

Antibacterial Screening of Methanol, Ethyl Acetate, Chloroform and Hexane Leaves Extracts of *Calotropis Procera*

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ABSTRACT

Objective: To determine the antibacterial activity of methanol, ethyl acetate, chloroform and hexane leaves extracts of *C. procera* using agar well diffusion method.

Method: Using agar well diffusion method, the determination of antibacterial activity of methanol, ethyl acetate, chloroform and hexane leaves extracts of *C. procera* was conducted.

Key results: The results obtained from antibacterial studies of the various leaves extracts of *C. procera* showed that, the highest zone of inhibition was on *S. pneumoniae* with mean zone of inhibition of 32.10 ± 0.20 mm as observed in chloroform leaves extract. The most active extract chloroform leaves extract showed lowest MIC value of 6 mg/ml and highest MBC value of 25 mg/ml.

Conclusion: The antibacterial screening of leaves extracts of *C. procera* showed that the various leaves extracts possessed antibacterial properties against the five bacterial species tested.

Keywords: Antibacterial, *Calotropis procera*, methanol, ethyl acetate, chloroform and hexane.

Introduction

The antimicrobial feature of medicinal herb is its capacity to effectually resist the growth of microorganisms such as bacteria and fungi (Sravanhi and Giri, 2021). Due to the rising incidences of microbial resistance against various antimicrobial agents and simultaneous evolution of many Multiple Drug Resistant (MDR) bacterial strains have further aggravated the present

situation, therefore scrutinizing for some alternative yet effective antimicrobial therapeutics like herbs have become need of the day (Sravanhi and Giri, 2021, Silvia *et al.*, 2021). The selected analeptic herb *Calotropis procera* is used by traditional healers and general population in the healing of skin, intestinal and urinary tract contaminations and the treatment have been effective. This study scientifically unfolds the antibacterial property of *C. procera*. The herb *calotropis procera* (binomial name (Aiton) W.T. Aiton). It is of the descendant *asclepiadaceae*

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(*apocynaceae*). It is usually known as Sodom apple and provincially or regionally named tumfafiya in Hausa. The separate indigenous organisations in Nigeria classify the plant by a succession of other native denominations. The Yoruba of western Nigeria dubbed it bomubomu and the Kanuri of north western Nigeria recognised it as kayou (Amna *et al.*, 2022).

Analeptic herbs are source of valuable medication due to presence of important antimicrobial properties, immunomodulatory activities, maintenance of general health, precious therapeutic properties and healing potentials, thus, they ensure prevention and cure for several illness and disorders of humans (Silvia *et al.*, 2021). Analeptic herbs are utilized as an origin of beneficial medication due to existence of outstanding or noteworthy antimicrobial characteristics, immune-modulatory exercises, preservation of diseases, provision of total wellbeing, invaluable ameliorative qualities and restoring or soothing power when used in disease treatment (Emad *et al.*, 2023).

Phytochemicals procured from analeptic plants may govern or manage microbial multiplication in different disease circumstances and in definite occasion of particular disease therapy. The curative and therapeutic qualities of these herbs were very much acknowledged by our predecessors and have been scientifically verified today. Abstracts procured from different medicinal plants for prophylaxis and treatment of different illness yielded therapeutic modalities with vast spectrum of antimicrobial property on different pathogenic microorganisms (Sravanhi and Giri, 2021).

Materials and Methods

All chemicals and reagents used are of analytical grade and purchased from reputable companies' sigma and merck chemicals.

Collection of Plant Material

Fresh leaves of the medicinal plant *Calotropis procera* was collected in Sokoto North Local Government of Sokoto State. The sample was washed thoroughly 2-3 times with tap water and once with sterile distilled water then the leaves material was shade-dried until completely dried.

Identification of Plant Material

For identification, the plant material was identified by a Taxonomist at the Herbarium of Botany Unit, Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria and their voucher specimen was deposited at the department.

Preparation of Plant Material

The plant leaves sample was pulverized to powder using pestle and mortar. The pulverized plant leaves material of *Calotropis procera* (100 g) was extracted with methanol, ethyl acetate, chloroform and hexane. The extracts were kept at room temperature for the period of 72 hours then filtered with a Whatman filter paper No. 1 of pore size 11 µm. The filtrates were evaporated at 45 °C using rotary evaporator and the crude extracts were obtained.

Antimicrobial Assay

Collection, Growth and Maintenance of Test Microorganism for Antibacterial Studies

The microorganisms associated with skin, intestinal and urinary tract infections, also microorganisms abundantly found in unhealthy and dirty environment were selected and used for this assay. The skin, intestinal and urinary tract infections are common human infectious diseases and are sometimes difficult to treat completely either due to re-exposure of patients to the microbes as these bacterial species are abundant in the environment and/or due to resistance of the microbes to antibiotic drugs. The high incidence of microbial infection and antibiotic resistance is

one of the biggest threats to global health today. Antibiotics are medications used to prevent and treat microbial infections. The burden of microbial infection is already substantial in developed countries and some parts of Africa (WHO, 2018). Bacterial isolates were collected from the specialist hospital in Sokoto, Sokoto state. Bacterial isolates of gram-positive bacteria *Salmonella typhi*, *Streptococcus pneumoniae* and *Staphylococcus aureus* and gram-negative bacteria *Escherichia coli* and *Pseudomonas fluorescence* were collected and maintained on nutrient agar.

Antibacterial Screening Using Agar Well Diffusion Method

The prepared concentration of the methanol, ethyl acetate, chloroform and hexane extract 12.5, 25, 50, 100 and 200 mg/ml was carefully placed into each hole while the hole containing ciprofloxacin served as positive control. The petri dish was left for some minutes for diffusion of extract before incubation at 37 °C for 24 hours. The length of the zone of inhibition was measured using a transparent meter rule.

Determination of Minimum Inhibitory Concentration (MIC)

Procedure: A serial dilution concentration of the extracts was prepared and used for the test. Each concentration (5 ml) of Mueller Hinton Broth was pipetted into sterile test tubes and 0.1 ml of the bacterial suspension was added into to each test tube. The test tubes were then incubated at 37 °C for 24 hours and was examined for evidence of turbidity. The MIC values was identified as the least concentration that inhibited visible growth of the tested bacteria.

Determination of Minimum Bactericidal Concentration (MBC)

A serial dilution concentration of the extracts were prepared and used for the test, a loop of broth was collected from those plates which did not show any growth and was inoculated on sterile nutrient agar plates. Then the petri dish were incubated at 37 °C for 24 hours, MBC values was the least concentration of the extract that failed to show any visible growth.

Data Analysis

The data obtained was expressed as mean $\pm$ standard deviation. Homogeneity and normality test were performed. The difference between the groups was determined using one-way analysis of variance (ANOVA) and $p < 0.05$ was considered statistically significant. The difference between continuous variables among the groups was compared by Duncan's multiple comparison test using the statistical package SPSS version 20 software. A p -value of 0.05 was considered statistically significant. Only antibiotic ciprofloxacin was used.

Results

Antimicrobial Studies

The various extracts of *C. procera* significantly ($p < 0.05$) inhibited the growth of bacterial species tested respectively and in an increasing concentration dependent manner as showed in table 1. Furthermore, the highest antibacterial activity on *S. aureus* was significantly ($p < 0.05$) observed in chloroform leave extract of *C. procera* at concentration of 200 mg/ml (32.1 ± 0.20 mm). The extracts also showed minimum MIC value of 6 mg/ml, but, MBC value of 25 mg/ml.

Table 1: Antibacterial Activities of Methanol, Ethyl acetate, Chloroform and Hexane Leaf Extracts of *C. procera* and Standard Antibiotic

Zone of inhibition (mm)						
(i)Bacterial species	Methanol	Ethyl acetate	Chloroform	Hexane	Ciprofloxacin	Distilled Water
<i>S. typhi</i>	7.30 ±0.45 ^a	7.20 ±0.01 ^a	8.50±0.45 ^a	7.20±0.45 ^a	21.40±0.02 ^b	0.00
<i>Strep.</i>	7.50±0.01 ^a	7.50±0.45 ^a	8.50±0.06 ^a	7.20±0.67 ^a	20.40±0.45 ^b	0.00
<i>S. aureus</i>	7.30 ±0.45 ^a	7.50±0.01 ^a	8.90±0.03 ^a	7.50±0.45 ^a	21.8±0.02 ^b	0.00
<i>Pseudo</i>	7.20±0.06 ^a	7.50±0.20 ^a	8.80±0.45 ^a	7.90±0.20 ^a	21.40±0.04 ^b	0.00
<i>E. coli</i>	7.90±0.03 ^a	7.50±0.45 ^a	8.50±0.58 ^a	7.80±0.20 ^a	20.80±0.01 ^b	0.00
(ii)Bacterial species	Methanol	Ethyl acetate	Chloroform	Hexane	Ciprofloxacin	Distilled Water
<i>S. typhi</i>	10.30±0.12 ^a	11.20±0.20 ^a	11.20±0.12 ^a	9.80±0.02 ^b	22.90±0.45 ^c	0.00
<i>Strep.</i>	11.20± 0.58 ^a	10.30±0.02 ^a	11.20±0.58 ^a	9.60±0.67 ^b	23.50±0.12 ^c	0.00
<i>S. aureus</i>	10.20±0.01 ^a	10.40±0.45 ^a	11.30±0.45 ^a	8.90±0.45 ^b	20.90±0.01 ^c	0.00
<i>Pseudo</i>	10.60±0.45 ^a	11.20±0.06 ^a	10.50±0.45 ^a	9.70±0.02 ^b	23.70±0.02 ^c	0.00
<i>E. coli</i>	10.20±0.10 ^a	11.30±0.03 ^a	10.90±0.10 ^a	8.70±0.04 ^b	22.10±0.45 ^c	0.00
(iii)Bacterial species	Methanol	Ethyl acetate	Chloroform	Hexane	Ciprofloxacin	Distilled Water
<i>S. typhi</i>	14.30±0.01 ^a	13.60±0.58 ^b	15.90±0.02 ^a	12.00±0.01 ^b	24.20±0.02 ^c	0.00
<i>Strep.</i>	14.30±0.58 ^a	13.50±0.45 ^b	15.80±0.10 ^a	12.20±0.45 ^b	25.70±0.67 ^c	0.00
<i>S. aureus</i>	14.20±0.45 ^a	13.30±0.02 ^b	15.60±0.45 ^a	12.30±0.12 ^b	24.10±0.45 ^c	0.00
<i>Pseudo</i>	14.40±0.04 ^a	12.70±0.06 ^b	14.90±0.58 ^a	11.90±0.58 ^b	25.40±0.20 ^c	0.00
<i>E. coli</i>	14.40±0.45 ^a	12.60±0.02 ^b	15.70±0.04 ^a	11.70±0.45 ^b	26.80±0.02 ^c	0.00
(iv)Bacterial species	Methanol	Ethyl acetate	Chloroform	Hexane	Ciprofloxacin	Distilled Water
<i>S. typhi</i>	20.20±0.04 ^a	19.80±0.45 ^a	24.20±0.12 ^b	17.60±0.45 ^c	34.20±0.04 ^d	0.00
<i>Strep.</i>	20.30±0.20 ^a	19.50±0.20 ^a	28.50±0.58 ^b	18.20±0.10 ^c	33.80±0.01 ^d	0.00
<i>S. aureus</i>	20.40±0.20 ^a	19.10±0.45 ^a	28.40±0.04 ^b	18.20±0.20 ^c	32.70±0.20 ^d	0.00
<i>Pseudo</i>	21.00±0.04 ^a	19.50±0.04 ^b	25.20±0.45 ^c	17.40±0.45 ^d	34.40±0.45 ^c	0.00
<i>E. coli</i>	20.20±0.02 ^a	19.80±0.03 ^a	25.40±0.04 ^c	18.60±0.04 ^b	34.50±0.58 ^d	0.00
(v)Bacterial species	Methanol	Ethyl acetate	Chloroform	Hexane	Ciprofloxacin	Distilled Water
<i>S. typhi</i>	27.10±0.02 ^a	19.50±0.04 ^b	29.60±0.58 ^c	20.40±0.01 ^b	38.40±0.04 ^d	0.00
<i>Strep.</i>	26.10±0.20 ^a	19.30±0.10 ^b	32.80±0.58 ^c	21.90±0.45 ^a	38.80±0.01 ^d	0.00
<i>S. aureus</i>	26.20±0.45 ^a	19.40±0.45 ^b	32.80±0.58 ^c	20.80±0.02 ^b	37.20±0.20 ^d	0.00
<i>Pseudo</i>	26.00±0.58 ^a	19.30±0.06 ^b	27.50±0.45 ^c	20.40±0.58 ^b	39.10±0.04 ^d	0.00
<i>E. coli</i>	26.40±0.04 ^a	19.60±0.03 ^b	28.80±0.67 ^c	21.80±0.02 ^d	37.40±0.58 ^c	0.00

Values are mean inhibition zone (mm) minus the size of cup borra (6 mm) ± S.D of three replicates. Mean values having different superscript letters along the rows are significantly different ($p < 0.05$) (One-way ANOVA followed by Duncan's multiple comparison test). *Strep.* = *S. pneumoniae*, *Pseudo.* = *P. Fluorescence*. i, ii, iii, iv and v represent 12.5, 25, 50, 100 and 200 mg/ml

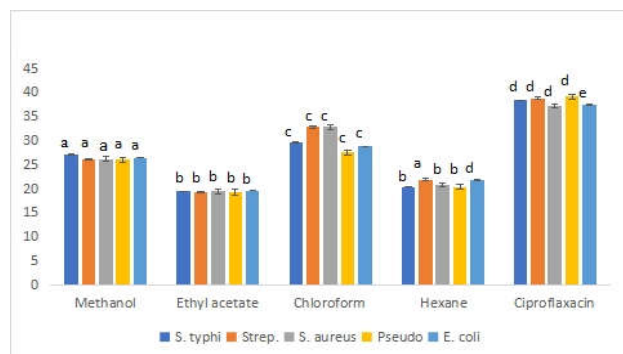


Figure 1: Antibacterial Activities of Methanol, Ethyl acetate, Chloroform and Hexane Leaves Extracts of *C. procera* (200 mg/ml) and Standard Antibiotic Ciprofloxacin (100 mg/ml) Tested by Agar Well Diffusion Method. (Values are mean inhibition zone (mm) minus the size of cup borra (6 mm) $\pm$ S.D of three replicates. Mean values having different superscript letters along the rows are significantly different ($p < 0.05$) (One-way ANOVA followed by Duncan's multiple comparison test). Strep.=*S. pneumonia*, Pseudo. = *P. Fluorescence*. i, ii, iii, iv and v represent 12.5, 25, 50, 100 and 200 mg/ml.)

Table 2: Minimum Inhibitory Concentration (MIC) of Methanol, Ethyl acetate, Chloroform and Hexane Leaves Extracts of *C. procera*.

Sample	Bacterial species	Concentration of Extracts mg/ml					MIC
		200	100	50	25	12.5	
Methanol	<i>S. typhi</i>	-	-	-	-	+	12.5
	<i>Strep.</i>	-	-	-	-	+	12.5
	<i>S. aureus</i>	-	-	-	-	+	12.5
	<i>Pseudo</i>	-	-	-	-	+	12.5
	<i>E. coli</i>	-	-	-	-	+	12.5
Ethyl acetate	<i>S. typhi</i>	-	-	-	-	+	12.5
	<i>Strep.</i>	-	-	-	-	+	12.5
	<i>S. aureus</i>	-	-	-	-	+	12.5
	<i>Pseudo</i>	-	-	-	-	+	12.5
	<i>E. coli</i>	-	-	-	-	+	12.5
Chloroform	<i>S. typhi</i>	-	-	-	-	+	12.5
	<i>Strep.</i>	-	-	-	-	+	6
	<i>S. aureus</i>	-	-	-	-	+	6
	<i>Pseudo</i>	-	-	-	-	+	6
	<i>E. coli</i>	-	-	-	-	+	6
Hexane	<i>S. typhi</i>	-	-	-	-	+	6
	<i>Strep.</i>	-	-	-	-	+	12.5
	<i>S. aureus</i>	-	-	-	-	+	12.5
	<i>Pseudo</i>	-	-	-	-	+	12.5
	<i>E. coli</i>	-	-	-	-	+	12.5
Ciprofloxacin	<i>S. typhi</i>	-	-	-	-	+	6
	<i>Strep.</i>	-	-	-	-	+	6
	<i>S. aureus</i>	-	-	-	-	+	6
	<i>Pseudo</i>	-	-	-	-	+	6
	<i>E. coli</i>	-	-	-	-	+	6

Key: Sign (-) indicates on visible growth of the microorganisms while sign (+) indicates the visible growth of the microorganisms. Strep.=*S. pneumonia*, Pseudo. = *P. Fluorescence*.

Table 3: Minimum Bactericidal Concentration (MBC) of Methanol, Ethyl acetate, Chloroform and Hexane Leaf Extracts of *C. procera*.

Sample	Bacterial species	Concentration of Extracts mg/ml					MBC
		200	100	50	25	12.5	
Methanol	<i>S. typhi</i>	-	-	-	+	+	25
	<i>Strep.</i>	-	-	-	+	+	25
	<i>S. aureus</i>	-	-	-	+	+	25
	<i>Pseudo</i>	-	-	-	+	+	25
	<i>E. coli</i>	-	-	-	+	+	25
Ethyl acetate	<i>S. typhi</i>	-	-	-	+	+	25
	<i>Strep.</i>	-	-	-	+	+	25
	<i>S. aureus</i>	-	-	-	+	+	25
	<i>Pseudo</i>	-	-	-	+	+	25
	<i>E. coli</i>	-	-	-	+	+	25
Chloroform	<i>S. typhi</i>	-	-	-	+	+	25
	<i>Strep.</i>	-	-	-	+	+	25
	<i>S. aureus</i>	-	-	-	+	+	25
	<i>Pseudo</i>	-	-	-	+	+	25
	<i>E. coli</i>	-	-	-	+	+	25
Hexane	<i>S. typhi</i>	-	-	-	+	+	25
	<i>Strep.</i>	-	-	-	+	+	25
	<i>S. aureus</i>	-	-	-	+	+	25
	<i>Pseudo</i>	-	-	-	+	+	25
	<i>E. coli</i>	-	-	-	+	+	25
Ciprofloxacin	<i>S. typhi</i>	-	-	-	+	+	25
	<i>Strep.</i>	-	-	-	+	+	25
	<i>S. aureus</i>	-	-	-	+	+	25
	<i>Pseudo</i>	-	-	-	+	+	25
	<i>E. coli</i>	-	-	-	+	+	25

Key: Sign (-) indicates on visible growth of the microorganisms while sign (+) indicates the visible growth of the microorganisms.



Strep.=*S. pneumonia*, Pseudo. = *P. Fluorescence*.

Figure 2: Antimicrobial Studies; Petri Dish Showing Zone of Inhibition of Microorganisms by Plant Extracts.

Plates A and B: Zone of inhibition of bacterial species.
Plates C and D: Minimum inhibitory concentration (MIC).
Plates E and F: Minimum bactericidal concentration (MBC).

Discussion

The antibacterial property of the analeptic herb *C. procera* was investigated by agar well diffusion method and the results obtained showed that the various extracts of the analeptic herb possessed antibacterial property on the tested microorganisms which are bacterial species *S. pneumoniae*, *S. aureus*, *S. typhi*, *P. fluorescence* and *E. coli*. Results obtained from antibacterial investigation are showed in Table 1. The antibacterial activities of the four extracts tested which are methanol, ethyl acetate, chloroform and hexane leave extracts of *C. procera* at the concentrations of 12.5, 25, 50, 100 and 200 mg/ml significantly ($p < 0.05$) inhibited the growth of bacterial species tested respectively and in an increasing concentration dependent manner. Furthermore, the highest antibacterial activity against *S. pneumoniae* and *S. aureus* was significantly ($p < 0.05$) observed in chloroform leave extract of *C. procera* at concentration of 200 mg/ml (32.8 ± 0.50 mm) when compared to the other plant extracts. The crude chloroform leaves extract of *C. procera* also inhibited the growth of *S. typhi* followed by inhibition on *E. coli*. While a non-significantly ($p > 0.05$) different in high antibacterial activity against bacteria *S. aureus*, *S. pneumoniae*, *P. fluorescence* and *E. coli* followed by *S. typhi* was observed in methanol extract. And a non-significantly ($p > 0.05$) different in high antibacterial activity against bacteria *S. pneumoniae* and *E. coli* was observed in hexane extract followed by inhibition on the other three bacterial species. A non-significantly ($p > 0.05$) different in lowest antibacterial activity against the five tested bacteria species was observed in ethyl acetate extract of *C. procera*. Significantly ($p < 0.05$) highest antibacterial activity was observed in the standard antibiotic ciprofloxacin when compared with the four leaves extracts.

The results of minimum inhibitory concentration (MIC) of methanol, ethyl acetate, chloroform and hexane leaves extracts of *C. procera* showed that

highest inhibition compared to that of ciprofloxacin was observed in chloroform leaves extracts of *C. procera*. Furthermore, methanol, ethyl acetate and hexane leaves extracts of *C. procera* equally inhibited the growth of all the five bacteria species tested *S. typhi*, *S. pneumoniae*, *S. aureus*, *P. fluorescence* and *E. coli* with MIC values of 12.5 mg/ml when compared with the standard antibiotic ciprofloxacin. The minimum bactericidal concentration (MBC) of methanol, ethyl acetate, chloroform and hexane leaves extracts of *C. procera* against bacteria species tested showed that the various leaves extracts equally inhibited the growth of all the five bacteria species tested *S. typhi*, *S. pneumoniae*, *S. aureus*, *P. fluorescence* and *E. coli* with MBC values of 25 mg/ml. The MIC and MBC results further highlighted the antibacterial activity leaves extracts of *C. procera*.

The four leaves extracts of *C. procera* studied, that is methanol, ethyl acetate, chloroform and hexane leave extracts of *C. procera* possessed antibacterial activities. The highest antibacterial activity recorded was on *S. pneumoniae* and *S. aureus* with mean zone of inhibition value of (32.8 ± 0.50 mm) observed in chloroform leave extract of *C. procera* at concentration of 200 mg/ml. Therefore, chloroform leaves extract of *C. procera* is the most active extract when compared with the other three extracts, then followed by antibacterial activity of methanol extract, followed by antibacterial activity hexane extract and lastly the antibacterial activity ethyl acetate extract.

The results of this study conform to the findings of Naser *et al.*, 2018; He reported that the leaves extract of *C. procera* had strong antibacterial activity. In his research Naser *et al.*, 2018; reported that leaves extracts of *C. procera* at different concentration inhibited the growth of bacterial species. The results of this study also conform to the findings of Amna *et al.*, 2022. The report stated that the leaves extract of *C. procera*

showed antibacterial activity and the antibacterial activity is attributed to the presence of secondary metabolites. The report of Amna *et al.*, 2022 experiments indicated promising antibacterial activity of *C. procera* against some bacterial species. The findings in this study also agree with the earlier report of Muhammad *et al.*, which revealed that leaves extract of *C. procera* had high antibacterial activity.

Recent study on qualitative and quantitative phytochemical analysis of the extract *C. procera* have revealed the presence of phytochemicals (Amna *et al.*, 2022). Antimicrobial mechanisms of action of analeptic herbs are multi-fold and mostly rely on their phytochemical composition and concentration, bioactive principles and their synergistic and antagonistic actions (Sravanthi and Giri, 2021, Emad *et al.*, 2023). Analeptic herbs use broad spectrum of secondary metabolites as specialized metabolites or natural products that mediate interactions with the environment of the host (Emad *et al.*, 2023). Every analeptic plant species might be endowed with hundreds of different secondary metabolites and these phytochemicals obviously have beneficial role in defence from herbivores animals, pathogenic microbes, viruses or competing plants and signal compounds to attract pollinating or seed dispersing animals (Silvia *et al.*, 2021). Therefore, the observed antimicrobial activities of the medicinal plant highlighted that these plant extracts are antimicrobial agents.

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Conclusion

The results of antibacterial activity obtained from this research highlighted that the methanol, ethyl acetate, chloroform and hexane leaves extracts of *C. procera* exhibited antibacterial activities on the five tested microbes, therefore, the plant extract can be an important source of useful compounds for the development of new therapeutic drugs for the treatment of diseases caused by these microorganisms.

Recommendation

This research identified that methanol, ethyl acetate, chloroform and hexane leaves extracts of *C. procera* had antimicrobial property therefore, the plant extract can be an important source of useful compounds for the development of new therapeutic drugs for the treatment of diseases caused by these microorganisms. However, it is important that the plants should be further investigated; Research should be performed on the antimicrobial mechanisms of action of chloroform leaves extracts of *C. procera*.

Conflict of Interest

There are no conflicts of interest.

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