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Antibacterial and Antifungal Analysis of Betulinic Acid, Sitosterol and Stigmasterol from *Calotropis procera* Root

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ABSTRACT

This study investigated the antimicrobial properties of the root of *Calotropis procera*. The root was subjected to cold maceration for 48 hours using 1.5 liters of ethyl acetate, and the resulting extract was concentrated via rotary evaporation. The antimicrobial activity of the ethyl acetate extract was assessed against two bacterial species (*Escherichia coli* and *Helicobacter pylori*) and two fungal species (*Aspergillus niger* and *Candida albicans)* using the disk diffusion method. The results demonstrated that the ethyl acetate extract exhibited significant antimicrobial activity, inhibiting the growth of all tested bacterial and fungal strains. The minimum inhibitory concentration (MIC) of Sitosterol and Stigmasterol (CRE-85) against *E. coli* and *H. pylori* was 50 µg/mL, while for *A. niger* and *C. albicans*, it was 75 µg/mL. The minimum bactericidal concentration (MBC) ranged from 75 to 100 µg/mL for the bacterial and fungal isolates. Additionally, betulinic acid (CRE-110) exhibited an MIC and MBC of 50 - 75 µg/mL. These findings highlight the potent inhibitory effects of *C. procera* root extract against the tested microorganisms, supporting its traditional use in herbal medicine for treating microbial infections.

Key words: *Calotropis procera*, bacteria, fungi, activity, zone of inhibition, herbal drugs.

INTRODUCTION

Calotropis procera, a member of the Asclepiadaceae family, is predominantly found in dry tropical and subtropical regions, as well as semi-arid and inland areas [1]. It is commonly recognized as a weed in disturbed regions, waste areas, roadsides, and grasslands [2]. Despite its

classification as a weed, *C. procera* has been extensively documented for its medicinal properties, with various parts of the plant exhibiting anti-inflammatory, analgesic, and antioxidant activities [3].

The therapeutic potential of medicinal plants is of global significance, especially in developing regions where over 80% of the population relies on traditional herbal remedies for healthcare [4]. Historically, medicinal plants have played a crucial role in treating various ailments based on empirical knowledge and experience [5]. Several medicinal plants, such as *Artemisia nilagrica*, *Vaccinium microcarp*, *Senellgalia visco*, and *Calotropis procera*, are used to treat dermatological conditions and are known to contain bioactive compounds like hydroquinone and epicatechin, which have been used to treat skin pigmentation disorders and skin cancer [6].

More than half of the pharmaceuticals available today are derived from traditional medicinal plants, highlighting their significance in modern medicine [6]. These plants owe their biological activity to secondary metabolites, which are often responsible for their antimicrobial and therapeutic properties [7]. In recent years, increasing antibiotic resistance in pathogenic bacteria has raised public health concerns, urging the search for alternative antimicrobial agents from natural sources [8].

Ethnobotanical studies of *C. procera* have shown its widespread use in treating various ailments, including piles, cholera, asthma, scabies, cough, and pain. Different parts of the plant are utilized for their analgesic, antibacterial, antimalarial, and hepatoprotective effects, either alone or in combination with other substances [9]. The roots, in particular, are known to treat colds, coughs, elephantiasis, syphilis, and other ailments, while the flowers have cytostatic properties useful in treating asthma and piles.

In the search for bioactive compounds, betulinic acid, isolated from various plants, has demonstrated diverse biological activities, including antibacterial and cytotoxic properties. However, its efficacy against microbial species varies. For instance, betulinic acid isolated from *Caesalpinia paraguariensis* showed no significant activity against *Escherichia coli* and *Candida albicans* [10], while crude extracts containing betulinic acid from *Syncarpia glomulifera* exhibited antibacterial properties [11]. The stem bark of *Zizyphus joazeiro* was also found to contain betulinic acid with notable activity against Gram-positive bacteria [12]. However,

Ghaffari et al [10] reported that isolated betulinic acid from *Vitex negundo* leaves showed minimal inhibition against *Escherichia coli* but was effective against *Bacillus subtilis*.

In spite of significant research in to *Calotropis procera*, not much has been reported on antibacterial and antifungal analysis of betulinic acid, sitosterol and stigmasterol from *calotropis procera* root. This study explores the antimicrobial potential of *C. procera* root extracts, particularly focusing on their effects against common bacterial and fungal pathogens, in order to evaluate their potential use in traditional and modern medicine.

MATERIALS AND METHODS

Plant collection

Calotropis procera root was collected from North-Eastern University, Gombe, Nigeria, on latitude 10.2881^0 N and longitude 11.0537^0 E and was identified by Mr Muhammad Chindo of Department of Botany, Gombe State University, Gombe State, Nigeria. A voucher specimen was deposited at the Herbarium with specimen number GSU/H/365.

Extraction

The pulverized plant material (1 kg) was macerated for 48 hours using 1.5 L of ethyl acetate. Superfluous filtrates were collected using a winchester bottle, a glass funnel, and fluted Whatman No. 1 filter paper. The crude extract was concentrated using a rotary evaporator [22].

Preparation of Isolates for Microbial Analysis

Bacterial and fungal isolates collected from Federal Medical Centre, Gombe State, Nigeria were sub-cultured on Mannitol salt agar (MSA) and MacConkey agar (MAC) and incubated at 37 °C for 24 hours to obtain pure isolates of bacteria. After incubation, pure bacterial isolates were stored on NA and prepared in Bijou bottles respectively. The stock cultures were then preserved in a refrigerator at 4 °C until used for further microbiological analyses [13].

Characterization and Identification of Bacterial Isolates

Bacterial isolates were characterized and identified by observation of colonial, and morphological characteristics, Gram reaction and biochemical tests. The various biochemical tests that were carried out for identification were catalase, coagulase, indole and oxidase test [14].

Catalase Test

Hydrogen peroxide was poured into the bottle and the organism was emulsified in it with the sterile wire loop. It produced bubbles of oxygen, the organism was catalase positive [13].

Coagulase Test

A drop of normal saline was dropped on a clean slide using inoculating wire loop, the loop was then flamed and allowed to cool before being used and was added to the normal saline which was used to make a smear that gives a creamy coloration. A drop of plasma was then added to it and mixed properly to observe agglutination. Agglutination was observed [2].

Indole Test

Peptone water was pipetted into the epidoff tube with the help of a micropipette. The organism to be tested was emulsified in the medium and incubated overnight. After incubation a drop of Kovacs reagent was added. If there is a pinkish ring in the solution was observed [13].

Oxidase Test

A few drops of oxidase reagent (1% tetra methyl p-phenylene-diamic dihydrochloride) were dropped on a filter paper, a clean slide was used to pick colonies of the organism to be tested and smear on a paper. A positive test was indicated [2].

Agar Plate and Inoculum Preparation

All aspects of the Kirby–Bauer procedure was standardized to ensure consistent and accurate results. The media used in Kirby–Bauer testing was Mueller–Hinton Agar (MHA) at only 4 mm deep, poured into 100 mm Petri dishes. Bacterial inocula were prepared by diluting a broth culture to match a 0.5 McFarland turbidity standard [15].

Inoculation and Incubation

Using the aseptic technique, broth culture of all organisms was collected with a sterile swab. Excess liquid was removed from the swab by gently pressing it against the inside of the tube. The swab was then streaked across a Mueller–Hinton agar plate to form a bacterial lawn. The agar plate was streaked with the swab in one direction, rotated 120° and streaked again, rotated another 120° and streaked again to obtain uniform growth. Using an antibiotic disk dispenser, filter paper disks containing specific antibiotics were then applied to the plate within 15 minutes

of inoculation. Flame-sterilized forceps were used to gently press each disk onto the agar and ensure it was attached. Plates were then incubated overnight at a temperature of 35 °C. Plates were incubated within 15 minutes of applying antibiotic disks [15].

Agar Disk diffusion Method

In this procedure, agar plates were inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (about 6mm in diameter), containing the crude extract and isolated compounds at different concentrations were placed on the agar surface. The petri dishes were incubated at 35 °C. Antimicrobial agents would diffuse into the agar and inhibit growth of the organisms (*E. coli, H. pyroli,* Yeast *(Candida albicans), A. niger*). The diameters of inhibition were measured in (mm) [15].

RESULTS AND DISCUSSION

Antimicrobial sensitivity of betulinic acid against all the tested organisms and also standard antimicrobial drugs are presented in Table 1.

Table 1. Antimicrobial Sensitivity Test of Fraction CRE-85 of *Calotropis Procera* Root

S/N	Organism	Concentrations(µg/mL) /zone of inhibition(mm)				Control (µg/mL)zone of inhibition(mm)
		100	75	50	25	Aug /Keto
1	*E. coli sp*	17	11	10	6	Augmentin 30/25
2	*H. pylori sp*	18	13	9	7	Augmentin 30 /23
3	*Yeast sp*	19	12	7	5	Ketoconazole100 /32
4	*A. niger*	16	8	6	4	Ketoconazole100 /22

Key: mm = millimeter, Keto = Ketoconazole 100µg/mL, Aug = Augmentin, 30µg/mL = microgram (µg). Values are means of triplicate experiments.

As shown in Table. 1 and Fig. 1, for *E. coli*, the concentrations at 100 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL showed inhibition zones of 17 mm, 11 mm, 10 mm, 6 mm and control showed 25 mm. For *H. pyroli* concentrations at 100 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL showed inhibition zones of 18 mm, 13 mm, 9 mm, 7 mm respectively while control showed 23 mm. For *Yeast* concentrations at 100 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL, showed inhibition zones of 19 mm, 12 mm, 7 mm, 5 mm respectively, while control showed inhibition zone of 3 mm. For *A.*

niger concentrations at 100 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL showed inhibition zones of 16 mm, 8 mm, 6 mm and 4 mm respectively. while control showed 22 nm zone of inhibition. Augmentin and Ketoconazole showed significant activity against *E.coli, H. pyroli, Yeast and A. niger*. *E. coli* showed its highest inhibition zone at a concentration of 100 µg/ml and lowest at 25 µg/mL. *H. pyroli* showed highest activity at 100 µg/mL and lowest at 25 µg/mL, *Yeast* had highest inhibition zone at 100 µg/mL and lowest at 25 µg/mL. Finally *A. niger* showed highest at 100 µg/mL and lowest at 25 µg/mL. The crude flavonoid fraction of *Calotropis procera* showed diameter of inhibition zones ranging from 15.5 and 28.5 mm against the tested bacterial strains [16].

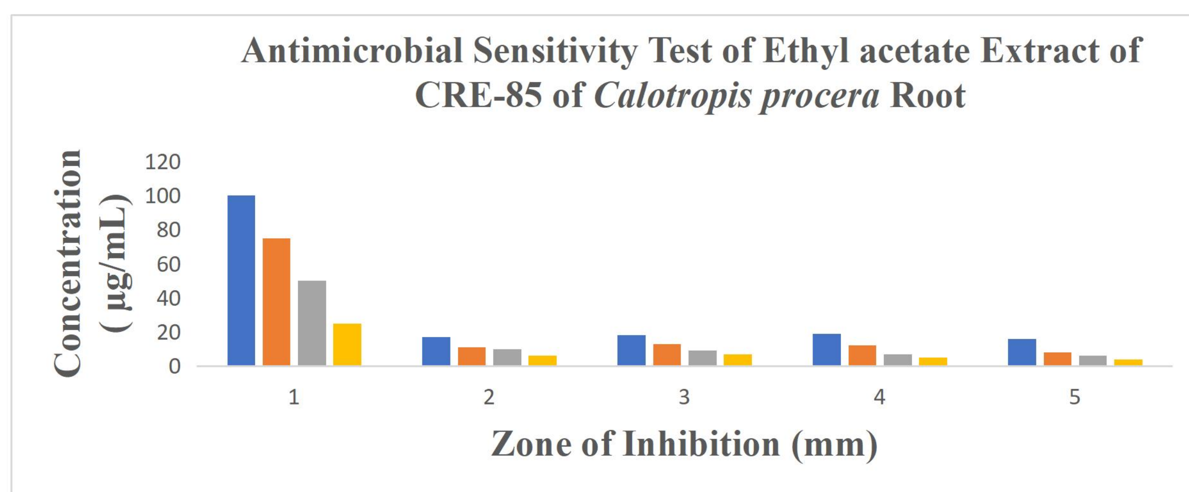

Figure 1: Antimicrobial Sensitivity Test of Ethyl acetate Extract of CRE-85 of *Calotropis procera* root

The minimum inhibitory concentration of sitosterol and stigmasterol are as shown in Table 2.

Table 2. Minimum Inhibitory Concentration (MIC) of Fraction CRE-85 of *Calotropis Procera*

S/N	Organism	Concentrations(µg/ml)/ Inhibition			
		100	75	50	25
1	*E. coli sp*	-	-	-	+
2	*H. pylori sp*	-	-	-	+
3	*Yeast sp*	-	-	+	+
4	*A. niger*	-	-	+	+

Key: = Negative not detected, = Positive Detected, S/N = Serial Number.

The concentration at 25µg/mL showed no effect on the growth of all the tested organisms. The concentration at 50 µg/mL inhibited the growth of *E. coli, H. pyroli*. It had no effect on the growth of *Yeast and A. niger*. The concentrations at 100 µg/mL, 75 µg/mL inhibited the growth of all the organisms. Therefore, the minimum inhibition concentration of extract against *E. coli* and *H. pyroli* was 50 µg/mL, that of *Yeast* and *A. niger* was 75 µg/mL as shown in Table 2. The minimum inhibitory concentration for the root of *Calotropis procera* was 200 µg/mL [17]. The minimum inhibitory concentration (MIC) of aqueous and organic extract of *Calotropis procera* varies from 5-20 mg/mL [18].

The minimum bactericidal concentrations of betulinic acid (CRE-85) against bacterial and fungal isolates are expressed in Table. 3.

Table 3: Minimum Bactericidal Concentration (MBC) of Fraction CRE-85 of *Calotropis procera*

S/N	Organism	Concentrations(µg/mL)/Inhibition			
		100	75	50	25
1	*E. coli sp*	-	-	+	+
2	*H. pylori sp*	-	-	+	+
3	*Yeast sp*	-	-	+	+
4	*A. niger*	-	-	+	+

Key: Negative not detected, Positive Detected, S/N = Serial Number

As shown in Table 3, concentrations of CRE-85 fraction at 100 µg/mL and 75 µg/mL inhibited the growth of E. *coli*, H. *pyroli*, *Yeast* and *A. niger respectively*. The minimum bactericidal concentration (MBC) of CRE-85 as shown in Table 3, was 75 µg/mL. The minimum bactericidal concentration for the root of *Calotropis procera* was 200 µg/mL [17] which is in line with the present study.

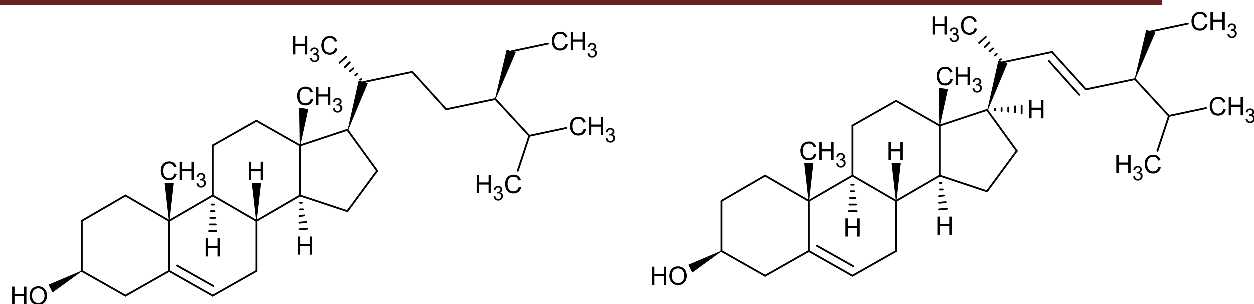

Figure 2: Stigmasterol and Sitosterol (CRE-85) [20, 21].

Antimicrobial sensitivity of betulinic acid (CRE-110) against microorganisms are shown in Table. 4.

Table 4. Antimicrobial Sensitivity Test of Fraction CRE-110 of *Calotropis procera*

S/N	Organism	Concentrations(µg/mL) /zone of inhibition(mm)				Control (µg/mL)zone of inhibition(mm)
		100	75	50	25	Aug /Keto
1	*E. coli sp*	18	12	10	8	Augmentin 30/24
2	*H. pylori sp*	16	13	8	7	Augmentin 30 /25
3	*Yeast sp*	17	10	7	6	Ketoconazole100 /33
4	*A. niger*	15	8	6	5	Ketoconazole100 /24

Key: mm = millimeter, Keto = Ketoconazole 100 µg/mL, Aug = Augmentin, 30 µg/mL = microgram (µg). Values are means of triplicate experiments

From Table 4 and Fig. 3, for E. *coli* the concentrations at 100 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL showed different zone of inhibition of 18 mm, 12 mm, 10 mm, 8 mm respectively while the control showed 25 mm inhibition zone. For *H. pyroli* the concentrations at 100 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL, showed different zone of inhibition of 16 mm, 13 mm, 8 mm and 7 mm respectively, while control showed 25 mm. For *Yeast*, the concentrations at 100 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL showed distinct zone of inhibition of 17 mm, 10 mm, 7 mm, 6 mm. Respectively while control showed 33 mm. For *A. niger* concentrations at 100 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL showed inhibition zones of 15 mm, 8 mm, 6 mm, and 5 mm respectively. while control showed 25 mm. The crude flavonoid fraction of *Calotropis procera* showed diameter of inhibition zones ranging from 15.5 and 28.5 mm against the tested bacterial strains [16]. The result is in line with the present study.

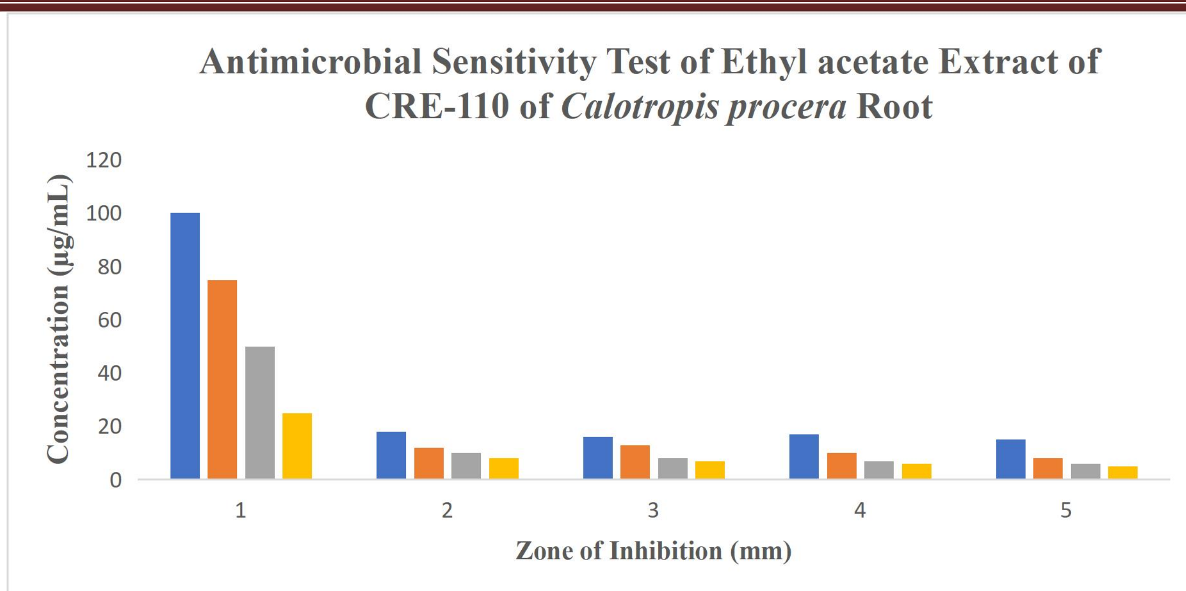

Figure 3: Antimicrobial Sensitivity Test of Ethyl acetate Extract of CRE-110 of *Calotropis procera* Root

The minimum inhibitory concentration of betulinic acid against fungal and bacterial isolates are as shown in Table 5.

Table 5. Minimum Inhibitory Concentration (MIC) of Fraction CRE-110 of *Calotropis procera*

S/N	Organism	Concentrations(µg/mL)/ Inhibition			
		100	75	50	25
1	*E. coli sp*	-	-	+	+
2	*H. pylori sp*	-	-	+	+
3	*Yeast sp*	-	-	-	+
4	*A. niger*	-	-	+	+

Key: = Negative not detected, Positive Detected, S/N = Serial Number.

As shown in Table 5. concentration of 25 µg/mL does not inhibit the growth of all the tested microorganisms. At 50 µg/mL only the growth of *Yeast* was inhibited, all other organisms resisted the effect of the extract. At concentrations of 75 µg/mL and 100 µg/mL all the organisms's growth were inhibited. It showed that MIC of *E. coli, H. pyroli* and *A. niger* was 75 µg/mL, and that of *Yeast* was 50 µg/mL. The minimum inhibitory concentration for the root of *Calotropis Procera* was 200 µg/mL [17]. The minimum inhibitory concentration (MIC) of aqueous and organic extract varies from 5-20 mg/mL [18]. Betulinic acid showed MIC of 0.025

and 0.056 mg/mL against *Staphylococcus aureus* and *P. aeruginosa* and this was comparable to the standard drug neomycin with an MIC of 0.072 mg/mL [19].

The minimum bactericidal concentration of betulinic acid against microorganisms are shown in Table. 6.

Table 6. Minimum Bactericidal Concentration (MBC) of Fraction CRE-110 of *Calotropis procera*

S/N	Organism	Concentrations(µg/ml)/Inhibition			
		100	75	50	25
1	*E. coli sp*	-	-	-	+
2	*H. pylori sp*	-	-	+	+
3	*Yeast sp*	-	-	+	+
4	*A. niger*	-	-	-	+

Key: (-) = Negative not detected, (+) = Positive Detected, S/N = Serial Number

As shown in Table. 6, at concentration of 25 µg/mL of extract of fraction CRE-110, the growth of all the organisms were not inhibited. At 50 µg/mL the growth of *E. coli and A. niger* were inhibited while that of *H. pyroli* and *Yeast* were not inhibited. At 75 µg/mL and 100 µg/mL all the organism's growth were inhibited. Therefore, minimum bactericidal concentration of *E. coli, and A. niger* was 50 µg/mL, and that of *H. pyroli* and *Yeast* was 75 µg/mL. The minimum bactericidal concentration of CRE-110 as shown in Fig. 4? structure is shown, isolated from the root of *Calotropis procera* was 200 µg/mL [17]. The ethyl acetate extract of *Calotropis procera* inhibited the growth of *Micrococcus aeruginosa* with zone of inhibition of 21 mm [18].

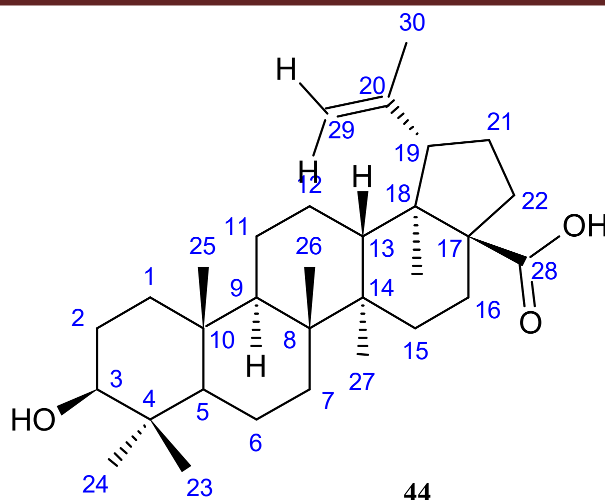

Figure 4: Betulinic acid. (CRE-110)[20]

CONCLUSION

Betulinic acid and a mixture of sitosterol and stigmasterol, isolated from the root of *Calotropis procera*, demonstrated potent antibacterial and antifungal activity against *Escherichia coli*, *Helicobacter pylori*, *Saccharomyces cerevisiae*, and *Aspergillus niger*. These findings highlight the potential of *C. procera* as a valuable source of bioactive compounds with significant antimicrobial properties. Further research is essential to fully explore the antimicrobial potential of *C. procera* root, with a view to its application in the pharmaceutical production of antibiotics. This research can provide a cost-effective alternative to expensive modern drugs, particularly in developing countries like Nigeria, where the majority of the population are low-income earners and access to affordable healthcare is limited.

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