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Evaluation of Antifungal Activity and Phytochemical Analysis of Leaves, Roots and Stem Barks Extracts of *Calotropis procera* (Asclepiadaceae)

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Abstract: The aqueous and organic solvents extracts of leaves, stem barks and roots of *Calotropis procera* were screened for antifungal (agar dilution method) and phytochemical properties. Organic solvents extracts of the stem bark significantly ($p<0.05$) inhibited growth of *Trichophyton rubrum* and *Microsporum gypseum*. The petroleum ether (PE) extract however was significantly ($p<0.05$) active on only *Trichophyton rubrum* at concentrations ranging from 0.66 to 5.33 mg mL^{-1}. All the leave extract fractions completely inhibited the growth of the tested organisms. The roots fractions of hexane (HX) and PE extracts showed significant ($p<0.05$) growth inhibitions of *Microsporum gypseum* and *Aspergillus niger*. All the aqueous extract fractions of the plant parts showed complete growth inhibition of all the tested organisms. Alkaloids, flavonoids, tannins, steroids, triterpenoids, saponins and saponin glycosides were detected in the leaves and roots extract fractions, with only flavonoids, triterpenoids and saponins in the stem bark extracts. The results obtained lend scientific credence for the use of the plant against fungal diseases.

Key words: *Calotropis procera*, antifungal, *In vitro* screening

INTRODUCTION

Most plant-derived medicines have been developed on the basis of traditional knowledge in health care and in many cases there is a correlation between the indications of pure substances and those of respective crude extracts used in traditional medicine (Farnsworth *et al*., 1985). Plants provide abundant resources of antimicrobial compounds and have been used for centuries to inhibit microbial growth (Zhang *et al*., 2006). The increasing incidences of fungal infections and gradual rise in azole resistance and available antibiotics had highlighted the need to find more alternative antifungal agents from other sources (Hammer *et al*., 1999; Fostel and Lartey, 2000). Grasela *et al*. (1990) also reported that, despite advances in antifungal therapies, many problems remained to be solved for most antifungal drugs available. In northern Nigeria the leaves, roots and stem barks of *Calotropis procera* are used in indigenous practice to treat fungal diseases (for topical application), convulsion, asthma, cough and inflammation. Aliero *et al*. (2001) reported the use of fresh follicles of the plant (soaked in cold water) for the treatment of asthma, the burnt dry stem for wound healing and the plant is also used in the treatment of several diseases of domestic animals.

Calotropis procera (family Asclepiadaceae) is commonly called Sodom apple. It is un-branched with soft wooden trunk, yellowish brown stem bark and the slash exudes caustic latex that turns yellow on exposure to air (Aliero *et al*., 2001). Dermatophytes and *Aspergillus niger* cause superficial infections of the skin and internal organs, respectively (Robert, 1978). Flavonoids, triterpenoids, steroids and other phenolic compounds in plants have been reported to have antimicrobial activity (Rojas *et al*., 1992; Hostetmann *et al*., 1995). *Calotropis procera* has not been thoroughly evaluated for its antifungal properties. This study therefore was aimed at investigating the phytochemical contents and antifungal activities of different parts of *Calotropis procera* by preliminary bioassay screening using aqueous, hexane, petroleum ether and chloroform extracts. This study would contribute to the acceptance of traditional medicine and to the solution of the growing problems of drug resistance by fungi.

MATERIALS AND METHODS

Plant material: The leaves, roots and stem barks of *Calotropis procera* were collected within Usmanu Danfodiyo University campus, Sokoto, Nigeria. The plant

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Table 1: Amount of residues obtained after extraction

Extracts	Fractions	Amount recovered (g)
Leaves	HX	3.10
	PE	3.50
	CHL	1.72
	LR	7.25
	W	17.50
Stem	HX	2.10
	PE	0.37
	CHL	1.10
	LR	1.90
	W	6.70
Root	HX	2.60
	PE	1.92
	CHL	3.76
	LR	1.30
	W	10.30

HX = Hexane, PE = Petroleum ether, CHL = Chloroform, W = Water and LR = Last remaining water ethanol fractions

parts were botanically authenticated at the herbarium, Botany Unit of the same Institution where voucher specimens were kept. The parts collected were room-dried and pulverized into powder and the powdered parts were subjected to aqueous and organic solvent extraction (Matawalli *et al*., 2004).

Extraction and fractionation procedure: Fractionation of the extract was done by activity-guided fractionation using ethanol-water (1:1) and different (hexane, petroleum ether and chloroform) organic solvents. The powdered extracts of the leaves, stem barks and roots of the plant (40 g) were extracted with ethanol-water (1:1,500 mL) separately at room temperature overnight (Morris and Aziz, 1976). Each extract was filtered; the different filtrates were partitioned in hexane separately (250 mL) and clarified by further filtrations. Evaporation of each hexane fraction to dryness in an oven at 45°C yielded residues (Table 1). The aqueous filtrates (ethanol-water) of each extract fractions were further partitioned with petroleum ether (250 mL) and chloroform separately (250 mL). Evaporation of the petroleum ether, chloroform (CHL) and the last remaining aqueous filtrates of leaves, stem barks and roots yielded residues (Table 1). A separate portion of each powdered plant part (40 g) was extracted with 500 mL distilled water at room temperature overnight and filtered. The filtrates of the leaves, roots and stem barks were then evaporated to dryness yielding residues of 17.50, 10.30 and 6.70 g, respectively. All the residues obtained were reconstituted in sterilized distilled water and screened for antifungal and phytochemical properties. The above procedure enabled us expunge the possible contributory antifungal effect of the organic solvents.

ORGANISMS

The fungal species used: *Trichophyton rubrum, Microsporum gypseum* and *Aspergillus niger,* were clinical isolates obtained from Usmanu Danfodiyo University Teaching Hospital, Sokoto, Nigeria. They were maintained on Sabouraud Dextrose Agar (SDA) medium and re-identified by microscopic examination of a portion of the colony for spores and characteristic hyphae (Cheesbrough, 1982).

Antifungal activity: The antifungal activities of the crude and organic solvent extracts were evaluated according to reported procedures of Zacchino *et al*. (1999), using the agar dilution method with some modifications. The fungal species were cultivated on Sabouraud Dextrose Agar (SDA) medium in 90 mm petridishes. Five mililiter of filter-sterilized reconstituted water solution of each plant extract (HX, PE and CHL) at concentrations of 10 to 80 mg mL^{-1} for stem barks and leaves and 10 to 60 mg mL^{-1} for roots were aseptically mixed with 15 mL of SDA (liquified and maintained at melting point in waterbath at 45°C) to give final concentrations of 0.67 to 5.33 and 0.66 to 4.00 mg mL^{-1}, respectively. The crude water extracts of the plant parts at 10 to 80 mg mL^{-1} were also prepared using the above procedure to give final concentrations of 0.67 to 5.33 mg mL^{-1}. The petri dishes (90 mm) were filled to 20 mL final volume with Sabouraud Dextrose Agar containing the requisite amounts of diluted extract solution. The petri dishes were then inoculated at their center with a disk (2×2 mm) cut from the periphery of a 14-day-old (*T. rubrum* and *M. gypseum*) and 7-day-old (*A. niger*) fungal colonies. Griseofulvin (0.67 mg mL^{-1}), Clarion Medicals Ltd. Lagos, Nigeria, measured from the pulverized 500 mg tablet was also included to serve as positive control. Water (5 mL) in place of the extract and media (15 mL) were mixed together as negative control. The treated and the control Petri dishes were incubated at room temperature for 14 days for superficial mycosis (dermatophytes) and 48 h for *A. niger*. Growth was observed each day to the last day. From these, the percentage inhibitions were calculated using the following formula:

$$I(\%) = \frac{d_c - d_t}{d_c} \times 100$$

dc: Diameter of colony of control culture.
dt: Diameter of colony of treated culture.

The significance of the difference of the antifungal activities of the extracts was tested by student t-test.

Phytochemical analysis of extracts: This was carried out using standard procedures as described by Persinos and Quimby (1967), Harbone (1973), Trease and Evans (1978) and El-Olemyl *et al.* (1994). The various extracts were analysed for the presence of volatile oil, alkaloids, cardiac

glycosides, anthraquinone glycosides, cyanogenic glycosides, saponin glycosides, flavonoids glycosides, tannins, saponins, flavonoids, steroids and triterpenoids.

RESULTS AND DISCUSSION

The *in vitro* assays of antifungal activity and phytochemical analysis of the residues obtained are presented in Table 2 to 6. The aqueous extract fractions at 0.66-5.33 mg mL^{-1} concentrations tested exhibited 97.80% growth inhibition on all the organisms tested. The stem bark extracts of HX, PE and CHL did not show activity against *A. niger* (0%). However, we noticed significant ($p<0.05$) growth inhibition of *M. gypseum* and *T. rubrum* (97.80%), except for PE extract against *M. gypseum* (0%). The leaves extracts of the plant at concentrations of 0.66 to 5.33 mg mL^{-1} produced 97.80% growth inhibition on all the organisms tested. Root extract of HX and PE inhibited the growth of all the organisms significantly ($p<0.05$) except *T. rubrum* (20-88%). Its CHL extract inhibited *M. gypseum* to a fair extent at 0.66 mg mL^{-1}, but at 2 and 4 mg mL^{-1} it was active on all the isolates. Stem bark extracts revealed the presence of flavonoids, triterpenoids and saponin glycosides. While flavonoids, saponins, tannins, alkaloids, steroids, triterpenoids and saponin glycosides only were found in roots and leaves extracts.

Results from our antifungal activity present an easy *in vitro* system that can be used for assessing the antifungal activities of plants. The organic solvents extracts exhibited significant ($p<0.05$) antifungal activities against some of the isolates tested, whereas aqueous extracts showed complete inhibition of all the fungal isolates used. Different solvent extracts of some plants may exhibit different pharmacological properties (Freiburghaus *et al*., 1996). From our findings (Table 2) water extracts of leaves, root and stem is the most effective solvent for extracting the pharmacologically active compounds from *Calotropis procera*. This corroborates the report by traditional healers and herbalists that water is the most commonly used solvent to extract biologically active compounds due to its easy availability (Shale *et al*., 1999). Our results of near complete growth inhibition of the isolates by the aqueous extracts of the plant did not contradict this assertion. The extraction method used (bioassay-guided fractionation) may be responsible for the inactivity of the CHL (root), PE (stem, with exception of *T. rubrum*) and last remaining water-ethanol extracts of root and stem. The significant ($p<0.05$) antifungal activities of the extracts of *Calotropis procera* confirmed its pharmacological and therapeutic potentials. Our results are comparable to those of Alade and Irobi (1993), who indicated that the alcoholic extract of *Argemone mexicana*, *Baccharis sordescens* and *Acalypha wilkesiana*, although belonging to a different family, has antifungal activities.

Results from our phytochemical analysis (Table 6) suggest that the presence of biologically active compounds (alkaloids, flavonoids, tannins, saponins, steroids, triterpenoids and saponin glycosides) in the plant extracts could be correlated to the antifungal effects of substances known to posses antimicrobial properties as shown by Tschesche (1971), Scalbert (1991) and Favel *et al.* (1994). Thus, it is probable that these molecules are the principal antifungal agents in the leaves, roots and stem bark extracts of *Calotropis procera*. The mechanism(s) of action of the constituents of *Calotropis procera* could be by inhibition of fungal cell wall, protein and amino acid, sphingolipid biosynthesis and electron transport chain (Lartey and Moehle, 1997; Ueki and Taniguchi, 1997; Dominguez and Martin, 1998). The antifungal agent(s) in the extracts of *Calotropis procera* may act via some of the

Table 2: Percentage inhibitions of fungal isolates by leaves, stem barks and roots water extracts of *Calotropis procera*

Plant parts	Concentration (mg mL^{-1})	*Microsporum gypseum*	*Trichophyton rubrum*	*Aspergillus niger*
Leaves	0.67	97.80	97.80	97.80
	1.33	97.80	97.80	97.80
	4.00	97.80	97.80	97.80
	5.33	97.80	97.80	97.80
Stem	0.67	97.80	97.80	97.80
	1.33	97.80	97.80	97.80
	4.00	97.80	97.80	97.80
	5.33	97.80	97.80	97.80
Root	0.67	97.80	97.80	97.80
	1.33	97.80	97.80	97.80
	4.00	97.80	97.80	97.80
	5.33	97.80	97.80	97.80
Griseofulvin	10.00	87.22	75.83	63.06

Diametre of water control petri dishes of all the organisms used is 90 mm, Values are percentage inhibitions, 0-25% = None or little inhibition; 25-50% = Average inhibition; 50-75% = Strong inhibition; 75-100% = Very strong inhibition

Table 3: Percentage inhibitions of fungal isolates by leaves organic solvent fractions of *Calotropis procera*

Plant parts	Concentration (mg mL^{-1})	*Microsporum gypseum*	*Trichophyton rubrum*	*Aspergillus niger*
Leaves	0.67 HX	97.80	97.80	97.80
	1.33 HX	97.80	97.80	97.80
	4.00 HX	97.80	97.80	97.80
	5.33 HX	97.80	97.80	97.80
	0.67 PE	97.80	97.80	97.80
	1.33 PE	97.80	97.80	97.80
	4.00 PE	97.80	97.80	97.80
	5.33 PE	97.80	97.80	97.80
	0.67 CHL	97.80	97.80	97.80
	1.33 CHL	97.80	97.80	97.80
	4.00 CHL	97.80	97.80	97.80
	5.33 CHL	97.80	97.80	97.80
	0.67 LR	89.44	97.80	97.80
	1.33 LR	93.33	97.80	97.80
	4.00 LR	97.80	97.80	97.80
	5.33 LR	97.80	97.80	97.80
Griseofulvin	10.00	87.22	75.83	63.06

Diametre of water control petri dishes of all the organisms used is 90 mm, HX = Hexane, PE = Petroleum ether, CHL = Chloroform and LR = Last remaining Water ethanol fractions, Values are percentage inhibitions, 0-25% = None or little inhibition; 25-50% = Average inhibition; 50-75% = Strong inhibition; 75-100% = Very strong inhibition

Table 4: Percentage inhibitions of fungal isolates by stem barks organic solvent fractions of *Calotropis procera*

Plant parts	Concentration (mg mL^{-1})	*Microsporum gypseum*	*Trichophyton rubrum*	*Aspergillus niger*
Stem	0.67 HX	97.80	97.80	0.00
	1.33 HX	97.80	97.80	0.00
	4.00 HX	97.80	97.80	0.00
	5.33 HX	97.80	97.80	0.00
	0.67 PE	0.00	97.80	0.00
	1.33 PE	0.00	97.80	0.00
	4.00 PE	0.00	97.80	0.00
	5.33 PE	0.00	97.80	0.00
	0.67 CHL	97.80	97.80	0.00
	1.33 CHL	97.80	97.80	0.00
	4.00 CHL	97.80	97.80	0.00
	5.33 CHL	97.80	97.80	0.00
	0.67 LR	0.00	0.00	0.00
	1.33 LR	0.00	0.00	0.00
	4.00 LR	0.00	97.80	0.00
	5.33 LR	0.00	97.80	0.00
Griseofulvin	10. 00	87.22	75.83	63.06

Diametre of water control petri dishes of all the organisms used is 90 mm, HX = Hexane, PE = Petroleum ether, CHL = Chloroform and LR = Last remaining Water ethanol fractions. Values are percentage inhibitions, 0-25% = None or little inhibition; 25-50% = Average inhibition; 50-75% = Strong inhibition; 75-100% = Very strong inhibition

Table 5: Percentage inhibitions of fungal isolates by roots organic solvent fractions of *Calotropis procera*

Plant parts	Concentration (mg mL^{-1})	*Microsporum gypseum*	*Trichophyton rubrum*	*Aspergillus niger*
Root	0.67 HX	97.80	20.00	97.80
	2.00 HX	97.80	30.00	97.80
	4.00 HX	97.80	87.78	97.80
	0.67 PE	97.80	97.80	97.80
	2.00 PE	97.80	97.80	97.80
	4.00 PE	97.80	97.80	97.80
	0.67 CHL	32.00	18.33	4.17
	2.00 CHL	76.67	19.33	63.33
	4.00 CHL	97.80	75.78	82.78
	0.67 LR	47.50	30.56	8.89
	2.00 LR	97.80	81.33	97.80
	4.00 LR	97.80	90.00	97.80
Griseofulvin	10.00	87.22	75.83	63.06

Diametre of water control petri dishes of all the organisms used is 90 mm, HX = Hexane, PE = Petroleum ether, CHL = Chloroform and LR = Last remaining Water ethanol fractions. Values are percentage inhibitions, 0-25% = None or little inhibition; 25-50% = Average inhibition; 50-75% = Strong inhibition; 75-100% = Very strong inhibition

above mechanisms. The results of the present study justify the traditional use of the plant for infectious fungal diseases. Further isolation and identification and toxicological studies of the anti fungal compounds in the crude aqueous and organic solvent extracts of the plant are recommended.

Table 6: Phytochemical analysis of water and organic solvent extracts of *Calotropis procera*

Plant parts	Extract fractions	VLO	ALK	CG	ATG	CYG	SAG	FG	TA	SAP	FL	STR	TRT
Leaves	I	-	+++	-	-	-	+++	-	+	+++	+++	+++	+++
	II	-	+++	-	-	-	+++	-	+	+++	+++	+++	+++
	III	-	+++	-	-	-	+++	-	+	+++	+++	+++	+++
	IV	-	+++	-	-	-	+++	-	+	+++	+++	+++	+++
	V	-	+	-	-	-	+	-	+	+++	+	+	+
Stem	I	-	-	-	-	-	+++	-	-	-	+++	-	+++
	II	-	-	-	-	-	+++	-	-	-	+++	-	+++
	III	-	-	-	-	-	+++	-	-	-	+++	-	+++
	IV	-	-	-	-	-	+++	-	-	-	+++	-	+++
	V	-	-	-	-	-	+	-	-	-	+	-	+
Roots	I	-	+++	-	-	-	+++	-	+++	+++	+++	+++	+++
	II	-	+++	-	-	-	+++	-	+++	+++	+++	+++	+++
	III	-	+++	-	-	-	+++	-	+++	+++	+++	+++	+++
	IV	-	+++	-	-	-	+++	-	+++	+++	+++	+++	+++
	V	-	+++	-	-	-	+++	-	+++	+++	+++	+++	+++

- = Absence, + =Trace amounts, +++ = Presence, I = Water, II = Hexane, III = Petroleum ether, IV = Chloroform, V = Last remaining Water-ethanol extract, VLO = Volatile oil, ALK = Alkaloids, FG = Flavonoid glycosides, FL = Flavonoids, ATG = Anthraquinone glycosides, CG = Cardiac glycosides, TRT = Triterpenoids, CYG = Cyanogenic glycosides. STR = Steroids, TA = Tannins, SAP = Saponins and SAG = Saponin glycosides

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