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PHYTOCHEMICAL PROPERTIES AND ANTIBACTERIAL ACTIVITIES OF THE LEAF AND LATEX EXTRACTS OF *CALOTROPIS PROCERA* (AIT.F.) AIT.F.

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ABSTRACT
A comparative preliminary study on the phytochemistry and antibacterial effects of ethanolic and aqueous extracts of the leaves and latex of Calotropis procera on four pathogenic clinical bacterial isolates namely Escherichia coli, Staphylococcus aureus, Salmonella species and Pseudomonas species was carried out using paper-disc diffusion and broth dilution techniques. The results obtained revealed that ethanol was the best extractive solvent for a fraction with antibacterial properties of the C. procera leaves and latex. Generally, the aqueous extracts showed no activity on the isolates. The minimum inhibitory concentration (MIC) for the leaf ethanolic extract was 1000 – 2000 µg/ml while the minimum bactericidal concentration (MBC) of the latex ethanolic extract was 2000 µg/ml. Phytochemical analysis of the leaf and latex extracts showed the presence of tannins, steroids, saponins and flavonoids while alkaloids were absent in both extracts. Generally, the antibacterial effects of the plant parts revealed that the leaf extracts had stronger activity in comparison with those of the latex.

Keywords: Phytochemistry, antibacterial effects, Calotropis procera, Leaf and latex extracts.

INTRODUCTION

Medicinal plants being an effective source of both traditional and modern medicines are genuinely useful for primary health care. World Health Organization (1978) has advocated traditional medicine as safe remedies for ailments of both microbial and non-microbial origin. It was further added that the use of plant extracts and phytochemicals with antimicrobial properties may be of importance in therapeutic treatments, whereas in the past few years, a number of studies have been conducted in different countries to prove such efficiencies (Ikram and Inamul, 1984; Sousa *et al*., 1991; Oyi *et al*., 2002; Uba *et al*., 2005; Oyi *et al*., 2007; Salihu and Garba, 2008). It is therefore pertinent to investigate such plants thoroughly to determine their pharmacological properties as well as the efficiency of their various parts for antimicrobial activities (Ellof, 1998). In Nigeria, *Calotropis procera* is either used alone or with other herbs to treat common diseases such as fever, rheumatism, indigestion, cold, eczema and diarrhoea. In addition, preparations from the latex with honey are used as antibodies and also in the treatment of toothaches and cough (Kew, 1985). The leaf extract, chopped leaf and latex of *C. procera* have shown great promise as nematicides *in-vitro* and *in-vivo* (Anver and Alam, 1992). Kuta (2006) reported the stem-bark of *C. procera* as a promising antifungal agent, which could be used against dermatophytes and suggests that *C. procera* could be a potential source of chemotherapeutic agents, thus could be used for the treatment of tinea diseases. The leaf and fruit extracts of *C. procera* when boiled together can be used in the extraction of guinea worm by immersion of the infected limbs, either for several hours of three consecutive days. The dry leaves in northern Nigeria are used as a remedy for asthma, cough, etc (Oke *et al*., 2004; Noatay, 2005). The dry leaves and pithy stems are burned for patients to inhale the smoke or the leaves are smoked like tobacco in a pipe for the treatment of paralysis, arthralgia, swellings and intermittent fevers (Agharkar, 1991). Different parts of *C. procera* have been reported to exhibit ethnomedicinal and nutritional properties while phytochemical evaluation of the plant parts revealed the presence of essential and trace elements in varied quantities (Abhay *et al*., 1997; Adoum *et al*., 1997; Awune, 2000; Olasupo *et al*., 2004). Its use in West Africa was first documented by Dalziel (1937), who reported its use as a local anaesthetic. Irvine (1961) also reported the use of the decoctions derived from the root bark for the treatment of syphilis. Adoum *et al* (1997) showed that the latex of *C. procera* possesses an antibacterial activity against *E. coli*, *S. aureus*, *Salmonella* and *Pasteurella* species. *C. procera* (Syn. *Asclepias procera* Aiton) is of the family Asclepiadaceae (Apocynaceae). It is a small to medium-sized shrub, up to 5.5 m high, occasionally branchless to a height of 2.5 m. The bark is fibrous, scaly, deeply fissured when old, grey to light brown. All parts of the plant exude white latex when cut or broken. It is commonly called the Sodom apple and locally known as 'tumfafiya' in Hausa.

The name 'aak' is vernacular. Its other local names are madar, akanda, arks, etc (Noatay, 2005). The different ethnic groups in Nigeria identify the plant by a series of other vernacular names. The Yorubas of western Nigeria call it 'Bomubomu' and the Kanuri of north-western Nigeria know it as 'Kayou' (Adoum *et al*., 1997). It is a plant of the dry savanna and other arid areas, mostly anthropogene occurring around villages (Aliyu, 2006). The only two species of this genus are *C. gigantea* and *C. procera*. The main difference between the two siblings is that while *C. gigantea* has white flowers, *C. procera* has pinkish white flowers (Noatay, 2005). The present work reports the phytochemical properties and comparative antibacterial activities of the aqueous and ethanolic extracts of the leaves and latex of *C. procera* with the view to further substantiate the earlier claims by various researchers on its potential use in traditional medicine.

MATERIALS AND METHODS

Collection and identification of the plant material

The leaves and latex of actively-growing *C. procera* plants were randomly and aseptically collected from around the female hostel (Nana Hall) at the old campus of Bayero University in Gwale Local Government Area of Kano State, northern Nigeria. The plant was first identified at the field using standard keys and descriptions (Dalziel, 1956; Gill, 1987; Keay, 1989). Its botanical identity was further confirmed and authenticated at the Herbarium Section of the Botany Unit of the Department of Biological Sciences, Bayero University, Kano, Nigeria. Voucher specimens (MA-AH2008) were preserved and stored at the Herbarium for future reference.

Preparation of the treatment samples

This was carried out in accordance with the method of Kareem *et al* (2003). Here, the latex of *C. procera* plant was obtained as exudates by hand plucking of fresh leaves of actively growing plant using aseptic techniques. The latex was collected into sterile, plastic containers by pressing and squeezing in-between fingers, the apex of the leaves to release as much as possible latex into the containers. After collection, the containers were cotton-plugged and stored at a temperature of 4ºC until required for use. Collections were made in the mornings on the days of each analysis. On the other hand, the leaves were obtained by hand-plucking. They were thoroughly washed under running tap water, rinsed with distilled water and finally air dried. The dried leaves were made into powder form using mortar and pestle as described by Fatope *et al* (1993). The content was then stored in air-dried containers until required for use.

Extraction protocols

This was carried out according to the method of Fatope *et al* (1993) using soxhlet extraction technique. A quantity (100 g) of the fine powder of the leaves was weighed and suspended into a 2500ml-capacity conical flask. This was percolated with 1000 ml of 95% ethanol while another (100g) was suspended into separate conical flask of 800 ml of distilled water. Each was allowed to stand for two weeks with constant shaking at regular intervals under room temperature. The percolates were then filtered and the solvents (ethanol and water) were evaporated using rotar evaporator (R110) to obtain the ethanolic and aqueous extracts of the leaves and latex respectively. These served as the stock solutions, which were stored in a refrigerator at 4ºC until needed for analysis.

Phytochemical screening

(a) Test for reducing sugars

One gram of the aqueous extract was weighed and placed into a test tube. This was diluted using 10 ml of de-ionised distilled water. This was fallowed by the addition of Fehling's solution. The mixture warmed to 40ºC in water bath. Development of brick-red precipitate at the bottom of the test tube was indicative of the presence of a reducing sugar. Same procedure was repeated using dimethylsulphoroxide (DMSO) as the diluent for the ethanolic extract (Brain and Turner, 1975).

(b) Test for resins

Two grams of the ethanolic extract was dissolved in 10ml of acetic anhydride. A drop of concentrated sulphuric acid was added. Appearance of purple colour, which rapidly changed to violet, was indicative of the presence of resins. Same procedure was repeated using the aqueous extract of the plant material (Cuilel, 1994).

(c) Test for tannins

Two grams of the aqueous extract was weighed and placed in a test tube. Two drops of 5% ferric chloride solution was then added. The appearance of a dark-green color was indicative of the presence of tannins. The same procedure was repeated using the ethanolic extract (Cuilel, 1994).

(d) Test for steroid glycosides

One gram of the ethanolic extract was weighed and placed in a test tube. This was dissolved in 2 ml of acetic anhydride, followed by the addition of 4 drops of chloroform. Two drops of concentrated sulphuric acid were then added by means of a pipette at the side of the test tube. The development of a brownish ring at the interface of the two liquids and the appearance of violet colour in the supernatant layer were indicative of the presence of steroid glycosides. Same procedure was repeated using the aqueous extract (Cuilel, 1994).

(e) Test for flavonoids

Two grams of the ethanolic extract was weighed, placed in a test tube, followed by the addition of 10 ml of DMSO.

The mixture was heated, followed by the addition of magnesium metal and 6 drops of concentrated hydrochloric acid. The appearance of red colour was indicative of the presence of flavonoids. Same procedure was repeated using aqueous extract (Sofowora, 1993).

(f) Test for alkaloids

One gram each of the ethanolic extract was weighed and placed into two separate test tubes. To the first test tube, 2-3 drops of Dragendoff's reagent was added while 2-3 drops of Meyer's reagent were added to the second test tube. The development of an orange-red precipitate (turbidity) in the first test tube (with Dragendoff's reagent) or white precipitate (turbidity) in the second test tube (with Meyer's reagent) was indicative of the presence of alkaloids. Same procedure was repeated using aqueous extract (Cuilel, 1994).

(g) Test for saponins

Five grams of the aqueous extract was weighed and placed in a test tube. This was followed by the addition of 5 ml de-ionised distilled water. The content was vigorously shaken. The appearance of a persistent froth that lasted for 15 minutes was indicative of the presence of saponins. Same procedure was repeated using DMSO for the ethanolic extract (Brain and Turner, 1975).

Test organisms

Clinical isolates of bacteria were used for the bioassay studies. The isolates included *Escherichia coli*, *Staphylococcus aureus* and the species of *Salmonella* and *Pseudomonas*. The isolates were obtained from the Pathology Department of Murtala Mohammed Specialist Hospital (MMSH), Kano, Nigeria. They were further confirmed using standard biochemical tests (citrate utilization, coagulase, oxidase and catalase) as described by Cheesbrough (2002). The isolates were maintained on freshly-prepared nutrient agar (oxoid) slants and kept in a refrigerator at 4ºC until required for use.

Preparation of extract concentrations

This was carried out using standard method (Cheesbrough, 2002). Stock solution of the ethanolic extract was prepared by weighing 10 mg of it and dissolved in 1ml of dimethylsulphoroxide (DMSO) in Bijou bottle. This gave an extract concentration of 10,000 µg/ml (stock solution). Three varied extract concentrations (1000 µg/ml, 2000 µg/ml and 5000 µg/ml) were prepared from the stock solution (10,000 µg/ml) using 10-fold serial dilution. The same procedure was repeated using de-ionised distilled water for the aqueous extract.

Preparation of sensitivity discs

The sensitivity discs were prepared using sterile Whatman's No. 1 filter paper (Cheesbrough, 2002). The discs (6.0 ± 1.0 mm in diameter each) were prepared by punching the filter paper appropriately. Ten discs were dispensed into each concentration (impregnation) by means of sterile forceps. DMSO and ciprofloxacin (30 µg/g) were used as negative and positive controls respectively (Jawetz and Adelberg, 2004).

Preparation and standardization of the inoculum

An overnight broth culture of the test bacterium was used to prepare a standard inoculum of 3.30 x 10^6 cfu/ml. This value was arrived at by appropriate dilutions of the broth culture in 0.85% sodium chloride solution to match with the standard turbidity of 1% barium sulphate suspension (Cheesbrough, 2002).

Sensitivity testing

Agar diffusion method was employed (Kirby *et al*., 1966). The freshly-prepared nutrient agar (oxoid) plates were dried in a dryer (CH 306 model; Gallenkamp, England) for about 10 minutes to remove surface moisture. The plates were aseptically inoculated uniformly with the test organism by streaking method. With the aid of a sterile forceps, impregnated paper discs containing the leaf extract of *C. procera* at different concentrations were arranged radially and pressed firmly onto the inoculated agar surface to ensure even contact. Each disc was sufficiently spaced out and kept at least 15mm from the edge of the plate to prevent overlapping of zones. The same procedure was repeated for the latex extracts and the plates were allowed a pre-diffusion time of 15minutes. Control tests were done on the same plates by placing a standard antibiotic disc of ciprofloxacin (30 µg/g) for bacteria and disc containing only DMSO on the inoculated plates. The plates were incubated aerobically at 37ºC for 18 hours. Diameters of zones of inhibition were measured using millimeter rule, recorded and interpreted in accordance with Cheesbrough (2002).

Determination of minimum inhibitory and bactericidal concentrations of the extracts

The MIC and MBC were determined in accordance with the method of Cheesbrough (2002). Varied extract concentrations were prepared to arrive at 2000, 1000, 500 and 250 µg/ml. A quantity (0.1 ml) of the suspension of the test bacterium (standardised inoculum) was inoculated onto fresh nutrient agar (oxoid) plates at the different extract concentrations. The plates were incubated at 37±1ºC for 18 hours. The lowest concentration of the extract that inhibited the growth of the test bacterium was noted and recorded as the MIC while the MBC was determined by taking a loopful from each negative (no growth) tube in the MIC assay and inoculated onto fresh nutrient agar (oxoid). The plates were incubated at 37±1ºC for 24 hours after which they were observed for growth or otherwise of the test organism.

RESULTS AND DISCUSSION

Many naturally-occurring compounds found in plants have been shown to possess antimicrobial functions and could thus serve as a source of both traditional and orthodox medicine (Kim *et al*., 1995; Abhay *et al*., 1997; Adoum *et al*., 1997; Awune, 2000; Musa *et al*., 2000; Kalamba and Kuneika, 2003; Akinyemi *et al*., 2007; Yusha'u *et al*., 2008). For example, flavonoids are known to inhibit bacterial growth (Lutterodt *et al*., 1994; Mbuh *et al*., 2007).

A yield of 12 g and 11 g of the ethanolic and aqueous extracts from the initial crude weights of 100 g and 80 g respectively were recovered from the leaf while 4 ml and 4.5 ml from the initial volume of 75 ml each were recovered from the ethanolic and aqueous extracts of the latex of *C. procera* respectively. Physical characteristics of the extracts indicate that there were variations in the colour, odour and texture of the extracts (Table 1). Table 2 presents the phytochemical compounds recovered from the extracts of the plant parts screened in which only alkaloids were absent. Tables 3-6 present the antibacterial activity pattern of the extracts. The results show that the ethanolic extracts of both the leaf and latex of *C. procera* have antibacterial activities on *E. coli* and *S. aureus* but with no activity against *Salmonella* sp and *Pseudomonas* sp at all concentrations. However, the antibacterial effect was more pronounced against *E. coli*, which was seen to be more sensitive to the both the leaf and latex ethanolic extracts at a concentration of 10,000 µg/ml with zones of inhibition of 15 mm (Table 4) and 10 mm (Table 6) respectively. In the case of *S. aureus*, it was only inhibited by the leaf ethanolic extract with a highest recorded zone of inhibition (10 mm) at 10,000 µg/ml (Table 4) while no activity was observed at 500 µg/ml. The antibacterial activities observed could be due to the presence of secondary metabolites (Tables 1-2), which have been reported as active constituents responsible for antimicrobial activities (Sofowora, 1993; Adoum *et al*., 1997; Oyi *et al*., 2002; 2007; Salihu and Garba, 2008). These observations contradict Adoum *et al* (1997) who reported that the aqueous latex extracts of *C. procera* possess an antibacterial activity against *E. coli*, *S. aureus* and *Salmonella* species. On the other hand, the aqueous extracts of both the leaf (Table 3) and latex (Table 5) tested at all concentrations had no activity against all the test micro-organisms. This could be due to the presence of the dissolved phytochemicals at low concentrations in the aqueous extracts; hence the phytochemicals were more dissolved in the ethanol and thus responsible for the antibacterial activity exhibited in this study.

The apparent resistance of the test bacteria against these extracts at almost all concentrations may be a result of transfer of resistance plasmids or indiscriminate and sub-therapeutic use of the extracts. Of particular recognition in this study is the non-susceptibility of *Pseudomonas* and *Salmonella* species to all the extracts tested at all concentrations. This may partly be explained by some earlier reports that *Pseudomonas* species exhibited strong resistance against a host of antibiotics including plant extracts (Normansell, 1982; Nwachukwu, 2000; Nwachukwu *et al*., 2001; Bibitha *et al*., 2002). The results of the present study further support Bibitha *et al* (2002) who reported variation in the antibacterial activities of different plant extracts. On the other hand, the variations observed in potency of the plant parts screened to inhibit bacterial growth are in conformity with the reports of Duke (1992) and Yusha'u *et al* (2008) that antibacterial activity may vary from one plant part to another. Previous studies have shown that tannins bind the cell wall of bacteria, preventing growth and protease activity and can also be toxic to filamentous fungi, yeasts and ruminal bacteria (Jones *et al*., 1994; Oyewole *et al*., 2004). Cardiac glycosides, which have been reported to have antimicrobial properties (Rucker *et al*., 1992; Murakami *et al*., 1993), were found in all the extracts. Saponins were detected in all the extracts. They are effective in the treatment of syphilis and certain skin diseases (Trease and Evans, 1989; Oyewole *et al*., 2004). Flavonoids are known for their anti-allergic effect as well as a wide variety of activity against Gram-positive and Gram-negative bacteria, fungi and viruses (Afolayan and Meyer, 1997). The properties of the phytochemical ingredients (Tables 1-2) could have attributed to the results of the antibacterial activities observed in the present study (Tables 3 and 6). The high MICs (Table 7) and MBCs (Table 8) of the extracts could be due to high resistance rate of the test isolates.

Table 1: Physical characteristics of the leaf and latex extracts of *Calotropis procera*

Plant part	Solvent	Initial weight (g)	Final weight (g)	Colour	Odour	Texture
Leaf	Ethanol	100.0	12.0	Dark-brown	Slightly repulsive	Oily
	Aqueous	80.0	11.0	Black	Pleasant fruity	Oily
Latex	Ethanol	75.0	4.0	Orange	Pleasant fruity	Gummy
	Aqueous	75.0	4.5	Milky-white	Tungent	Soft

Table 2: Phytochemical characteristics of the leaf and latex extracts of *Calotropis procera*

Ingredient	Ethanol	Aqueous
Reducing sugar	+	-
Tannins	+	+
Steroid glycosides	+	+
Resins	-	+
Alkaloids	-	-
Saponins	+	+
Flavonoids	+	+

Key: + = present; - = absent.

Table 3: Antibacterial activity of the aqueous extract of *C. procera* leaf

	Diameter of zone of inhibition (mm) / Extract concentration (µg / ml)					
Isolates	**500**	**1000**	**2000**	**5000**	**10,000**	**Control**
Escherichia coli	00	00	00	00	00	27.0
Staphylococcus aureus	00	00	00	00	00	28.0
Pseudomonas sp	00	00	00	00	00	43.0
Salmonella sp	00	00	00	00	00	33.0

Table 4: Antibacterial activity of the ethanolic extract of *C. procera* leaf

	Diameter of zone of inhibition (mm) / Extract concentration (µg / ml)					
Isolates	**500**	**1000**	**2000**	**5000**	**10,000**	**Control**
Escherichia coli	07	09	11	12	15	24.0
Staphylococcus aureus	00	07	08	08	10	26.0
Pseudomonas sp	00	00	00	00	00	40.0
Salmonella sp	00	00	00	00	00	37.0

Table 5: Antibacterial activity of the aqueous extract of *C. procera* latex

	Diameter of zone of inhibition (mm) / Extract concentration (µg / ml)					
Isolates	**500**	**1000**	**2000**	**5000**	**10,000**	**Control**
Escherichia coli	00	00	00	00	00	24.0
Staphylococcus aureus	00	00	00	00	00	25.0
Pseudomonas sp	00	00	00	00	00	36.0
Salmonella sp	00	00	00	00	00	35.0

Table 6: Antibacterial activity of the ethanolic extract of *C. procera* latex

	Diameter of zone of inhibition (mm) / Extract concentration (µg / ml)					
Isolates	**500**	**1000**	**2000**	**5000**	**10,000**	**Control**
Escherichia coli	00	08	09	09	10	28.0
Staphylococcus aureus	00	00	00	00	00	24.0
Pseudomonas sp	00	00	00	00	00	42.0
Salmonella sp	00	00	00	00	00	34.0

Table 7: Minimum Inhibitory Concentration of the ethanolic extract of *C. procera* leaf

	Concentration of extract (µg / ml)			
Isolates	**2000**	**1000**	**500**	**250**
E. coli	-	-	+	+
S. aureus	-	+	+	+

Key: + = Growth not inhibited; - Growth inhibited

Table 8: Minimum Bactericidal Concentration of the ethanolic extract of *C. procera* latex

	Concentration of extract ((µg / ml)			
Isolate	**2000**	**1000**	**500**	**250**
E. coli	-	+	+	+

Key: + = Growth not inhibited; - Growth inhibited

CONCLUSIONS AND RECOMMENDATIONS

Based on the pharmacological results of the present study, it could be said that the plant extracts contain chemical constituents of pharmacological significance. The presence of these chemical constituents in this plant is an indication that the plant, if properly screened using additional solvents, could yield drugs of pharmaceutical significance. Further research is therefore recommended to isolate, purify and characterize these chemical constituents with a view to supplementing conventional drug development especially in developing countries.

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