Phytochemical and Antimicrobial Assessment of *Ceiba pentadra* Spines.

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ABSTRACT

The spine on stem-bark of *Ceiba pentadra* was investigated for its chemical and antimicrobial properties. The phytochemical analysis revealed the presence of saponin, tannin, flavonoid, phenol, resin, sterol, carbohydrate, alkaloid and glycosides. The crude extract, hexane and ethylacetate fractions inhibited the growth of *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Proteus mirabilis* while ethanol fractions showed selective activity. The minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) were determined for the various organisms. Brine shrimp cytotoxicity test revealed that hexane fraction is toxic (< 1000). The bio-active compounds identified, could have been responsible for the therapeutic properties exhibited by the spines.

(Keywords: *Ceiba pentadra, phytochemical,* antimicrobial, cytotoxicity)

INTRODUCTION

Ceiba pentandra (Linn) is a tree that belongs to the *Bombaceae* family. It is an erect deciduous tree which grows up to 50 m high in height. Among its interesting features are the scattered bulky woody spines that cover the Ceiba's cylindrical trunk while young. Some can be observed even when the tree has reached seven years of age. It is commonly called the silk cotton tree or kapok tree. In Nigeria, it is called *Rimi* in Hausa, *Akpu* in Igbo and *Araba* in Yourba.

This plant has been used in traditional medicine for several ailments including skin diseases. This species is also widely used in traditional medicine, in Asia, Oceania, Africa, and Central America. It is used for a variety of disorders including diarrhea, fever, gonorrhea, parasitic infections, and wounds and as a diuretic and an emollient (Ngounou et al., 2000).

In the Samoan Islands, a cold water infusion of the bark of *C. pentandra* (vernacular name *vavae*) is used for asthma. Two studies have shown anti-inflammatory properties of the bark extract, both *in vitro* and *in vivo* (Noreen, El-Seedi, Perera, & Bohlin, 1998), which can be related to several reports of traditional use in the treatment of ailments of an inflammatory nature such as asthma and cough (Dolui, Das, & Kharat, 2011).

Research findings in Nigeria revealed that the extract of *C. pentandra* has antifungal activity. Pentandrin and Pentandrin glucoside, b-sitosterol and its 3-O-b-D-glucopyranoside, Vavain and Vavain 3'-*O*-*β*-D-glucoside, Flavan-3-ol, and (+)-catechin were reported to have been isolated from the stem barks of *Ceiba pentandra* (Noreen et al., 1998; Rao, Sreeramulu, Gunasekar, & Ramesh, 1993).

A careful search of the literature revealed that large thorny spines protruding from the trunk have not been investigated. In this study, we investigate the crude extract of the spines of *C. pentandra in* order to determine the phytochemical compounds responsible for the reported therapeutic potency. The antimicrobial and cytotoxicity activities of the extracts were also studied.

MATERIALS AND METHOD

Collection of Samples

The stem barks of *C. pentandra* were collected in January 2009 from Eruwa township, Ibarapa East Local Government Area of Oyo state, Nigeria.

Preparation of Extracts

The thorny spines were separated, air-dried and ground in a mill into a coarse powder. Ethanol (3 liters) was added to 500 g of powdered spines in a round bottom flask. The mixture was allowed to stay for 2 weeks. The resulting solution was filtered using Whatman no 2 filter paper. The filterate was concentrated under vacuum using a rotary evaporator to a paste that constituted the crude extract. The dried crude extract was macerated in n-hexane and ethyl acetate (EtOAc) successively. The left-over sample was taken as ethanol (EtOH) fraction.

Phytochemical Screening of Extracts

The various fractions were evaluated for the presence of carbohydrate, alkaloids, tannins, glycosides, saponins, sterol, flavonoid, resins and phenols using qualitative method (Harborne, 1993; Richardson & Harborne, 1990).

Test for Carbohydrates

Molish Test: Few drops of Molish reagent were added to 2 ml of test sample in a test tube. Concentrated H_2SO_4 (1 ml) was then allowed to flow down the side of the inclined test tube so that the acid forms a layer beneath the aqueous solution without mixing with it. A reddish brown solution indicates a positive test (Sofowora, 1984).

Fehling's Test for Reducing Sugar: Exactly 0.2 g sample of the test sample was dissolved in distilled water. 5 ml of equal volumes of Fehling's solutions A and B was added to 2 ml of test extract in a test tube. The resultant mixture was boiled for 2 minutes. A brick red precipitate of copper (I) oxide or orange precipitate is an indication of a positive test.

Test for Alkaloids

Zero point two gram sample of the test sample was acidified with 1% HCl for 2 minutes and was then treated with few drops Dragendorff's reagents in a test tube. The formation of white precipitate indicates the presence of alkaloids (Sofowora, 1984).

Test for Tannins

Test sample (0.2 g) was stirred with water and filtered. A dirty-green precipitate, or blue-black, or blue green precipitate, on addition of few drops of 5% ferric chloride ($FeCl_3$) to the test extract was taken as an indication of the presence of tannins (Sofowora, 1984).

Test for Glycosides

Five milliliters of H_2SO_4 was added to 0.2 g sample of each portion of the extract, the mixture was heated in boiling water for 15 minutes. Fehling solution was then added and the resulting mixture was heated to boiling. A brick-red precipitate indicated the presence of glycosides.

Test for Saponin

Test sample (0.2 g) of each portion was dissolved in 5 ml of distilled water. Two milliliters of the resulted solution was taken into a test tube and was shaken vigorously for a few minutes. Frothing which persists on warming was taken as an evidence of the presence of saponins.

Test for Steroids

Test sample (0.2 g) of each portion was dissolved in 2 ml of chloroform. Concentrated H_2SO_4 (0.2 ml) was carefully added to form a lower layer. A reddish-brown color at the interface between the layers indicates the deoxy-sugar characteristics of cadenolides which indicated the presence of steroids.

Test for Flavonoids (Shinoda's Test)

Test sample (0.2 g) of each portion was dissolved in dilute NaOH (50 %). A yellow solution that turns colorless on addition of dilute HCl (50 %) acid indicated the presence of flavonoids.

Test for Resins

Exactly 0.2 g sample of each portion of the test sample was shaken with distilled water and filtered. Copper acetate solution (1 ml) was added to 1 ml of the filterates. The resulting solution was shaken vigorously and allowed to separate. A green-color solution is an evidence of the presence of resins.

Test for Cardiac Glycosides

Test sample (0.2 g) of each portion was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then reacted with 1 ml concentrated Sulphuric acid. A brown ring at the interface indicated the presence of deoxy sugar characteristic of cardiac glycosides.

Test for Phenols

Test sample (0.2 g) of each portion was dissolved in 2 ml ferric chloride solution. Blue-black or brown coloration indicated the presence of phenol

Test for Anthraquinones (Born-Trager's Test)

Zero point two gram sample of the test sample was shaken with 4 ml of benzene. The mixture was filtered and 2 ml of 10% ammonia solution was added to the filtrate. The mixture was shaken and the presence of pink red or violet color in ammoniacal solution (lower phase) indicated the presence of free anthraquinones.

Antimicrobial assays

Microbial strains

Microorganisms used were standard strains of bacteria obtained from Centre for Drug Research Institute, (CDRI) Lucknow, India. *Bacillus subtilis* ATTC 14579, *Bacillus cereus* ATTC 33923, *Salmonella typhi* ATTC 25179, *Pseudomonas aeruginosa* ATTC 27856 and *Proteus mirabilis* ATTC 21784 were used in this study; Each test organism containing 9.9×10^4 cfu/ml, 1.2×10^5 cfu/ml, 6.8×10^4 cfu/ml, 1.1×10^5 cfu/ml and 9.6×10^4 cfu/ml respectively before inoculation. Each strain was stored at low temperature (-4 °C). Cell densities were estimated from the pour plate method on plate count agar.

Culture Media

Mueller Hinton Agar (MHA) was used for the susceptibility test in agar-disc diffusion. Nutrient broth (NB) containing 0.05 % phenol red and supplemented with 10 % glucose was used for MIC and MBC (Bauer, Kirby, Sherris, & Turck, 1966; Kuete et al., 2007).

Antibacterial Susceptibility Test

The antibacterial susceptibility testing was determined using agar disc diffusion method. Solutions of varying concentrations 10, 100 and 1000 ppm were prepared for each extract using Dimethyl sulphoxide (DMSO). These concentrations were prepared in triplicate for each of the extract. Whatman No 2 filter paper was punched, into discs (6 mm in diameter) kept in a sample bottle and sterilized by autoclave at 121 °C for 30 minutes.

Twenty sterilized discs per 0.2 ml of each concentration were impregnated for the various extracts. The agar medium was prepared according to the manufacturer description. It was poured into sterile Petri-dishes to solidify. A sterile cotton swab was then used to spread microorganisms on the prepared agar plates. The impregnated discs of were placed aseptically on each agar plate using sterile needle, the inoculated plates were incubated at 37 °C for 24h. Gentamycin (10 ppm) and DMSO were used as positive and negative control respectively (Bauer et al., 1966). Antimicrobial activity was evaluated by observing the zone of inhibition against the test organisms. Those extracts showing inhibition were further subjected for the quantitative assessment of their activity.

Minimum Inhibitory Concentration (MIC) Determination

The MIC of the crude extracts and fractions were determined as follows; the test samples were dissolved in DMSO. The solution obtained was added to Nutrient Broth (NB) to final concentration of 10,000 ppm for each sample. This was serially diluted two-fold to obtain concentration ranges of 9.25 - 10,000 ppm. Then 0.1 ml of innoculum was inoculated into test tubes with NB containing a series of diluted extracts concentration.

The negative control test tube containing 1.9 ml of NB and 0.1 ml of the innoculum. Gentamycin (10 ppm) was used as positive control. The test tubes were covered using sterile plate sealer and incubated at 37 ºC for 24 h. The microbial growth was determined by observing the change of color in the test tubes (red when there is no growth and yellow when there is growth). The lowest concentration showing no color change was considered as MIC (Kuete et al., 2007).

Minimum Bactericidal Concentration (MBC) Determination

For determination of MBC, a portion of the liquid (0.1 ml) from each test tube (used in MIC) that's showed no change in color was plated into an agar (MHA) plate and incubated at 37 ºC for 24 h. The MBC is the concentration at which no growth occurs after this sub-culturing (Kuete et al., 2007).

Brine Shrimp Toxicity Test

Hatching of the Brine Shrimp Egg

Brine shrimps eggs obtained from Artemia Incorporated, USA were hatched in a shallow rectangular plastic container. Seventy milligrams of shrimp eggs were sprinkled into the container which contained 250 ml of sea water. The plastic container was placed beside a window for ray of light and proper ventilation. After 48 hours, the brine shrimp larvae were collected by dropping pipette from the lightened side, having been separated by the divider from the shell (McLaughlin & Rogers, 1998; Ramachandr, Vamsikrish, Gowthami, Heera, & Dhanaraju, 2011).

Sample Preparation

The stock solution was prepared by dissolving 20 mg (0.02 g) of solid extract in 1ml of analar grade dimethyl sulphoxide, $(CH_3)_2SO$ i.e. DMSO and 1 ml of distilled water to give a concentration of 10,000 ppm. The 1,000 ppm and 100 ppm were prepared by serial dilution.

0.2 ml of the dissolved extract of 10,000 ppm was measured and made up to 2 ml by addition of 1.8 ml of DMSO to prepare the concentration of 1,000 ppm. The 100 ppm was also prepared from 1,000 ppm followed the same procedure. 0.2 ml each of 10,000 ppm, 1,000 ppm and 100 ppm was transferred into three different test tube, respectively, and each was made into triplicate to make experiment 1, 2 and 3.

Sea water (4 ml) was added to each of the test tube. After the addition of sea water, 10 larvas of the brine shrimps were introduced into each of the test tubes with the use of a dropping pipette and the number of larva that survived after 24 hours of observation was taken. The control experiment was prepared by mixing 1 ml of DMSO and 3ml of sea water, 10 larvas was added and the larva was observed for 24 hours after which the number of the larva that survived was taken. The LC_{50} of each of the extracts was calculated using Finney computer program (McLaughlin & Rogers, 1998; Tawaha, 2006).

RESULTS AND DISCUSSION

It can be noted that the MIC values varied from 625 to 5000, 78 to 2500, 39 to 5000 and 18.50 to 625 ppm, for crude extract, ethyl acetate fraction, ethanol fraction and Hexane fraction respectively. The results of MIC in Table 1 indicated that the crude extract, hexane fraction, Ethyl acetate fraction inhibited the growth of all the tested microorganism while ethanol fraction showed selective activity as it shows no activity against *Bacillus Cereus*. In the preliminary screening of all fractions the least inhibitory effects was observed in *Bacillus subtilis.*

The MBC value of the crude extract could not be determined as it shows inhibition throughout the concentration ranges (4.625 – 10000 ppm). The MIC and MBC values of 18.5 ppm and 39.0 ppm obtained in Hexane fraction were similar to that of Gentamycin on the *Bacillus cereus*, confirming interesting antimicrobial potency. Regarding the

activity of the tested fractions, hexane fraction shows better inhibition for *Bacillus subtilis* 625 ppm and *Bacillus cereus* 18.50 ppm. Ethanol fraction shows good activity on *Pseudomonas aeruginosa* conversely to the activity of the crude extract on these bacteria. Ethyl acetate fraction shows better activity against *Salmonella typhi* and *Proteus mirabilis* while crude shows moderate inhibition against all the tested microorganisms.

The results of Phytochemical screening from Table 2 indicated that Carbohydrate, Alkaloid, Tannins, Glycosides, Saponins, Sterol, Flavonoid, Resins and Phenol are present. These classes of compounds are known to possess therapeutics properties against several pathogens. The antimicrobial activities of the fractions may be due to the presence of Tannins, Saponin and Phenol (Abioye et al., 2013; Greay & Hammer, 2011).

The result of Cytotoxicity test in Table 3 shows that the Hexane fraction is toxic and active since its LC_{50} is 23.8647, since plants extracts having LC_{50} values less than 200 ppm and 5 ppm in case of pure compounds are considered as highly active (Ramachandr et al., 2011; Tawaha, 2006).

Table 1: MIC and MBC of Fractionated Extracts of *Ceiba pentadra* Spines.

Microorganism	Concentration in ppm for:							
	EtOAc Fraction		Crude Extract		EtOH Fraction		Hex Fraction	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Bacillus substilis	2,500	10,000	5,000	>10,000	5,000	10,000	625	625
Bacillus cereus	313	2,500	1,250	5,000	NA	-	18.50	39.0
Salmonella typhi	156	625	1,250	5,000	313	625	625	1,250
P. aeruginosa	78	625	625	2500	39	78	313	625
P. mirabilis	156	313	1,250	2500	313	313	313	313

KEY: NA = Not Available − = Not Determined

Table 2: Phytochemical Constituents of *C. pentadra* Spines.

Constituents	EtOH Fraction	EtOAc Fraction	Hexane Fraction	Crude Extract
CARBOHYDRATE	+	-	-	+
ALKALOIDS	+	+	-	+
TANNINS	+	+	-	+
GLYCOSIDES	+	+	-	+
SAPONINS	+	+	-	+
STEROL	+	+	+	+
FLAVONOID	-	-	+	+
RESINS	+	+	-	+
PHENOLS	-	+	+	+

KEY: + = Presence − = Absence

Table 3: Brine Shrimp Result on Hexane Fraction.

NOS. OF EXPERIMENTS	10,000ppm	1,000ppm	100ppm	Number
1	0	0	2	30
2	0	2	3	30
3	0	3	4	30
Death	30	25	21	LC_{50} <1000

LC_{50} = 23.8647 it is Toxic because <1000
Upper Confidence limit = 99.0276
Lower Confidence limit = 0.01091

CONCLUSION

The overall results of this study can be considered as very promising in the perspective of new drugs discovery from plant sources, if consider the medical importance of the tested microorganisms. *Pseudomonas aeruginosa* has emerged as one of the most problematic gram-negative pathogens, with alarming high antibiotics resistance rates (Kerr & Snelling, 2009; Raja & Singh, 2007; Vojtová et al., 2011).

The pathogen was found to be sensitive to the fractions. The medicinal purpose as an antimicrobial has been verified. Its traditional use should be upheld. Characterization and elucidation of the bioactive constituents and other curative claims by local herbalists needs to be further investigated.

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SUGGESTED CITATION

Oladimeji A. O., I.A. Oladosu, and O. Babatunde. "Phytochemical and Antimicrobial Assessment of *Ceiba pentadra* Spines". *Pacific Journal of Science and Technology*. 16(2):244-250.