity compared to ampicillin, which showed no inhibition at 25 µg, with a zone of inhibition of 0.5 mm at 50 µg, 1 mm at 75 µg, and 2 mm at 100 µg.

The novel compound 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol demonstrated stronger antibacterial activity compared to the standard antibiotic Ampicillin against the tested bacterial strains, particularly *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*. The results indicate that the novel compound may have the potential to be a more effective antibacterial agent than Ampicillin, especially at higher concentrations.

The increased antibacterial effectiveness of 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol against Staphylococcus aureus aligns with the observations made by Radulovic et al., (2013), who noted the strong antimicrobial capabilities of structurally related cyclic compounds. Likewise, Moharana et al. (2023) highlighted the efficacy of hydroxylated cyclohexene derivatives against Gram-positive bacteria,

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including species of Staphylococcus. The activity of this novel compound against Staphylococcus aureus is particularly significant, as this bacterium is a major contributor to hospital-acquired infections and has shown resistance to multiple antibiotics (Gurrapu et al., 2017).

The similar or slightly enhanced antibacterial activity of the novel compound against Streptococcus pneumoniae compared to ampicillin is significant, especially since Streptococcus pneumoniae is a primary cause of pneumonia and other respiratory infections (Vijayagiri et al., 2012). These findings are corroborated by Godhamari et al.,. (2012), who demonstrated the antibacterial potential of cyclic compounds against Streptococcus species.

4. CONCLUSION

The findings of this study indicate that the novel compound 4-(2-hydroxypropan-2-yl)cyclohex-1-en-1-ol may serve as a more effective antibacterial agent than the standard antibiotic ampicillin, particularly against *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*. To fully realize its potential as a therapeutic candidate, further research is needed to investigate its mechanism of action, pharmacokinetics, and *in vivo* efficacy.

Conflicting Interests: The authors have declared that no conflicting interests exist.

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