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The Screening of Phytoconstituents, Antibacterial and Antifungal Properties of *Smilax kraussiana* Leaves

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ABSTRACT

The Preliminary phytoconstituent analysis of the hexane, ethylacetate and methanol extracts of Smilax kraussiana leaves revealed the presence of glycosides, flavonoids and anthraquinones. The three extracts were evaluated invitro to determine inhibition of human pathogenic microorganisms made up of six bacteria and six fungi. The extracts inhibited the growth of the twelve test organisms significantly. Hexane and methanol extracts showed higher inhibition on Staphylococcus aureus and Bacillus subtilis (gram positive) than ethylacetate extract at concentrations between 25 and 200mg/mL, while all extracts possess lower antibacterial properties on Escherichia coli, Pseudomonas aeruginosa, Klebsiellae pneumonae and Salmonellae typhii (gram negative).However, hexane, ethylacetate and methanol extracts of Smilax kraussiana leaves exhibited higher antifungal activities on Candida albicans, Aspergillus niger, Rhizopus stolon, Penicillum notatum, Tricophyton rubrum and Epidermophyton floccosum with activity comparable to that of the reference drug Tioconozole.

Keywords: *Smilax kraussiana*, Smilacaceae, phytochemical screening, antibacterial, antifungal, ethnomedicine.

INTRODUCTION

Medicinal plants have played essential roles since early times in the treatment of all kinds of diseases in Africa and other part of the world, owing to the challenges confronting the appropriate delivery of official health care to millions of people in remote and rural communities. Medicinal plants which serve as herbal remedies constitute a strong traditional, complementary and alternative medicine. In realization of the inherent value of herbal remedies to primary care and the fact that over three quarters of the world's population rely on plants for medicinal care, the World Health Organization (WHO) has called for the identification, sensible exploitation, scientific development and appropriate utilization of herbal medicines which provides save and effective remedies in medicare [1]. Hence, therapeutic evaluations are critical in drug development. Results obtained from the research work reveal the leaf extracts of *Smilax kraussiana* as potential and reliable herbal remedy for the treatment of veneral diseases like gonorrhea, syphilis etc, and fungal infections (skin, mouth diseases etc). Smilacaceae comprises of 10 genera. One of

the genera is *Smilax* L. [2]. *Smilax* is the genus of about 300-350 species [3]. They are found in tropical, subtropical and warm temperate regions worldwide. One of these species is *Smilax kraussiana. S.kraussiana* is a climbing, thorny, flowering and ornamental plant [3,4,5]. It is exploited for its medicinal properties in Africa and other regions of the world as a cure for gout in Latin American countries, to ease labour, diuretic, ophthalmia, for the treatment of fever , infertility in eastern Tanzania, veneral and skin diseases [3,6,7,8,9].

Smilax kraussiana

The pharmacological properties of *S.kraussiana* have been reported by various researchers. Iwu and Anyanwu[10] reported the anti-inflammatory properties of the plant. The antihepatotoxic effects of the methanolic extract of *S.kraussiana* leaves have been established [11], but the antibacterial, antifungal and other biological activities of the plant have not been reported. The phytochemistry of *S.kraussiana* leaves or twigs have not been also investigated fully. In continuation of our studies on biological activities on medicinal plants, we report the phytochemical, antibacterial and antifungal properties of leave extracts of *Smilax kraussiana.*

MATERIALS AND METHODS

Collection and authentication of the plant material.

The whole plant material of *Smilax kraussiana* was collected from Ibadan, Oyo State of Nigeria, November 2009. Botanical identification and authentication was done by Mr. A.W. Ekundayo of the Forestry Research Institute of Nigeria (FRIN), Ibadan where a voucher specimen (FHI108799) was deposited.

Preparation of plant extracts

Fresh whole plant of *Smilax kraussiana* was air-dried, weighed and separated into twigs and leaves (twigs 505g and leaves 646g). The dried leaves of *S. kraussiana* was successively extracted in hexane, ethylacetate and methanol for 10 days respectively using cold extraction method. The resultant hexane (7g), ethylacetate (9g) and methanol (11g) extracts were obtained by evaporation and stored in the refrigerator for further use.

Phytochemical studies

The Preliminary phytochemical screening of the hexane, ethylacetate and methanol extracts of *S. kraussiana* was done using standard procedures [12,13,14,15,16].

1) Saponins: Small quantity of each extract was boiled with 5 ml of distilled water, filtered and cooled.
a). Frothing: To the filtrate (2.5 ml) about 10 ml of distilled water was added and shaken vigorously for

2 minutes. Frothing observed indicates a positive test. **b). Emulsification:** To the filtrate (2.5 ml) added 3 drops of olive oil and shaken vigorously for 2 minutes. An emulsified layer indicates a positive test.

2) Alkaloids: Small quantity of each extract was stirred with 5 mL of 1% hydrochloric acid for five minutes on a water bath and then filtered. Of the filtrate of each extract was divided into two portions. Mayer's reagent was added to one portion; occurrence of creamy white precipitate was taken as positive. To the second portion few drops of Dragendorff's reagent was added and appearance of orange red precipitate was regarded as positive for the presence of alkaloids.

3) a.Glycosides (Keller-killiani Test): Small quantity of each extract was diluted in 5 ml of distilled water. Added 2 ml of glacial acetic acid containing one drop of ferric chloride solution (3.5%) to each. This was underlay with 1 ml of concentrated sulfuric acid. A radish brown ring is formed at the interface and upper layer turns bluish green on standing indicates the presence of a deoxy sugar characteristic of cardiac glycosides. **b) Method-2:** Small quantity of each extract moistened with 5 ml in distilled water and filtered.Few drops of chloroform were added to each (to enhance enzymatic activity). A sodium picrate–saturated filter paper strip was hanged at the neck of the flask with the help of the cork and warmed the flask. The filter paper strip turned brick-red or maroon is indicated the presence of cyanogenetic glycosides.

4) Tannins: a) Ferric Chloride Test: Small quantity of each extract was boiled in 10 ml of water in a test tube and then filtered while hot and a few drops of 0.1% ferric chloride solution were added to the filtrate. A brownish green or a blue-black coloration indicates as a positive test. **b) Lead Acetate Test:** Small quantity of each extract was taken in a test tube and diluted with 5 ml of distilled water. Add few drops of a 1% solution of lead acetate to each. A yellow or red precipitate indicates a positive test.

5) Flavonoids: Three methods were used to determine the presence of flavonoids in the extracts. **a) Method-1:** Dilute ammonia solution (5 ml) was added to aqueous filtrate of each extract followed by addition of concentrated H_2SO_4 acid (1 ml). A yellow colouration that disappears on standing indicates the presence of flavonoids. **Method-2:** Few drops of 1% aluminium solution were added to aqueous filtrate of the each extract. A yellow coloration indicates the presence of flavonoids. **Method -3:** A small portion of the each extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration indicates the presence of flavonoids.

6)Steroids: Liebermann-burchard's Test: Small amount of each extract was dissolved in 1 ml of chloroform. Added 2 ml of acetic anhydride and 1 ml of concentrated H_2SO_4 acid to each portion. A greenish color is produced which turns blue on standing indicates the presence of steroids. **b) Salkowski's Test:** Small amount of the extract was dissolved in 2ml of chloroform. Concentrated sulphuric acid was carefully added to a lower layer. A reddish-brown colour at the interphase indicates the presence of deoxysugar characteristics of cardenolides. A violet ring may form just above the ring and gradually spread throughout the layer.

7) Reducing sugars (Fehling's Test): A small portion of each of the extract was shaken with distilled water and filtered. The filtrate was boiled with drops of Fehling's solution A and B for 2 minutes. An orange-red precipitate on boiling with Fehling's solution indicates the presence of reducing sugars.

8) Anthraquinones: A small portion of each extract was boiled with 10 ml of sulfuric acid, traces of ferric chloride solution was added and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was taken into another test tube and 1 ml of dilute ammonia was added to each portion. Rose-pink color in the aqueous layer indicates the presence of anthraquinones.

Antimicrobial Assay

Microorganisms: Cultures of six human pathogenic bacteria made up of four gram negative and two gram positive were used for the antibacterial assay. These were; *Salmonella typhii* (UCH 4801), *Escherica coli* (UCH 00260), *Pseudomonas aeruginosa* (UCH 1102) and *Klebsiellae pneumonae* (UCH 2894) belong to the gram-negative while *Bacillus subtilis* (UCH 74230) and *Staphylococcus aureus* (UCH 2473) belong to the gram-positive. For the Antifungal assay, six fungi were also utilized. These were; *Candida albicans, Aspergillus niger, Rhizopus stolon, Penicillum notatum, Tricophyton rubrum and Epidermophyton floccosum.* All the microorganisms used were clinical strains from the Medical Microbiology (University College Hospital, Ibadan) and screened in the Laboratory of Pharmaceutical Microbiology Department, University of Ibadan, Ibadan, Nigeria.

Media: Nutrient agar, Sabouraud dextrose agar, nutrient broth and tryptone soya agar were used in this study. Hexane, ethylacetate and methanol were also used in solubilizing the extracts and as negative controls in the assays.

Antimicrobial agents: Gentamycin (10 µg/mL) and Tioconazole (0.7 mg/mL) were included as standard reference drugs in the study.

Antimicrobial activity determination

Agar diffusion-pour plate method (bacteria): An overnight culture of each organism was prepared by taken two wireloop of the organism from the stock and inoculated each into the sterile nutrient broth of 5ml, each incubated for 18-24hr at 37^{o}C. From overnight culture, 0.1 mL of each organism was taken and put into the 9.9mL of sterile distilled water to obtained 10^{-2} inoculum concentration of the organism.

From the diluted organism (10^{-2}), 0.2mL was taken into the prepared sterile nutrient agar cooled to about 40-45^{o}C, then poured into sterile Petri dishes and allowed to solidify for about 45-60min. Using a sterile cork-borer of 8mm diameter, the wells were made according to the number of the test tubes for the experiment. For this work 8 wells were made. The graded concentrations of the extracts were put into the wells accordingly including the controls. The studies were done in duplicates to ascertain the results obtained. The plates were left on the bench for about 2hrs to allow the extract diffuse properly into the nutrient agar i.e. pre-diffusion. The plates were incubated for 18-24hr at 37^{o}C .

Agar diffusion-surface plate method (fungi): A sterile sabouraud dextrose agar was prepared accordingly and aseptically poured into the sterile plates in triplicates and solidified properly. 0.2mL of the 10^{-2} inoculum concentration of the organism was spread on the surface of the agar using a sterile Petri-dish cover to cover all the surface of the agar. Eight wells were bored using a sterile cork-borer of 8mm diameter. The graded concentrations of the extracts were put into the including the controls. All the plates were left on the bench for 2hr to allow the extract diffuse properly into the agar i.e. pre-diffusion. The plates were incubated at 25^{o}C for 72hr [17,18,19].

RESULTS AND DISCUSSION

The result of the phytochemical studies of the hexane, ethylacetate and methanol extracts of leaves of *Smilax kraussiana* is presented in Table 1. Preliminary phytochemical screening of all the extracts revealed the presence of glycosides, flavonoids and anthraquinones. There was presence of saponins in methanol extract but not found in hexane and ethylacetate extracts of *Smilax kraussiana* leaves. Steroids was also revealed in hexane extract but absent in ethylacetate and methanol extracts of the plant. However, tannins, reducing sugar and alkaloids were all absent in all the three of the extracts of *Smilax kraussiana* leaves.

Table 1: Phytochemical constituents of the hexane, ethylacetate and methanol extracts of *Smilax kraussiana* leaves

Secondary metabolites	Extracts (whole plant)		
	Hexane	Ethylacetate	Methanol
Alkaloids	-	-	-
Saponins	-	-	++
Tannins	-	-	-
Reducing sugars	-	-	-
Steroids	++	-	-
Glycosides	++	++	++
Flavonoids	++	++	++
Anthraquinones	++	++	++

- *Absent* ++ *Present*

The antibacterial result of hexane, ethylacetate and methanol extracts at concentrations ranging from 6.25 to 200 mg/ml is presented in Table 2.

The bacteria used were clinical strains of *Salmonella typhii* (UCH 4801), *Escherica coli* (UCH 00260), *Pseudomonas aeruginosa* (UCH 1102) and *Klebsiellae pneumonae* (UCH 2894) (gram negative), *Bacillus subtilis* (UCH 74230) and *Staphylococcus aureus* (UCH 2473) (gram positive). All extracts showed inhibition on the six test bacteria. Hexane and methanol extracts effectively inhibited the growth of *Staphylococcus aureus* and *Bacillus subtilis*(gram positive) than ethylacetate extract at concentrations between 25 and 200mg/ml, while all extracts possess lower antibacterial properties on *Salmonella typhii, Escherica coli, Pseudomonas aeruginosa* and *Klebsiellae pneumonae* (gram negative).

Table 11: Antibacterial activities of the hexane, ethylacetate and methanol extracts of *Smilax kraussiana* Leaves.

Extracts	Extract conc/Ref./ Control (mg/ml)	Diameter of well = 8 mm					
		Diameter of zone of inhibition of bacteria(mm)					
		S.a	E.coli	B.sub	Ps.a	Kleb	Sal
Hexane	6.25	-	-	-	-	-	-
	12.5	-	-	-	-	-	-
	25	11	-	-	-	-	-
	50	13	10	12	-	-	-
	100	15	12	14	10	-	-
	200	19	15	17	12	10	13
	Hexane	-	-	-	-	-	-
	Gentamycin	38	34	35	36	35	34
Ethylacetate	6.25	-	-	-	-	-	-
	12.5	-	-	-	-	-	-
	25	-	-	-	-	-	-
	50	10	-	-	-	-	-
	100	12	10	10	-	-	-
	200	15	12	13	10	12	12
	Ethylacetate	-	-	-	-	-	-
	Gentamycin	38	34	35	36	35	34
Methanol	6.25	-	-	-	-	-	-
	12.5	10	-	-	-	-	-
	25	12	-	10	-	-	10
	50	14	-	12	-	-	12
	100	18	10	14	11	10	14
	200	23	13	16	13	14	17
	Methanol	-	-	-	-	-	-
	Gentamycin	38	34	35	36	34	35

The result of the antifungal activities of the hexane, ethylacetate and methanol extracts of *S.Kraussiana* leaves at concentrations between 6.25 and 200 mg/ml is presented in Table 3.

Six clinical strains of human pathogenic fungi were also used in this study. *Candida albicans, Aspergillus niger, Rhizopus stolon, Penicillum notatum, Tricophyton rubrum and Epidermophyton floccosum.* The test fungi were sensitive to the hexane, ethylacetate and methanol extracts. The three extracts exhibited higher antifungal properties on *Candida albicans, Aspergillus niger, Rhizopus stolon, Penicillum notatum, Tricophyton rubrum and Epidermophyton floccosum;* In fact the activity is comparable to that of the reference drug Tioconazole against the six test organisms at concentrations between 6.25 and 200mg/ml.

Table 111: Antifungal activities of the hexane, ethylacetate and methanol extracts of *Smilax kraussiana* Leaves.

Extracts	Extract conc/Ref./ Control (mg/ml)	Diameter of well = 8 mm					
		Diameter of zone of inhibition of fungi(mm)					
		C.a	A.n	Rhiz	Pen	T.r	E.f
Hexane	6.25	10	-	-	-		
	12.5	12	-	-	-	10	11
	25	15	12	10	10	12	13
	50	17	14	12	12	16	15
	100	19	18	17	17	20	18
	200	23	23	21	19	23	23
	Hexane	-	-	-	-	-	-
	Tioconazole	26	24	24	22	24	24
Ethylacetate	6.25	10	-	10	-	-	-
	12.5	12	10	11	10	10	10
	25	14	12	14	10	12	11
	50	16	14	16	14	14	15
	100	18	17	19	17	18	20
	200	20	19	21	19	21	22
	Ethylacetate	-	-	-	-	-	-
	Tioconazole	26	24	23	22	24	24
Methanol	6.25	10	-	-	-	-	-
	12.5	11	-	10	10	10	10
	25	14	10	12	12	12	11
	50	16	14	14	14	14	14
	100	20	17	18	16	17	16
	200	24	23	22	19	20	20
	Methanol	-	-	-	-	-	-
	Tioconazole	26	25	24	22	23	23

S.a		*Staphylococcus aureus*
E.coli		*Escherichia coli*
B.sub		*Bacillus subtilis*
Ps.a		*Pseudomonas aeruginosa*
Kleb		*Klebsiellae pneumonae*
Sal		*Salmonellae typhii*
C.a		*Candidas albicans*
A.n		*Aspergillus niger*
Rhiz		*Rhizopus stolon*
Pen		*Penicillum notatum*
T.r		*Tricophyton rubrum*
E.f.		*Epidermophyton floccosum*

CONCLUSION

The phytochemical screening of the extracts revealed the presence of glycosides, flavonoids, anthraquinones, saponins and steroids. The antibacterial and antifungal activities of the hexane, ethylacetate and methanol extracts of leaves of *S. kraussiana* suggest the use of the plant in ethnomedicine for the treatment of infertility, veneral and skin diseases [6,7,8,9]. Studies are ongoing in our laboratory to isolate, identify, characterize and elucidate the structure of bioactive compounds responsible for the observed pharmacological activities.

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