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Antifungal Activity and Phytochemical Analysis of Column Chromatographic Fractions of Stem Bark Extracts of *Ficus sycomorus* L. (Moraceae)

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Abstract: The *in vitro* antifungal activity and phytochemical properties of column chromatography fractions of Hexane (HX), Petroleum Ether (PE) and Chloroform (CHL) of stem bark of *Ficus sycomorus* were studied. The bioactive agent in the most potent fraction with antifungal properties was isolated and identified by preparative Thin Layer Chromatography (TLC) and phytochemical analysis. The hexane fractions (HX2 and HX3) were significantly active ($p<0.05$) at 0.31 to 5.63 mg mL^{-1} on *Microsporum gypseum*, *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans*. The fractions revealed the presence of Steroids (STR), Condensed Tannins (CDT), Cardiac Glycosides (CG) and Saponins (SAP). The HX4 fraction was the most potent, exhibiting complete inhibitory activity against all the fungal isolates tested. The fraction contained Anthraquinone Glycosides (ATG) with Retention factor (R_f) value of 0.87. All the PE fractions (PE1, PE2 and PE3) did not exhibit significant activity on the fungal isolates at 0.31 to 3.75 mg mL^{-1}. The CHL fractions (CHL1 and CHL2) showed very high inhibitory activity on only *Trichophyton mentagrophytes* and *Trichophyton rubrum*. The phytochemical analysis of these fractions revealed the presence of CG, SAP, CDT and STR. The results of the present study add credence to the ethnomedicinal uses of *Ficus sycomorus* for the treatment of fungal diseases.

Key words: *Ficus sycomorus*, column and thin layer chromatography, antifungal activity, phytochemical analysis

INTRODUCTION

Limitations of synthesized compounds in the treatment of chronic diseases and the potential of plant-based medicine as a more effective and cheaper alternative, was probably responsible for the fast growing industry of herbal medicine (Rojas *et al*., 1992). Resistance to antibiotics and toxicity during prolonged treatment with present-day drugs have been the reasons for an extended search for newer drugs to treat opportunistic microbial infections (Forstel and Lartey, 2000). In northern Nigeria, the stem bark of *Ficus sycomorus* is used traditionally to treat fungal diseases, jaundice and dysentery. The leaves have been reported to have calcium, phosphorus, iron, magnesium and zinc (Keay, 1989; Umar and Azare, 2006). The stem bark extracts were reported to contain tannins, saponins, reducing sugars, flavone aglycones, anthraquinone glycosides, flavonoid glycosides and condensed tannins

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(Hassan, 2005; Sandabe *et al*., 2006). In an earlier work, *Ficus sycomorus* was shown to have sedative and anticonvulsant effects on rats (Sandabe *et al*., 2003). The plant was found to have partial inhibition on bacterial growth (Sandabe, 2002) and the stem bark organic solvent extracts possess higher antifungal activity compared to aqueous extract (Hassan *et al*., 2006). The aqueous stem bark extract was also reported to have inhibitory effect on smooth and skeletal muscle contractions in laboratory animals (Sandabe *et al*., 2006).

Ficus sycomorus L. is a rough leaved fig tree with trunk that can grow to about 20 ft. in diameter. It is found in semi-arid parts of Nigeria (Williams *et al*., 1980). The fungal isolates used in the present study are associated with the incidence of skin and internal organ infections (Robers, 1978). This study was aimed to evaluate the antifungal activity of column chromatographic fractions of stem bark extracts of *Ficus sycomorus* by preliminary bioassay screening using column chromatography, thin layer chromatography and phytochemical screening. This study would contribute to the development of plant-based antifungal drugs.

MATERIALS AND METHODS

Materials

Anthracene (BDH 34166 2F). Silica gel column (60 g, Si 60, 40-63 μm) from Merck, Germany. Commercially Prepared TLC plate (GmbH and Co. D-3440 Eschwege Germany).

Collection of Plant and Authentication

The stem bark of *Ficus sycomorus* was obtained from Kara, Sokoto, Nigeria. The plant was identified at Botany Unit, Usmanu Danfodiyo University, Sokoto, Nigeria. A voucher specimen was prepared and deposited in the herbarium of the same institution for reference. The stem bark was air dried and pulverized (using pestle and mortar) into moderately coarse powder and subjected to aqueous, hexane, petroleum ether and chloroform extractions.

Organisms

The fungal organisms used were *Candida albicans*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum gypseum*, *Aspergillus niger* and *Aspergillus flavus*. They were all clinical isolates obtained from Usmanu Danfodiyo University Teaching Hospital, Sokoto, Nigeria. The fungi were maintained on Sabouraud Glucose Agar (SGA) medium and re-identified by microscopic examination of a portion of the colony for spores and characteristic hyphae (Cheesbrough, 1982).

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 as described by Moris and Aziz (1976) and Springfield and Weitz (2006). The powdered stem bark (120 g) was extracted with ethanol-water (1:1, 1500 mL) separately at room temperature overnight. The extract was filtered and partitioned in hexane separately (750 mL) and clarified by further filtration. Evaporation of hexane fraction to dryness in an oven at 45°C yielded 1.58% (w/w) residue. The aqueous filtrate (ethanol-water) of the extract fraction was further partitioned (to obtain fractions of different polarities) with petroleum ether (750 mL) and chloroform separately (750 mL). Evaporation of the petroleum ether and Chloroform (CHL) yielded 2.00 and 2.25% (w/w) residues, respectively. The procedure was repeated to obtain more residues. All the residues obtained were reconstituted in ethanol-water and chromatographed over silica gel column. The fractions obtained were evaporated and the residues were screened for antifungal activity.

Column Chromatographic Separation

The HX, PE and CHL fractions of the stem bark extracts were chromatographed over silica gel column and eluted with ethanol-water (1:1) as described by Brain and Turner (1975). The residues obtained were: HX1 (0.35 g), HX2 (0.38 g), HX3 (0.43 g), HX4 (0.36 g), PE1 (0.63 g), PE2 (0.70 g), PE3 (0.68 g), CHL1 (0.76 g), CHL2 (0.70 g) and CHL3 (0.80 g). The column fractions were tested for antifungal activity against six fungal isolates. The most potent fraction (HX4 fraction) was further isolated and identified through TLC and phytochemical screening.

Isolation of Anthraquinone Glycosides

The method employed was as gentle as possible so that the glycosides are maintained as entire molecules (Brain and Turner, 1975). Ten grams of the sample were extracted with 50 mL of chloroform for 8 h. The clear liquid was decanted. The liquid was heated at 80°C for 3 min to denature proteins and was allowed to stand for 24 h. It was filtered and the filtrate was evaporated under reduced pressure at a temperature of 50°C to obtain residue. The purity of the substance was determined by means of TLC (Vanedr and Johan, 2001). Antifungal activity of the isolated anthraquinone glycosides was also determined.

TLC of Anthraquinone Glycosides

Ten microlitres of the bioactive anthraquinone glycosides were further fractionated by means of commercially prepared thin layer chromatography plates (GmbH and co. D-3440 Eschwege, Germany) using ethylacetate: methanol: water (77: 13: 10 v: v: v) as mobile phase. Ten milligrams of anthracene (reference standard) were dissolved in 10 mL of 50% ethanol with gentle heating and 10 μL was applied as a band beside the sample solution. After developing the plates, the positions of the compounds were determined by spraying 5% KOH in 50% ethanol and heated for 15 min at 105°C and inspected immediately in day light. The R_f values of the standard and the bioactive fraction were determined as described by El-Olemyl *et al*. (1994).

Phytochemical Screening

This was carried out using standard procedure as described by Wall *et al*. (1954), Persinos and Quimby (1967), Harbone (1973), Trease and Evans (1978) and El-Olemyl *et al*. (1994).

Antifungal Activity

The antifungal activities of the column fractions were evaluated according to Zacchino *et al*. (1999), using the agar dilution method with some modifications. The fungal species were cultivated on Sabouraud Glucose Agar (SGA) medium (Diagnostic Pasteur) in 90 mm petridishes. Five millilitres of filter-sterilized reconstituted water solution of each stem bark column fractions (4.65 to 84.45 mg mL^{-1}) were aseptically mixed with 15 mL of SGA (liquefied and maintained at melting point in water bath at 45°C) to give final concentrations of 0.31 to 5.63 mg mL^{-1}. The Petri-dishes were filled to 20 mL final volume with SGA 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*, *T. mentagrophytes* and *M. gypseum*) and 7-day-old (*A. niger*, *A. flavus* and *C. albicans*) fungal colonies. Griseofulvin (0.63 mg mL^{-1}, Clarion Medicals Ltd., Lagos, Nigeria), measured from the pulverized 500 mg tablet was included as positive control. Water (5 mL) in place of the extract and media (15 mL) were mixed together to serve as negative control. The treated and the control Petri-dishes were incubated at room temperature for 14 days for *T. rubrum*, *T. mentagrophytes* and *M. gypseum* and 48 h for *A. niger*, *A. flavus* and *C. albicans*. Growth was observed each day to the last day. From these, the percentage inhibitions were calculated using the following formula:

$$I(\%)=\frac{dc-dt}{dc}\times 100 \quad (1)$$

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 one way analysis of variance.

Statistical Analysis

The data collected in the study was subjected to one-way Analysis of Variance (ANOVA), Benferoni compare all columns using Graph Pad Instat Software (San Diego, USA).

RESULTS AND DISCUSSION

Fractionation of the organic solvent extracts by column chromatography on silica gel gave several fractions (HX1-HX4, PE1-PE3 and CHL1-CHL3). The *in vitro* assays of antifungal activity and phytochemical analysis of the column fractions are presented in Table 1 and 2, respectively. The column fractions (HX1 and HX3) indicated insignificant activity (0-25%) at 0.31 to 5.63 mg mL^{-1} on *M. gypseum*, *A. niger*, *A. flavus* and *C. albicans* (not shown in table). However, the HX4 fraction was found to be the most potent with complete inhibitory activity (98%) on the fungal isolates employed. All PE fractions (0.31-5.63 mg mL^{-1}) did not produce significant inhibitory activity (0-25%) against the isolates. The CHL1 fractions (0.31 mg mL^{-1}) exhibited significant activity (98%) on *T. mentagrophytes*, *T. rubrum* and *A. niger*, whereas CHL1 (3.75 and 5.63 mg mL^{-1}) produced significant activity (75-100%) on all the tested organisms. The CHL2 fractions (0.31-3.75 mg mL^{-1}) produced minimal inhibitory activity (0-25%) while CHL2 fraction (5.53 mg mL^{-1}) exhibited average activity (25-50%) on only *M. gypseum* (not shown in table). The CHL3 fraction (0.31 mg mL^{-1}) was only very active (98%) on *T. mentagrophytes* and *T. rubrum* but the CHL3 fraction (0.63 mg mL^{-1}) was very active (98%) on *T. mentagrophytes*, *T. rubrum* and *A. flavus*. CHL3 fractions (3.75 and 5.63 mg mL^{-1}) were very active (98%) on *T. mentagrophytes* and *T. rubrum*.

Table 1: Percentage inhibitions of fungal isolates by column chromatographic fractions of organic solvent extracts of *Ficus sycomorus*

Fraction conc. (mg mL^{-1})	*Microsporum gypseum*	*Trichophyton mentagrophytes*	*Trichophyton rubrum*	*Aspergillus niger*	*Aspergillus flavus*	*Candida albicans*
0.31 HX2	97.64	12.36	86.50	28.21	35.41	27.14
0.63 HX2	97.64	31.46	27.39	53.14	54.05	53.92
3.75 HX2	97.64	38.20	34.10	97.14	97.29	59.14
5.63 HX2	97.64	53.26	49.50	97.14	97.29	71.43
0.31 HX4	97.64	97.75	97.69	97.14	97.29	97.14
0.63 HX4	97.64	97.75	97.69	97.14	97.29	97.14
3.75 HX4	97.64	97.75	97.69	97.14	97.29	97.14
5.63 HX4	97.64	97.75	97.69	97.14	97.29	97.14
0.31 CHL1	9.41	97.75	97.69	52.14	52.70	45.71
0.63 CHL1	22.35	97.75	97.69	65.29	64.86	69.14
3.75 CHL1	95.29	97.75	97.69	74.29	73.18	97.14
5.63 CHL1	76.18	97.75	97.69	79.29	86.49	97.14
0.31 CHL3	2.06	97.75	97.69	2.86	5.41	1.07
0.63 CHL3	5.88	97.75	97.69	5.00	97.29	11.07
3.75 CHL3	32.94	97.75	97.69	13.93	97.29	12.50
5.63 CHL3	42.94	97.75	97.69	17.14	97.29	17.14
0.63 GS (Positive control)	88.76	87.64	81.50	62.14	67.57	97.14

Values are percentage inhibitions, 50-75% = Strong inhibition; 75-100% = Very strong inhibition; HX 2, 4 = Hexane fractions and CHL1, 3 = Chloroform Fractions; GS = Griseofulvin

Table 2: Phytochemical analysis of column chromatographic fractions of stem bark extracts of *Ficus sycomorus*

Extract fraction	VLO	ALK	CDT	PSEU	HT	ET	CG	FG	ATG	SAG	CYG	DG	SAP
HX1	-	-	+++	-	-	-	-	-	-	-	-	-	+++
HX2	-	-	+++	-	-	-	+++	-	-	-	-	-	+++
HX3	-	-	+++	-	-	-	-	-	-	-	-	-	+++
HX4	-	-	-	-	-	-	-	-	+++	-	-	-	-
CHL1	-	-	-	-	-	-	+++	-	-	-	-	-	+++
CHL2	-	-	+++	-	-	-	-	-	-	-	-	-	+++
CHL3	-	-	-	-	-	-	+++	-	-	-	-	-	-
PE1	-	-	-	-	-	-	-	-	-	-	-	-	-
PE2	-	-	-	-	-	-	-	-	-	-	-	-	+
PE3	-	-	+	-	-	-	+	+	-	-	-	-	+

	Steroids	Free anthraquinone	Balsams	Resins	Flavonoids
HX1	-	-	-	-	-
HX2	+++	-	-	-	-
HX3	-	-	-	-	-
HX4	-	-	-	-	-
CHL1	+++	-	-	-	+++
CHL2	+++	-	-	-	-
CHL3	+++	-	-	-	+++
PE1	+	-	-	-	-
PE2	-	-	-	-	-
PE3	+	-	-	-	+

- = Absence; + = Trace amounts; +++ = Presence; HX1-4 = Hexane fractions; CHL1-3 = Chloroform fractions; PE1-3 = Petroleum ether fractions; VLO = Volatile oil; ALK = Alkaloids; CDT = Condensed Tannins; PSEU = Pseudotannins; HT = Hydrolyzable Tannins; ET = Ellagitannins; CG = Cardiac Glycosides; FG = Flavonoid Glycosides; ATG = Anthraquinone Glycosides; CYG = Cyanogenic Glycosides; DG = Digitalis Glycosides; SAG = Saponin Glycosides and SAP = Saponins

Phytochemical analysis of the active fractions (Table 2) revealed the presence of STR, CDT, CG, SAP (HX2, HX3, CHL1 and CHL2) and ATG (HX4). Further isolation and identification of the active constituents (ATG) in HX4 fraction by TLC gave R_f value of 0.87. The reference standard (Anthracene) has R_f-value of 0.89. Isolated anthraquinone glycosides exhibited complete inhibitory activity (98%) against all the isolates employed.

Out of the entire organic fractions hexane fraction exhibited the most optimum inhibitory activity against the isolates followed by the chloroform fractions with petroleum ether fraction producing none or least activity.

The results of the present study present an easy *in vitro* system that can be used for assessing the antifungal activities of medicinal plants. Freiburghans *et al*. (1996) reported different solvent extracts of some plants to have different pharmacological properties. Hassan *et al*. (2006) reported organic stem extracts of *F. sycomorus* with higher antifungal activity than aqueous extracts. From our results (Table 1), HX4 fraction of the stem was most effective (98% inhibitory) and could be the best medium for extracting the pharmacologically active compound (s) from the stem of *F. sycomorus*. It is possible that the extraction method (bioassay-guided fractionation) employed could be responsible for the inactivity of the PE extracts. The results of the study suggest that most of the *F. sycomorus* organic solvent stem extracts were very active against most of the pathogenic fungi even at very low doses, thus validating its pharmacological and therapeutic potentials. The results also explains the use of this plant in folk medicine for the treatment of various diseases whose symptoms might involve microbial infections and underline the importance of ethnobotanical approach for the selection of *F. sycomorus* in the discovery of new bioactive compounds.

Present results are comparable to those of Subramanian *et al*. (2006) who showed ethanolic extracts of *Aloe vera* gel, although a different plant family, to have activity against most of the pathogenic fungi at very low doses. The antifungal activity of *F. sycomorus* may be attributable to

the presence of compounds such as ATG, CDT, STR, CG and SAP. The antimicrobial actions of most of these phytochemical substances have been well documented (Deeni and Hussain, 1991; Scalbert, 1991; Favel *et al*., 1994; Shale *et al*., 1999). The presence of ATG in the most active fraction (HX4) suggests that it is the principal antifungal agent in the stem bark extract of *F. sycomorus*. From the above results, it may be concluded that *F. sycomorus* stem bark extracts possess compounds with antifungal properties which, with further refining, can serve as basis for the development of novel antifungal agents.

The mechanism(s) of action of the constituent(s) of the organic fractions of the plant could probably be by already known mechanisms such as inhibition of electron transport chain, sphingolipid biosynthesis and fungal cell wall (Lartey and Moehle, 1997; Ueki and Taniguchi, 1997; Dominiguez and Martin, 1998), or yet some other undiscovered mechanism(s).

This research scientifically justifies the use of *F. sycomorus* stem bark extracts for antifungal therapy, thus presenting a potentially new cheap source of potent antifungal agent. Further studies on Minimum Bactericidal concentration, toxicological assessments and elucidation of the structure of the active compound (using spectroscopic technique) in the plant are in progress in our laboratory.

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