PHYTOCHEMICAL, ANTIOXIDANT AND CYTOTOXIC SCREENING OF THE LEAVES OF *Calotropis procera* EXTRACTS

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Abstract: The bioactive components of the leaves of *Calotropis procera* was extracted using aqueous, methanol, and n-hexane and investigated for the presence of secondary metabolites. All the extracts revealed the presence of steroid and saponins while flavonoids are present in aqueous and methanol extracts. The antimicrobial activities of the extracts were tested against fungi, gram positive and gram negative bacteria's such as *Candida albicans*, *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Salmonela typhi* using the agar disk diffusion method. The n-hexane extract demonstrated a significant broad-spectrum antimicrobial activity against *S. typhi* and *S. aureus* with the zone of inhibition of 7 and 11 mm. The methanol extract was only active against Gram negative *P. aeruginosa* with the zone of inhibition of 6, while the aqueous extract was inactive against all the organisms tested. The result of the antioxidant property showed that methanol extract has the highest activity of about 88% while aqueous extract is 37%. The flavonoid content of the extracts was also quantified to be 96.90 and 39.90 mg/g for methanol and aqueous extracts. The crude extracts were also subjected to brine shrimp lethality bioassay and hexane extract showed significant cytotoxicity having LC_{50} of 2.01 µg/ml.

Keywords: Phytochemicals, antimicrobial, flavonoids, cytotoxicity, *Calotropis procera*

Introduction

The relationship between humans and plants is as old as life on earth, and that relationship has remain till date as plants has continued to supply humans with oxygen, food, shelter and medicine (Iqbal and Jabbar, 2005). With the progress of civilisation, human learned to recognize and categorize plants materials suitable for use in meeting the necessities of life (Kumar and Basu, 1994). The use of herbs and herbal extracts by humans for their healing powers can be traced to earliest of myths, traditions and writings used to codify those plants that can ease pain and treat diseases (Setty *et al.,* 2007). Plant parts such as the leaves, flowers, twigs, seeds, fruits, exudates, roots and stems barks form important major constituents of drugs used in traditional herbal medicinal systems and their therapeutic efficacy depend on the use of proper and genuine raw materials (Imaga *et al.,* 2009).

World Health Organization (WHO, 2002), reported that about 80% of the world's population relies on traditional medicine for their primary health care needs and about 4.5 billion people in the World rely on plants as sources of drugs. Research has shown that some 35,000 to 70,000 plants species has been used as drugs, a figure corresponding to 14-28% of the 250,000 plants species estimated to occur around the World which is equivalent to 35-70% of all species used World-Wide (Doughari *et al.,* 2007).

Calotropis procera Linn belong to the family *Asclepiadaecae*. It is known by various vernacular names like Swallow wort in English, madar in Hindi and Tumpapiya in Hausa. It is an erect, soft wooded perennial shrub reaching 2.5 to 6 m in height. It is found in most parts of the world with a warm climate in dry, sandy and alkaline soils where it thrives luxuriantly in the wastelands. It also grows well on rubbish heaps, waste and fallow land, by the roadside and in sand dunes (Quazi *et al.,* 2013). *C. procera*is an Ayurvedic plant with important medicinal properties. The flowers of this plant have been reported to exhibit hepatoprotective activity, anti-inflammatory, antipyretic, analgesic, and antimicrobial effects and larvicidal activity (Yoganarasimhan, 2011). The root has been found to possessα-amyrin, β-amyrin, lupeol, β-sitosterol and quercetin-3-rutinoside which may be responsible for antifertility and anti-ulcer effects of the plant. The latex of the plant is also reported to possess analgesic and wound healing activity as well asanti-inflammatory and antimicrobial activity, while the leaves have been reported to have anthelmintic, antipyretic, anticancer and antioxidant (Ahmed *et al.,* 2006; Rajani and Gupta, 2009; Perwez and Mohammad, 2009).

Materials and Methods

Sample collection and authentication

The leaves of mature *C. procera* were collected at various locations within Lafia metropolis, Nasarawa State, Nigeria, West Africa. The samples were authenticated by the Botanists of National Institute of Pharmaceutical Research and Development (NIPRD), Abuja where a voucher specimen NIPRD/H/6893 was deposited.

Sample preparation

The samples were transported to the laboratory in air-tight polyethylene bags to avoid any form of contamination and air dried at room temperature for 21 days. The dried samples were then ground manually using pestle and mortar, sieved, weighed, stored in a tightly closed glass sample bottle with proper labelling and kept away from moisture for further analyses.

Extraction of the samples

The powdered samples were exhaustively extracted using cold extraction method with hexane, methanol and water. These were carried out by soaking 50 g of each sample in 200 ml of hexane, methanol and water. The extraction was done at room temperature for 48 h. The solvents were removed by rotary evaporation and the concentrates, weighed and stored in the refrigerator prior to analysis.

Phytochemical screening

The phytochemical components of the leaves extracts of *C. procera* were screened for the presence of tannins, saponins, alkaloids, steroids, flavonoids, carbohydrates and anthraquinone derivatives using standard procedures as described by Edeoga *et al.* (2005) with slight modification.

Test for alkaloids

Each powdered sample (0.2 g) was dissolved in 5 ml dilute hydrochloric acid in a steam bath and filtered. 1 ml of the filtrate was treated with 3 drops of Mayer's reagent, the formation of a cream or pale yellow precipitate, indicating the presence of alkaloids.

Test for tannins

About 0.2 g of each dried powdered samples was boiled in 10 ml of water and then filtered. Approximately 2 drops of 0.1% ferric chloride was added, the appearance of green or a blue colouration, indicate the presence of tannins.

Test for saponins (Frothing test)
Distilled water 20 ml was added to 0.5g of the extract in a test tube. The test tube was stoppered and shaken vigorously for about 30 seconds; it was allowed to stand for 10 minutes. The formation of honey-comb froth is an indication of the presence of saponins.

Test for anthraquinone
The extract 0.2g was dissolved in 10 ml of benzene. The mixture was shaken for 1 minute and filtered, followed by addition of 5 ml of 10% ammonia solution. The appearance of pinkish colour in the lower ammoniacal layer indicated the presence of anthraquinones.

Test for steroids
2 ml of acetic anhydride was added to 0.2 g of the extract, followed by 2 ml concentrated sulphuric acid. The change of colour from violet to blue or green in the sample extract indicates the presence of steroids.

Test for flavonoids
Dilute ammonia solution 2 ml was added to 2 ml filtrate of each plant extract, followed by addition of 1 ml concentrated sulphuric acid. The mixture was allowed to stand and the yellow colouration disappeared. The addition of 2 drops of 1% aluminium solution to each mixture resulted in the appearance of yellow colouration, and this indicate the presence of flavonoids.

Test for carbohydrate (Molisch's test)
The dried powdered sample of each extract (1 g) was added 15 ml of distilled water and boiled for 3 minutes on a hot plate. The mixture was filtered while hot and allowed to cool. The filtrate 1 ml was added 3 drops of Molisch's reagent, followed by 0.5 ml of concentrated hydrochloric acid. The formation of a purple ring at the interface indicates the presence of carbohydrate.

Antimicrobial activity screening (sensitivity test)

Collection and preparation of test micro-organisms
The six test microorganisms used in this study were type cultures of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, yeast (*Candida albican*) obtained from the Microbiology/Biotechnology Departments of NIPRD, Idu, Abuja and clinical isolates of *Salmonella typhi* obtained from the Diagnostics Unit of the same Institute and were all maintained on Mueller Hinton broth at 37°C for 24 h. The organisms viability control (OVC) and the media sterility control (MSC) were carried out by streaking each test culture on a sterile plate and incubated at 37^0C for 24 h according to Cheesbrough (2000). 0.2 ml of each test culture was sub-cultured in sterile broth in bijou bottles containing 22 ml of the prepared Mueller Hinton agar and was incubated for another 3 h before used.

Antibacterial potency and minimum inhibitory concentration
The antimicrobial effect of the hexane, methanol and aqueous extracts of the leaves extracts of *C. procera* was determined by the method of agar disk diffusion method alongside the minimum inhibitory concentration (MIC) (Cheesbrough, 2000). 12 sterile nutrient agar plates prepared according to manufacturer's specification were used, six for each extract; one in each case streaked with one of the six test microorganisms as labelled. Four 5 mm diameter cork borers were used to bore 5 holes on each of the 12 plates. 0.1 ml of different concentration of the prepared extract (40, 20, 10 and 5 mg/ml) were dispensed into the holes on the plates that were sealed with test microorganisms. A standard drug (chloramphenicol capsule) was use as a control. The concentration of the drug used was 6.125 mg/ml, and 0.1 ml was collected into one of the holes in all the plates.
These plates were then incubated at 37°C for 24 h. The following day, the plates were brought out from the incubator, observed and the results recorded. The zone of inhibition and bio-efficacy activity against the microorganisms were recorded. The experiment was conducted in duplicate.

DPPH-free radical scavenging activity
The analysis of the DPPH radical scavenging activity of the plant extracts was performed according to the method described by Koleva *et al.,* (2002). Stock solution was prepared by dissolving 100 mg of extract in 1 ml of methanol and five, two fold serial dilutions was made. 0.5 ml of each of the concentrations was measured into separate test tubes and 0.3 ml of 0.5 mM DPPH was added. The reaction mixtures were vigorously shaken for 30 s in a Vortex apparatus and allowed to stand in the dark at room temperature for 30 min. Ascorbic acid was used as a standard for the investigation of the antiradical activity and was prepared in a similar manner. The absorbance was read using spectrophotometer at 517 nm against the blank. The blank was prepared by mixing 0.5 ml of the extract or ascorbic acid with 3.3 ml of methanol. Similarly, the control solution was prepared by mixing 3.5 mL of methanol and 0.3 ml of DPPH radical solution. The percentage of scavenging activity (X %) was calculated according to the equation below:

$$X\% = \frac{absorbanceof\ sample - absorbanceof\ blank}{absorbanceof\ control} x100$$

Determination of flavonoid content
The level of flavonoid content in the aqueous and methanol extracts of the sample were determined spectrophotometrically using rutin as standard (Odonotez *et al.,* 2006). In this evaluation, 20 mg of the extract was dissolved in 1 ml of methanol. Two additional concentration (5 and 10 mg/ml) were prepared by 2-fold serial dilution. To obtain a standard curve usingrutin, 1mg of rutin was dissolved in 1 ml of methanol and seven, two-fold serial dilutions was made to obtain a concentration of 0.015625 to 1 mg/ml. Then, 100 µl of the extract and rutin standard concentrations were aliquoted into a cuvette and an equal volume of 2% aluminium chloride ($AlCl_3$) was subsequently added. The mixtures were incubated at room temperature for 1 h. The absorbance was measured at 415 nm, using the AT1 UNICAM UV/VIS spectrometer UV4 coupled to Vision V3.40 computer software. The sample, blank (methanol) and standards were prepared in triplicate for each analysis and the mean value of absorbance was obtained. From the absorbance readings, the total flavonoid content of each extract was calculated from the regression equation of the rutin standard curve ($y = 4.8736x + 0.2463$, $R^2 = 0.9988$ for methanol and $y = 2.7524x + 0.378$, $R^2 = 0.9797$ for aqueous extracts) and expressed as rutin equivalents.

Cytotoxicity activities
The cytotocixity screening of the crude extracts against *Artemia salina* was carried out in an in-vivo simplified assay as described by Meyer *et al.* (1982). In this experiment, 500 µg of the extracts were dissolved in 1 ml of DMSO and by serial dilution technique, solutions of varying concentrations such as 250, 125, 75, 37.5, 18.75, 9.375, 4.6875, 2.34375, 1.171875, 0.5859375 µg/ml were obtained. Then 0.5 ml each of these standard concentrations were added to test tubes containing 10 shrimps in simulated brine water. After 24 h, the median lethal concentration (LC_{50}) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration. Vincristine sulphate was used as positive control in this assay to compare the cytotoxicity of the extracts.

Results and Discussion
Aqueous, methanol and hexane extracts were prepared and screened for secondary metabolites. The yield obtained from 50 g of dry plant material was measured for each extract

(Table 1). Aqueous has the highest extract because it is a universal solvent and is generally used in traditional medicine settings to prepare the plant decoction and infusion for health remedies. However, hexane has the lowest extract because they are good solvent for extracting non-polar compounds.

Table 1: Yield of solid residue after extractions and evaporation from 50 g dried sample

Extract	Percentage yield
Hexane	3.50
Methanol	5.90
Aqueous	10.70

Table 2: Phytochemical screening of *Calotropis procera* leaves extracts

Phytochemicals	Hexane extract	Methanol extract	Water extract
Alkaloids	-	-	-
Tannins	-	-	-
Steroids	+	+	+
Flavonoids	-	+	+
Saponins	+	+	+
Anthraquinones	-	-	-
Carbohydrates	-	-	-

+ = Present; - = Absent

The phytochemical studies of *C. procera* leaves using different solvents revealed the presence of steroids, and saponins in all the extract of the leaves, while water and methanol extract also contains flavonoids as shown in Table 2. This study contradicts the reports of Murti *et al.* (2010), Begum *et al.* (2010) and Shrivastava *et al.* (2013) that report the presence of all the metabolites tested in this study. The presence of saponins in this plant sample is an indication that the plant may be useful in reducing blood cholesterol level and cancer risk due to its foaming ability that produce frothy effect (Okwu, 2004). Steroids are related to sex hormones and they also interact with androgen receptors to increase muscle and bone synthesis (Trease and Evans, 1989). Flavonoids are potent water soluble antioxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity (Del-Rio *et al.,* 1997; Okwu and Josiah, 2006). They are also found to be useful in the preparation of some antimicrobial compounds such as dettol and cresol (Sharmal and Bais, 2015).
The antimicrobial activity of aqueous, methanolic and hexane extracts of leaves of *C. procera* against *S. aureus, E. coli, B. subtilis, S. typhi, P. aeruginosa and C. albicam*was studied on agar disc diffusion method. Methanolic extract had inhibitory effect on the growth of Gram negative *P. aeruginosa* isolates while hexane extract inhibit the growth of *S. typhi* and *S. aureus.* Aqueous extract has no effect on all the growth isolates tested (Table 3). This report contradict the earlier report of Mako *et al.* (2012), Moronkola *et al.* (2011) and Bouratoua *et al.* (2013) that both polar and nonpolar solvents inhibit the growth of these microorganisms.

Table 3: Antimicrobial activity of *Calotropis procera* extracts (40 mg/ml) and chloramphenicol (6.125 mg/ml)

Test organism	Zones of Inhibition (mm)			
	Water extract	Methanol extract	Hexane extract	Chloramphenicol
Escherichia coli	NA	NA	NA	31
Salmonella typhi	NA	NA	7	29
P. aeruginosa	NA	6	NA	30
S. aureus	NA	NA	11	32
Bacillus subtilis	NA	NA	NA	30
Candida albicam	NA	NA	NA	NA

NA = No activity; ***P. aeruginosa*** = *Pseudomonas aeruginosa;* ***S. aureus*** = *Staphylococcus aureus*

Table 4: Result of antioxidant activities of methanol extract of *C. procera* leaves and ascorbic acid standard

Concentration (mg/ml)	Methanol extract	Aqueous extract	Ascorbic acid
2.0	88.01±0.8	37.11±0.5	85.11±1.3
1.5	85.04±1.7	35.96±0.3	80.05±3.2
1.0	73.12±2.1	25.21±0.2	83.33±1.6
0.5	69.28±1.5	18.62±0.4	85.03±2.0
0.25	50.22±1.3	21.13±0.3	85.37±2.9

Values are presented as mean (SD of three replicates)

The methanolic and aqueous extracts of leaves of *C. procera* were subjected to the 1,1,-diphenyl-2- picryl hydrazyl (DPPH) radical scavenging assay. The assay revealed substantial free radical scavenging activity (50.22 – 88.01%) in the methanol extract, which is concentration dependent. Conversely, the aqueous extract showed mild antioxidant activity (Table 4). This result is in agreement with the report of Mako *et al.* (2012), and it indicated the strong antioxidant activity of the plant which occurs by preventing the production of free radicals produced in the body or reducing and chelating the transition ion metal composition of food. The prevention of the chain initiation step by scavenging various reactive species such as free radicals is considered to be an important antioxidant mode of action (Dastmalchi *et al.,* 2007). The high DPPH radical scavenging ability of the methanol extract could be attributed to its high flavonoid content 96.90±2.50 mg/g while the flavonoid of the aqueous extract is 39.90±0.70 mg rutin equivalent/g (Fig. 1).

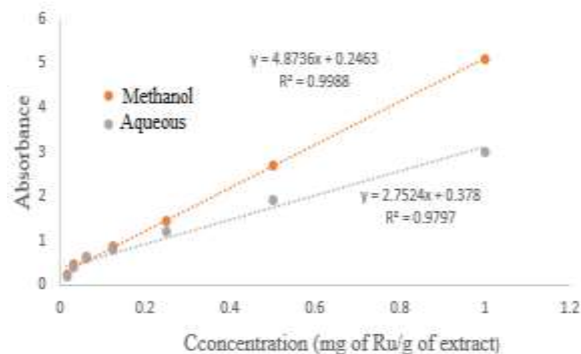

Fig. 1: Calibration curve for total flavonoid content in the aqueous and methanol leaves extracts

Table 5: Result of LC_{50} data of *C. procera* leaves extracts and vincristine sulphate standard

Sample extract	LC_{50}(μg/ml)
Vincrisine sulphate standard	0.41±0.01
Hexane extract	2.01±0.03
Methanol extract	10.60±0.20
Aqueous extract	12.10±0.40

Values are presented as mean (SD of three replicates)

The cytotoxic potentials of crude extract of hexane, methanol and aqueous extracts were screened by brine shrimp lethality bioassay for probable cytotoxic activity. The LC_{50} obtained from the best fit line slope were found to be 2.01, 10.60 and 12.10 μg/ml for hexane, methanol and aqueous extract respectively as shown in Table 5. In comparison with the 0.41 μg/ml of positive control (vincristine sulphate), the cytotoxicity exhibited by hexane extract was significant. Agreeing with the report that methanol extract is weekly cytotoxic (Van *et al.,* 2005).

Conclusion
The qualitative screening of *C. procera* leaves extracts shows that it has limited but very useful phytochemicals which were active against some test microorganisms, *P. aeruginosa, S. typhi* and *S. aureus.* These microorganisms are known to be involved in pneumonia and cutaneous diseases. The methanol extract has high flavonoids content which is responsible for the high anti- oxidant activity of the plant, while hexane extract showed significant cytotoxicity using brine shrimp lethality bioassay. Although, this plant part is used in the treatment of stomach ache in this environment, studies on the toxicity of the various extracts to ascertain its safety for therapeutic use is also suggested.

Conflict of Interest
Authors declare that there is no conflict of interest reported in this work.

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