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Research Paper

Phytochemical and Antibacterial Activity of Leaf Extracts of *Calotropis Procera* on some Selected Bacteria

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Bacterial infectious diseases are the most leading cause of death, accounting for approximately one half of all deaths. Hence, the study was designed to carry out the phytochemical screening and antibacterial potential of the ethanol, methanol and aqueous leaf extracts of *Calotropis procera.* Phytochemical analysis revealed the presence of tannins, saponins, alkaloids, flavonoids and cardiac glycosides. Using the Agar well diffusion method, the different extract showed visible inhibitory effect when compared to the positive control. The ethanol extract was observed to be more potent on *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Salmonella typhi* respectively than the methanol and aqueous extract. The best minimum inhibitory concentration (MIC) for the ethanol extract observed for *S. aureus, E. coli, P. aeruginosa* at 125 mg/ml; for methanol, it was also recorded at 125 mg/ml for *E. coli,* and *P. aeruginosa* while the aqueous extract showed the list MIC at 500 mg/ml for *E. coli* and *P. aeruginosa.* However, the ethanol extract showed the minimum bactericidal concentration (MBC) at 250 mg/ml for *S. aureus and E. coli* and methanol extract, at 250 mg/ml for *E.coli* while the aqueous extract showed MBC at 500 mg/ml on *E. coli* and no MBC result was obtained for the others. From these findings, it shows that the plant is a potential source of bioactive compounds that can be used in management of bacterial infections.

Keywords: Agar well diffusion, minimum bactericidal, bioactive potential, phytochemicals, inhibition

INTRODUCTION

Medicinal plants are used from the ancient time as the major sources of drugs. The fact is that we can obtain many of the presently available drugs, either directly in the extract form or in the modified synthetic form (Parihar and Balekar, 2016). Naturally, plants have the ability to synthesize products beneficial for us namely as phytoconstituents that are used to perform biological functions, which also protect us against predators such as virus fungi and other microorganisms. The phytoconstituents obtained from the natural products are one of the most successful strategies for the discovery of new drugs. *Calotropis procera* is a plant which is used in several traditional medicine and folklore systems to cure various ailments as reported in the Hindu literature (Parihar and Balekar, 2016). Phytomedicine derived from plants is more promising in the treatment of traceable infectious diseases. The World Health Organization (WHO) estimates that approximately 80% of the world`s population relies mainly on traditional medicine, predominantly originated from plants, for their primary healthcare (Igbinosa *et al.,* 2009). Natural products represent a rich source of antimicrobial agents with a low level of toxicity, a broad spectrum and sufficiently good pharmacokinetics to be clinically useful without chemical modification (Newman and Cragg, 2007). The plant, *Calotropis procera* is used in many African countries for the treatment of various ailment including bacterial diseases. *C. procera* commonly known as Giant Swallow wort, milkweed is a small to medium-sized shrub. *Calotropis procera* (Ait) R. Br. (Asclepiadaceae), is a plant commonly distributed throughout the tropics of Asia, South America, Africa and the Middle East (Nenaah, 2013; Kawo *et al*., 2013; Ahmed *et al.,* 2005; Mohanraj *et*

al., 2010). Various parts of the plant including the inner bark, stem, leaf, sap, roots, fruits and bark of the root have been found useful in the management of syphilis, cold, fever, rheumatism, indigestion, eczema and diarrhea. The dried root is used to cure bronchitis, asthma, leprosy, elephantiasis, hepatic and splenic enlargement and as eye tonic (Aworh *et al.,* 1994). The latex of *C. procera* is processed and used in treating vertigo, baldness, hair fall, toothaches, joints swellings and paralysis (Ahmed *et al*., 2005). *Calotropis procera* is propagated by seed, root suckers, root cuttings and stem cuttings. Each year hundreds to thousands of seeds may be produced per plant. There are about 100, 000 seeds/kg, half the seed weight being in the coma (seed floss). Seeds do not have dormancy; germination of fresh seed is 55-90% and varies from 1 to 9 weeks. All plant parts are toxic, due to the presence of cardiac glycosides (cardenolides) with 162 mg/g at dry weight, compared to 2 mg/g of the total leaf dry weight (Maroyi, 2012). There are several reports on the antimicrobial activities of *C. procera* from India, Saudi Arabia and some parts of Africa (Salem *et al*., 2014; Yesmin *et al*., 2008; Nenaah, 2013; Nenaah and Ahmed, 2011), but little is known about the Nigerian *C. procera.* Therefore, there is the need to investigate the antimicrobial activities of ethanol, methanol and aqueous extracts of *C. procera* to compare their activities on some pathogenic bacteria.

MATERIALS AND METHODS

Sample collection and preparation

Fresh leaves of *Calotropis procera* were collected from surrounding bush at the University of Abuja, F.C.T., Nigeria. They were authenticated at the herbarium Department of Biological sciences, University of Abuja. The sample was prepared according to the methods described by Oluduro *et al*. (2011); Anibijuwon and Udeze, (2009). The leaves of *C. procera* were air-dried to constant weight in the shade. The dried leaves were then pulverized to smaller granules using mortar and pestle and then powdered mechanically using a laboratory blender. The powdered sample was then stored in an air-tight plastic container for further use.

Extraction of the leaf extracts

Three different solvent were used for the extraction of the leaf sample. The method adopted was the one described by Ngulde *et al*. (2010) and Arekemase *et al*. (2011). Exactly 100 g of the powdered sample was weighed and extracted by cold extraction in 500 ml of the different solvents. The mixture was agitated at 30 min interval for 3 h and then soaked for 72 h. Subsequently the resulting mixture was then filtered using Whatman's No. 1 filter paper and the filtrate was evaporated to dryness using a water bath. The resulting extracts was weighed and stored in a sterile bottle and refrigerated at 4°C prior to testing.

Reconstitution of extracts and preparation of standard drug

This was done according to the methods described by Nascimento *et al*. (2000) and Arekemase *et al*. (2011). The extracts were reconstituted by measuring out 62.5,125, 250 and 500 mg of each extracts and diluting with 1 ml of dimethylsulphuroxide (DMSO) to give different concentrations of 62.5,125, 250 and 500 mg/ml respectively of each extracts. Also, 62.5,125, 250 and 500 mg of chloramphenicol was dissolved in 1 ml of sterile distilled water to obtain a concentration of 62.5, 125, 250 and 500 mg/ml, which was used as positive control and the DMSO, was used as negative control.

Determination of phytochemical constituents of the extracts

Chemical analysis of the dried extracts was carried out for the qualitative determination of phytochemical constituents as described by Akinyemi *et al.* (2005); Junaid *et al*. (2006) and Prashant *et al.* (2011).

Preparation of test organisms

The stock bacterial isolates used were obtained from the University of Abuja Teaching Hospital. Fresh purity plates of the test organisms were made from the isolate cultures obtained on agar slants. The isolates were authenticated in the laboratory of Microbiology by sub-culturing on selective and differential solid media and re-identified using colony morphology, gram reaction and biochemical tests namely catalase, oxidase and fermentation of sugars, mannitol and lactose (Cheesbrough, 2002; Baker *et al*., 1998). With the aid of sterile wire loop, colonies of fresh cultures of the different bacterial isolates were picked and suspended in 5 ml nutrient broth in a well-labeled sterile 10 ml bijou bottles. They were incubated at 37°C for 24 h. Five colonies were transferred into 5 ml of sterile nutrient broth in test tubes and incubated for 3 h at 37°C. The growth of bacterial suspension obtained was compared to that of freshly prepared barium sulphate solution (0.5 ml of a 1% barium in chloride to 99.5 ml of 1% H_2SO_4).The turbidity was adjusted by adding more sterile nutrient broth to match 0.5 McFarland standard (10^8 cfu/ml).

Determination of antibacterial activity of extracts

The antibacterial activities of the extracts were determined using agar well diffusion technique (Adeniyi *et al*., 1996). Mueller Hilton agar plates were each seeded with 0.05 ml of an overnight culture of each bacterial isolates (equivalent to 10^7-10^8 cfu/ml). The seeded

plates were allowed to set and then dried. A sterile cork borer of 6 mm diameter was used to bore four (4) uniform wells on the surface of the agar. Exactly 0.05 ml of different concentration of the extracts (500, 250, 125 and 62.5 mg/ml) was placed in the wells respectively. Also, two holes were bored on a plate at equidistant position and 0.2 ml of chloramphenicol was introduced into one of the holes to serve as positive control and 0.2 ml DMSO into the other hole to serve as negative control for each test organism. The plates were allowed on the bench for pre-diffusion for 40 min followed by an overnight incubation at 37°C for bacterial isolates. The degree of antibacterial activity of each extract was measured as the inhibition zone diameter in millimeters. The sensitivity test was done in duplicates. The mean of the two readings was taken to be the zone of inhibition of the bacterial concentration.

Determination of minimum inhibitory concentration (MIC)

MIC of the extracts was determined by dilution to various concentrations according to the macro broth dilution technique (Baron and Finegold, 1990; Akinyemi *et al.,* 2005). Standardized inoculum of the test organisms was added to series of sterile tubes of 5ml nutrient broth containing 500, 250, 125 and 62.5 mg/ml of the extracts and incubated at 37°C for 24 h. The MIC was read as the least concentration that inhibited the growth of the test organisms.

Determination of minimum bactericidal concentration (MBC)

The MBC was determined by collecting 1ml of broth culture from the tubes used for the MIC determination and sub-culturing onto fresh extract-free and drug-free solid agar plates. The plates were incubated at 37°C for 24 h. The least concentration that did not show any growth after incubation was regarded as the MBC according to Akinyemi *et al.* (2005).

RESULTS

The Phytochemical screening of the leaf extracts of *C. procera* presented in (Table 1) reveals the presence of some secondary metabolites such as alkaloids, saponins, tannins, cardiac glycosides and flavonoids but anthraquinones and phenols were not detected in the three extracts. Steroid was observed to be present in both methanol and aqueous extracts but absent in the ethanol extracts. The results of the antibacterial activity presented in (Table 2) revealed that the extracts produced visible inhibitory activity against all the test organisms as compared to the positive control, chloramphenicol which is a standard drug. The ethanol extract showed the highest visible inhibitory activity on all the test organisms with *S. aureus* been the most susceptible with zone of inhibition ranging from 7.0 - 23.0 mm while the methanol leaf extract showed highest inhibitory activity on *E. coli.* Meanwhile*,* the aqueous leaf extract showed less activity on the test organisms than both the ethanol and methanol leaf extracts. The control was more potent than all the extracts, comparing their zones of inhibition. It also showed that *S. aureus* was most susceptible with zones of inhibition ranging from 23 - 36 mm, followed by *P. aeruginosa* ranging from 21 – 35 mm, then *E. coli* ranging from 18 – 33 mm and *S. typhi* with the least susceptibility ranging from 15 - 31 mm. The MIC of ethanol, methanol and aqueous leaf extracts of *C. procera* on the test organisms is presented in table 3. For the ethanol leaf extract, the MIC of *S. aureus*, *E. coli* and *P. aeruginosa* was at the concentration of 125 mg/ml, while for *S. typhi* was 250 mg/ml. Also, the MIC of the methanol leaf extract for both *E. coli* and *P. aeruginosa* was 125 mg/ml, 250 mg/ml for *S. aureus*. However, the MIC of the aqueous leaf extract for *S. aureus* was 250 mg/ml, while the MIC for both *E. coli* and *P. aeruginosa* was 500 mg/ml. The MBC for the ethanol leave extract. *S. aureus* and *E. coli* was at the concentration of 250 mg/ml, *P. aeruginosa* was 500 mg/ml, while *S. typhi* had no MBC. For methanol, the MBC of *S. aureus* and *P. aeruginosa* was 500 mg/ml, *E. coli* was 250 mg/ml and *S. typhi* not active. For aqueous, the MBC for *E. coli* was 500 mg/ml, while *S. aureus* and *P. aeruginosa* had no MBC.

DISCUSSION

The phytochemical analysis for all the leaf extract of *C. procera* showed the presence of alkaloids, saponins, tannins, cardiac glycosides and flavonoids in all the extract. Terpenoids were seen to be absent in ethanol and methanol extract but present in aqueous extract while reducing sugars were seen to be present in both ethanol and methanol extract and absent in aqueous extract. Also steroids were present in both methanol and aqueous extract but absent in ethanol extract while anthraquinones and phenols were seen to be absent in the three solvent extracts. This is somewhat in agreement with the findings of Kareem *et al.* (2008) that used only the ethanol and aqueous solvent. The flavonoids and tannins are phenolic compounds and plant phenols are major group of compounds that act as primary antioxidants (Parihar and Balenkar, 2016). The presence of these phytochemicals maybe responsible for the reported antibacterial activities of the plants. The antibacterial screening indicated that the three (3) different extracts of *C. procera* had visible inhibitory effects on the test organisms. From the results obtained, all extracts of the leaf of *C. procera* were active against all the test bacteria. The findings of this study agree with the earlier studies of other investigators (Mainasara *et al*., 2012; Kareem *et al*., 2008; Yesmin *et al*., 2008). All the extracts demonstrated antibacterial activity with ethanol extract showing the highest potency than other solvents

Table 1. Phytochemical properties of *C. procera* leaf extract.

Parameters	Ethanol extract	Methanol extract	Aqueous extract
Tannins	+	+	+
Saponins	+	+	+
Alkaloids	+	+	+
Steroids	_	+	+
Flavonoids	+	+	+
Cardiac glycosides	+	+	+
Anthraquinones	_	_	_
Reducing sugars	+	+	_
Terpenoids	_	_	+
Phenols	_	_	_

Key: + = Present, - = Absent

Table 2. Antimicrobial activity of the three different extracts of *C. procera* leaf.

Test organisms	Concentration (mg/ml)	Mean zone of inhibition (mm) (x ± σ)*			
		Ethanol extract	Methanol extract	Aqueous extract	Chl. (control)
Staphylococcus aureus	500	23.0 ± 0.71	22.5 ± 0.35	19.0 ± 0.71	36
	250	16.5 ± 0.35	18.5 ± 0.35	16.5 ± 0.35	31
	125	11.0 ± 0.71	13.0 ± 0.71	13.0 ± 0.71	29
	62.5	7.0 ± 0.71	9.5 ± 0.35	8.0 ± 0.71	23
Escherichia coli	500	16.0 ± 0.71	19.5 ± 0.35	14.0 ± 0.71	33
	250	12.0 ± 0.71	16.5 ± 0.35	10.0 ± 0.71	29
	125	9.5 ± 0.35	11.0 ± 0.71	8.0 ± 0.71	24
	62.5	7.0 ± 0.71	8.5 ± 0.35	6.5 ± 0.35	18
Pseudomonas aeruginosa	500	22.0 ± 0.71	21.0 ± 0.71	18.0 ± 0.71	35
	250	17.5 ± 0.35	17.5 ± 0.35	12.5 ± 0.35	32
	125	14.0 ± 0.71	12.0 ± 0.71	9.5 ± 0.35	27
	62.5	10.5 ± 0.35	8.0 ± 0.71	7.5 ± 0.35	21
Salmonella typhi	500	18.0 ± 0.71	12.5± 0.35	12.5± 0.35	31
	250	13.0 ± 0.71	9.5 ± 0.35	11.0± 0.71	27
	125	10.5 ± 0.35	8.0 ± 0.71	8.5 ± 0.35	21
	62.5	7.5 ± 0.35	6.5 ± 0.35	6.5 ± 0.35	15

KEY: Chl = Chloramphenicol

used in this research work. The ethanol extract showed good zone of inhibition at higher concentrations of 125, 250 and 500 mg/ml with mean zones of inhibition ranging from 9.5 ± 0.35 to 23 ± 0.71 mm. The methanol and aqueous extract also showed a wide range of antibacterial activity against *S. aureus, E. coli, S. typhi* and *P. aeruginosa,* which is in partial agreement with the work of Yesmin *et al.* (2008). The methanol extract was more effective against *S. aureus,* (9.5± 0.35 – 22.5 ± 0.35mm) which is close to what was reported (20.0±0.75 mm) for methanolic extract of *C. procera* on *S. aureus* and (11.0 ± 0.70 mm) for *E. coli* (Nenaah, 2013). Also, it was observed to be more effective against *P. aeruginosa, E. coli,* but *S. typhi* (Table 2) been less susceptible as against the report of Salem *et al*. (2014) which they reported negative (no inhibition) and in contrast to the work of Mainasara *et al*. (2012). The aqueous extract showed more activity against *S. aureus* and *P. aeruginosa* which is in variance with the work of Kareem *et al.* (2008). Also the aqueous extract was effective against *S. aureus, E. coli, S. typhi and P. aeruginosa* in contrast to the findings of Adoum *et al*. (1997). Ali *et al*. (2014), in a different studies, using the ethanolic, methanolic and chloroform extracts of this plant exhibited good antibacterial properties against different pathogens and the flowers of the plant are highly effective against multidrug resistant organisms. Traditionally, crude plant extracts are prepared with water as infusions and decoctions; therefore, it is very likely that the herbalist is unable to extract all these compounds which are responsible for the activity observed in ethanol extract. It was also observed from this work that the higher the concentration, the more their activity and as the concentration decreases the lower the antibacterial activity. Hence an acceptable and effective dosage can be prepared by traditional healers for the control and eradication of bacterial pathogens. Nenaah and Ahmed, (2011) reported that regardless of the microorganism tested; the extraction solvent is a determinant factor for the extraction of microbial agents. All the test organisms

Table 3. Minimum inhibitory concentration of the different extracts of *C. procera* leaf on the tested organisms.

Test organisms	Concentration (mg/ml)	Ethanol extract	Methanol Extract	Aqueous Extract
Staphylococcus aureus	500	-	-	-
	250	-	-	-
	125	-	+	+
	62.5	+	+	+
Escherichia coli	500	-	-	-
	250	-	-	+
	125	-	-	+
	62.5	+	+	+
Pseudomonas aeruginosa	500	-	-	-
	250	-	-	+
	125	-	-	+
	62.5	+	+	+
Salmonella typhi	500	-	+	+
	250	-	+	+
	125	+	+	+
	62.5	+	+	+

KEY: + = growth (turbid), - = no growth (clear).

Table 4. The MBC of different extracts of *C. procera* leaf the tested organisms.

Test organisms	Concentration (mg/ml)	Ethanol extract	Methanol Extract	Aqueous Extract
Staphylococcus aureus	500	-	-	+
	250	-	+	+
	125	+	+	+
Escherichia coli	500	-	-	-
	250	-	-	+
	125	+	+	+
Pseudomonas aeruginosa	500	-	-	+
	250	+	+	+
	125	+	+	+
Salmonella typhi	500	+	+	+
	250	+	+	+

KEY: + = Growth on Agar plate, - = No growth on Agar plate.

were much more susceptible to the commercial antibiotics (Chloramphenicol) than to the crude plant extract in their different concentrations. This may be ostensibly due to the fact that the active ingredient in Chloramphenicol is in fine and purified form whereas the active ingredient in the plant extract is in a crude, impure, unrefined form and in a mixture with other unknown compounds. The MIC of the ethanol leaf extract, ranges from 125 - 500 mg/ml on *S. aureus, E. coli* and *P. aeruginosa* respectively and 250 - 500 mg/ml on *S. typhi*. The MIC of the methanol leaf extracts ranges from 125-500 mg/ml on *S. aureus* and *P. aeruginosa*, and 250-500 mg/ml on *E.coli*. On the other hand, the aqueous leaf extract produced an MIC value of 500 mg/ml on *E. coli* and *P. aeruginosa*. These values were higher than what was reported (5.0 – 10.5 mg/ml) by Shobowale and Olatope, (2013) and Nenaah and Ahmed, (2011) who reported MIC of the methanolic extract of the leaf to be 0.25 – 1.25 mg/ml. Doughari *et al*. (2007), reported that high MIC may be an indication of low efficacy or that the organisms have the potential for developing resistance to the bioactive compounds and low MIC is a good indication of high efficacy against this bacterium. This is in agreement with the present study as shown in (Table 3), with the extract having an MIC value of 125 mg/ml and above. For ethanol extract, the minimum bactericidal

concentration (MBC) showed 250 mg/ml for *S. aureus and E. coli* and 500 mg/ml for *P. aeruginosa.* For methanol extract, the MBC showed 500 mg/ml for *S. aureus* and *P. aeruginosa* and 250 mg/ml for *E.coli*. For aqueous extract, MBC is 500 mg/ml on *E. coli* and no MBC result was obtained for the others as shown in (Table 4).

Conclusion

In conclusion, this research work showed that the leaf extract of *C. procera* is rich in phytochemical compounds such as flavonoids, tannins, cardiac glycosides, alkaloids and saponins. These phytochemicals have been reported to be of pharmaceutical importance. This supports the use of this plant in folklore medicine for the herbal treatment of gastrointestinal infections. The extracts of *C. procera* used in this work were also found to possess antibacterial properties, making this plant a potential source of bioactive compounds that can be used in the management of bacterial infections. This can go a long way in contributing to the improvement of health care delivery in Nigeria if the active chemical compounds capable of inhibiting the growth of the test bacteria are analyzed and compounded into dosage forms for use. It is hoped these compounds will lead to the formulation of new and more potent antimicrobial drugs that will prove useful in the treatment of infections caused by microorganisms that have developed multiple resistance to currently available synthetic antimicrobial compounds.

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