Antidermatophytic Activity of Stem Back Extracts of *Khaya senegalensis* (Desr.) A Juss.

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Abstract: The high prevalence of Dermatomycoses coupled with the residual side effect associated with orthodox medicine, treatment failure and cost of the chemotherapeutics, have necessitated exploration of new avenues of controlling these diseases with the use of plant such as *Khaya senegalensis* which is cheap and available, so that potent and efficacious candidate drugs could be identified. Therefore, this study determined the antidermatophytic activity and the phytochemical constituents of the stem bark extracts of *Khaya senegalensis*. This was achieved through extraction of powdered stem bark of the plant with methanol, distilled water and chloroform using percolation method. The extracts were subjected to qualitative detection of plant secondary metabolites. The extracts were tested for antidermatophytic activity using agar well diffusion method against *Trichophyton mentagrophyte, Trichophyton verrucosum, Trichophyton terrestre and Microsporum canis*, followed by testing the extracts for toxicity using brine shrimp lethality assay. Some phytochemicals identified in the stem bark are alkaloids, glycosides, tannins, flavonoids, saponins, steroids and anthraquinone. The antifungal assay indicated that aqueous extract exhibited no activity against all the test organisms. Methanolic extract showed less activity (8mm at 78µg/ml) compared to chloroform extract (14mm at 78µg/ml) when tested against *T. mentagraphyte,* but when tested against *M. canis,* Methanolic extract showed greater activity (26mm at 78µg/ml) compared to the chloroform extract (24mm at 78µg/ml). Both methanolic and chloroform extracts showed similar activity on *T. verrucosum* and *T. terrastre.* The Minimum Inhibitory Concentration (MIC) of both methanolic and chloroform extracts ranged from 1.2-2.4 µg/ml while the Minimum Fungicidal Concentration (MFC) was generally 4.9 µg/ml. Toxicity study revealed that the extracts were non-toxic at LC_{50} values of 919.3 µg/ml and 370 µg/ml for methanolic and chloroform extract respectively. It could be concluded that the stem bark of *Khaya senegalensis* habours important phytochemicals and has methanolic and chloroform extracts that showed good potencies against some dermatophytes. The low toxicity results of the extracts indicate that the plant may not be toxic to human and could be a potential source for the production of antifungal drugs.

Keywords: Antidermatophyte, Activity, Extract, Fungi, Khaya senegalensis.

INTRODUCTION

Diseases due to pathogenic fungi in similar tune with bacteria, represent a critical problem to human health and are one of the main causes of morbidity and mortality worldwide, World Health Organization (WHO, 1998). The investigation of the efficacy of plant based drugs used in traditional medicine are being paid great attention because they are cheap and have little side effects (Dharmasiri *et al*., 2003). The need to study a local medicinal plant *Khaya senegalensis* cannot be over emphasized. The identification of certain metabolites, such as the steroid (Abdullahi, 2005), Anthraquinone glycosides which is the most potent antifungal compound from plants (Hassan, 2005) and the susceptibility of some known pathogens will serve as the basis for further research into the medicinal uses of *Khaya senegalensis* (Abdullahi, 2005).

Dermatophytes infections are common worldwide. The prevailing cases of fungal infections of the skin, hair and nails are disturbing (Havlickova *et al.,* 2008; Ameen, 2010). Dermatophytes are filamentous fungi belonging to the genera; *Trichophyton, Microsporum* and *Epidermophyton*. They cause infections of the skin, hair and nails, obtaining nutrients from keratinized materials (Midgley *et al*., 1994).

The prevalence of dermatophytes infections in human is increasing with an estimated 20-25 percent of the world population being infected and the incidence continue to increase steadily (Male, 1990; Nweema, 2010). About 62% of the Dermatophyte species isolated from humans are *Trichophyton rubrum*; 27% are *Trichophyton mentagrophytes*; 7% are *Trichophton verrucosum*; 3% are *Trichophyton tonsurans*; 1% are *Epidermophyton flocosum, Microsporum oudoini, Microsporum canis, Microsporum manum, Microsporum versicolor* e.t.c (Sequeira, 1906).

However, *T. rubrum and T. mentagrophytes* account together for 80% to 90% of all dermatophytosis worldwide (Havlickova *et al*., 2008; Ameen, 2010).

This study is based on the justifications that, Okafor *et al*. (2001) and Nweze *et al*. (2004) have screened some of plants used in Nigeria (i.e. *Zapotica portericensis, Cissus guodrangularis* Lin*., Trema guineensis and Moninda lucida* Benth) against dermatophytes and indeed found some to have good in vitro activity against dermatophytes. Therefore, the need to screen more medicinal plants so as to identify more plants with potential for antidermatophytic activity with less toxic effect, cheap and readily available.

The study determined the in-vitro activity of *Khaya senegalensis* stem bark extract against selected dermatophytes. The objectives of the study include; to extract stem bark components of *Khaya senegalensis*, to determine the *in-vitro* anti-dermatophytic activity of the extracts and the phytochemical constituents, to determine the toxicity of the active crude extracts (LC_{50}) and to isolate the chemical constituent (fractions) present in all the active crude extracts.

MATERIALS AND METHODS

Extraction of Plant Materials

The extraction was carried out using percolation method as described by Fatope and Hamisu (1993). One hundred grams (100g) of the powdered plant material was percolated in one thousand millitres (1000ml) of methanol at room temperature (20-25℃). The mixture was left to stand for two weeks with regular shaking. The mixture was then filtered using a Whatman No.1 filter paper ($11\mu m\ pore\ size$) and the filtrate obtained was transferred into a pre-weighed beaker and then evaporated to dryness in water bath at 45^0C until the solvent evaporates completely leaving behind the methanolic extract. Similarly, another one hundred grams (100g) of the powdered plant materials was percolated in one thousand millitre (1000ml) of sterile distilled water. The mixture was left to stand for one week with regular shaking. The aqueous solution was filtered using Whatman No.1 filter paper and the filtrate was evaporated to dryness in a water bath at 45^0C.

Determination of Some Physical Properties of the Plant Extract:

The colour of the extracts was assessed by looking at the colour of the extract immediately after the removal of the solvent by evaporation process. Texture was felt manually with the help of glass rod and feeling of the particulate nature of the resulting friction in between glove fingers as described by Adoum *et al.* (1997).

Preparation of Different Concentrations of the Extract:

Sterile distilled water was used for the aqueous extract while dimethyl sulfoxide (DMSO) was used for the methanolic and chloroform extract to prepare different concentrations of the extract by serial doubling dilution from an initial concentration of 100mg/ml to make the required concentrations of 50mg/ml, 25mg/ml and 12.5mg/ml respectively as demonstrated by Vaslika (2010).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The broth dilution method was used to determine the MIC as described by Rasooli and Abyanek (2004). A twofold serial dilution of the extract from the stock solution was made using 1ml of Sabouroud's dextrose broth (SDB) that was put in test tubes numbered one to nine. One milliliter (1ml) of 39μg/ml of the extract was added to tube one. This was thoroughly mixed and 1ml was transferred to tube -2. The dilution continued until tube seven (7), from which 1ml was discarded. Zero point one (0.1ml) of the standard inocula of the test organism was then added to tube 1 to 8. Tube eight and nine were used as positive and negative controls respectively. The procedure was repeated for all organisms susceptible to the extracts. The content of the tubes were well mixed and incubated at 37^0C for 72h.

The tubes were then examined visually for turbidity after 72h incubation by comparing with the control tubes. The lowest concentration of the extract showing no visible growth when compared with controls was considered as the minimum inhibitory concentration (MIC). The minimum fungicidal concentration is the lowest concentration of the extract that prevents the growth of the fungal organism

To determine this, the tubes that showed no visible fungal growth from the MIC were inoculated on to sterile Sabouraud's dextrose agar and incubated for 3 days at 37^0C. The lowest concentration in which no growth occurred was taken as the minimum fungicidal concentration (MFC) as described by Rasooli and Abyanek (2004).

Brine Shrimp Lethality Test

Solutions of the plant crude extract were made in DMSO at varying concentrations (i.e. 1000 ppm, 100ppm, 10ppm) and incubated in triplicate vials of 5ml volume. Ten brine shrimp larvae were then place in each of the triplicate vials. Others were placed in a mixture of DMSO (30μl) and sea water to serve as a negative control. After 24hrs of incubation, the nauplii were examined against a lighted background, with a magnifying glass and the average number of survived larvae was determined. The mean percentage mortality was plotted against the logarithm of concentrations and the concentration that kills fifty percent of the larvae (LC 50) was determined from the graph (Moshi *et al*., 2010)

Activity Guided Thin Layer Chromatography (TLC) of the Extract

The TLC was used to separate the crude extract into fractions as described by Hubpages and Hubbers (2017) based on their retention factor. The stationary phase (consisting of 2:1 mixture1 of water and TLC silica gel) was applied on to the glass plate uniformly and then allowed to dry and stabilized for 24hrs at 106^0C. With a pencil, a thin mark was made at the bottom of the plate to apply the sample. The crude extracts were applied on the mark drawned. The mobile phase was poured into the TLC chamber to a level few centimeters above the chamber bottom. A filter paper was soaked in mobile phase and placed on the inner wall of the chamber to maintain equal humidity. The prepared plate was placed in TLC chamber so that the side of the plate with sample line was facing the mobile phase. The plate was then immersed, such that the samples spots are well above the level of mobile phase for development. The chamber was closed with a lid. This was allowed until the solvent reached a distance of 2/3 of the plate before the plate was removed and allowed to dry. The sample spot was seen in a suitable UV light chamber to obtain the various fractions by determining the RF values of each fraction (Hubpages and Hubbers, 2017).

Determination of the Antidermatophytic Activity of the Extract Fractions

All the fractions of the extracts were tested individually for anti-dermatophytic activity using paper disc diffusion method. In this case, Whatman No1 paper discs of 6mm diameter were impregnated with the fraction of the extract and tested for activity against the test organisms (Jagessar *et al.,* 2008).

Statistical Analysis

The oneway Anova test was used to compare result among and within groups for any significant different in antidermatophytic activity of the extract and the control as demonstrated by Idu *et al*. (2014) using the Graph pad Instat 3 Statistical Software for windows 2006. Values were considered significant when $P<0.05$.

The activity of the extract against the tested organisms was analysed by oneway analaysis of variance (ANOVA) using Tukey – Kramar – Multiple comparism test, where p value less than 0.0001 (i.e. $P < 0.0001$) is considered extremely significant and P value less than 0.05 (i.e. $P < 0.05$) is also significant. Variation among columns is significantly greater than expected by chance.

RESULTS AND DISCUSSION

The results of this study have shown up to 32.32% of methanol soluble extract of the stem bark whereas, 2.13% anthraquinone was obtained using chloroform (Table 1). This may be due to differences in the quantities of the component or perhaps may be a bearing to the polarity of the solvent and the constituent of the stem bark of *Khaya senegalensis*.

The phytochemical analysis of the stem bark extracts of *Khaya senegalensis* revealed the presence of alkaloids, glycosides, tannins, flavonoids, saponins, steroids and anthraquinone (Table 2). This findings correlate with the finding of Abdullahi *et al.* (2016) who reported the presence of alkaloids, flavonoids, saponins

and tannins from the leaves and bark of *Khaya senegalensis* while Kankia and Zainab (2015) reported the presence of alkaloids, flavonoids, saponins, steroids, tannins and triterpenoids from the leaves, barks and roots of *Khaya senegalensis*. However, Olayinka *et al.* (1992) reported the presence of glycosides from the stem bark of *Khaya senegalensis*. Waterman (1992) also reported that, the main classes of phenolic predominant in plant extracts were alkaloids and flavonoids which are found useful in medicine as antimicrobial, anti-inflammatory and antioxidant agents. Bougard *et al.* (1994) reported that, the significant antifungal activity exhibited by the plant materials may be linked to the presence of steroids, flavonoids, alkaloids and saponins. The variation in phytochemical constituents resulted from the extraction ability of a particular component which appeared to depend on extraction medium polarity (solvent) and the ratio of solute to solvent as well as increase in temperature (Simon, 2015). The secondary plant metabolites are usually analyzed because of the established pharmacological activity of many like tannins, alkaloids, glycosides etc. (Gennaro, 1990). The alkaloids are secondary metabolites of amino acids and constitute the largest and most diverse group of natural products of vegetable origin (Tedder *et al*., 1979). Some alkaloids are analgesic e.g Morphines, anti-malaria e.g quinine, tranquilizers e.g reserpin which remains of value in treating hypertension in which low doses are effective (Trease and Evans, 1989). Colchicin however, is another group of alkaloids mainly used in the treatment of gout. The alkaloid has also been used to double the chromosome number of sterile hybrids produced by crossing widely separated species of plants. The cardiac glycosides have been used for over two centuries as stimulants in cases of cardiac failure and are still indispensable in this field (Olayinka *et al.*, 1992).

Tannins are polyphenols and have an antimicrobial property while the flavonoids are substances with phyenylbenzopyrone skeleton and are the most numerous class of naturally occurring oxygen heterocyclic compounds constituting an important group of yellow natural pigments (Tedder *et al*., 1979). The steroids and their modified derivatives have more diverse biological effect than any other natural products (Tedder *et al*., 1979). Tannins however, is used in styptic preparation which produces contractions of the blood vessels stopping bleeding and having the quality of retaining heamorhages when applied to the bleeding part (Abdullahi, 2005). This should be related to the application of *Khaya senegalensis* in the treatment of ulcers and wounds. The most significant function assigned to flavonoids is as regulators of seed germination and plants growth but have also been implicated in the protection of plants and animals against infections from microorganisms (Abdullahi, 2005). Saponin however has the property of causing hemolysis of cells when it comes in contact with it even at low dilution but are not absorbed by the normal epithelium of the alimentary canal (Abdullahi, 2005).

The result of the antifungal activity of this study indicated that both methanolic and chloroform extracts exhibited activity against all the tested isolates whereas the aqueous extract did not show any activity against any of the tested organisms (Table 3). The inhibitory activity exhibited by the crude extracts tend to agree with the report that antimicrobial properties of the plants are due to the presence of tannins, alkaloids, flavonoids terpenoids or essential oils (Bassole *et al.,* 2003; Erasto *et al.,* 2004). The zone of inhibition produced by the extracts against the test organisms indicated the potency of the active principles which have measurable activity against the isolates. The methanolic extract exhibited less activity on *T. mentagrophyte* (8mm at 78 $\mu g/ml$) compared to the chloroform extract (14mm at 78$\mu g/ml$). However, both methanolic and chloroform extracts showed similar activity against *Microsporum canis* at the concentration of 156$\mu g/ml$ (i.e 24mm) and at the concentration of 39 $\mu g/ml$ (i. e 26 mm) each. Similarly, the methanolic extract showed similar activity with the standard antifungal agent used as control (ketoconazole) against *M. canis* at a concentration of 39 $\mu g/ml$ and 25mg/ml respectively.

The activity of the chloroform extract against *T. mentagrophyte* is similar to that of the methanolic extract against *T. terrestre* at various concentrations. *T. verrucosum* showed similar susceptibility pattern to both chloroform and methanolic extracts at different concentrations. *M. canis* was the most susceptible among the isolates with mean zone of inhibition of 26 mm and *T. mentagrophyte* was less susceptible with mean zone of inhibition of 8mm both at 78 $\mu g/ml$.

The study revealed that the MIC for both methanolic and chloroform extracts ranged from 1.2 to 2.4$\mu g/ml$ and the MFC was found to be generally 4.9 $\mu g/ml$ for all the isolates (Table 4 and 5). This correlate with the report of David *et al. (2007*) who reported the MIC of griseofulvin (a standard antifungal agent) ranging from 1 to 2 $\mu g/ml$.

The observed antidermatophytic activity of *Khaya senegalensis* Stem bark extract on the isolates was interesting and encouraging since it showed that, the extract may indeed be effective *in-vivo* as claimed by traditional practitioners. It is however, not unusual for observed antimicrobial activity of plant extract to have been contributed by various solvents of extraction, separation or dilution. In this study, distilled water used to reconstitute the aqueous extract and the dimethylsulfoxide (DMSO) used to reconstitute the methanolic and anthraquinone extracts were not active against the tested isolates.

However, all the fractions from the TLC extracts exhibited no activity against the isolates. Therefore, the antidermatophytic activity of the crude extracts may be due to the synergy from the various phytochemical present in the crude extracts of the plant than the fractions.

Toxicity study of the methanolic and chloroform extracts using brine shrimp toxicity test shows that the LC_{50} were observed to be 919.3 $\mu g/ml$ and 370 $\mu g/ml$ respectively (Table 7). It therefore follows that the extracts may not be toxic to humans as the LC_{50} value are greater than 100 $\mu g/ml$ (LC_{50}>100 $\mu g/ml$). According to the Meyer *et al.* (1982) an LC_{50} value higher than 100 $\mu g/ml$ is considered not non-toxic.

The bioactive compounds were found to be carboxylic acid esters as shown by the GCMs results. Most of these compounds have been found to show interesting biological activity against certain pathogens. The anti-inflammatory, antioxidant and antibacterial activities reported for carboxylic acid may suggest the rationale for its traditional use against illnesses such as skin diseases as well as analgesics (Wel *et al*., 2014).

Table 1: Physical Properties of the Plant Extracts

Physical Properties	Aqueous extract	Methanolic extract	Anthraquinone extract
Colour	Reddish	Dark Reddish	Yellowish
Texture	Gummy	Gummy	Crystalline and oily
% yield	12.78	32.32	2.13

Table 2: Phytochemical Components of *K. senegalensis* Stem Bark

Test	Aqueous extract	Methanolic extract	Chloroform extract
Resin	-	-	0
Alkaloids	+	+	0
Glycosides	+	++	0
Tannins	++	++	0
Flavonoids	+	+	0
Saponins	+++	+	0
Steroids	+	+	0
Anthraquinone	+	++	+++

Key: + = Slightly Present (Trace)
++ = present, +++ = Abundantly present, - = Absent, 0 = Test was not carried out.

Table 3: Antifungal Activity of the Plant Extract

Test Organisms	Extracts	Zones of inhibition (mm) at various concentrations of the extracts.					
		625μl/ml	313μg/ml	156μg/ml	78μg/ml	39μg/ml	Control 25mg/ml
Trichophyton mentagrophyte	Methanolic	00	00	00	08	10	20
	Aqueous	00	00	00	00	00	18
	Chloroform	10	12	12	14	19	18
Trichophyton Terrestre	Methanolic	10	12	12	14	16	16
	Aqueous	00	00	00	00	00	16
	Chloroform	12	14	14	18	24	15
Trichophyton verrucosum	Methanolic	16	18	18	20	21	26
	Aqueous	00	00	00	00	00	31
	Chloroform	16	19	20	20	21	30
Microsporum canis	Methanolic	20	22	24	26	26	26
	Aqueous	00	00	00	00	00	30
	Chloroform	18	20	24	24	26	28

Key: Control = Ketoconazole (25mg/ml)

Table 4: Minimum Inhibitory Concentration (MIC) of the Extract on the Organisms

Test organisms	**Extracts**	**Various Concentration (μg/ml) using two-fold dilution**						**MIC (μg/ml)**
		19.5	**9.8**	**4.9**	**2.4**	**1.2**	**0.6**	
Trichophyton mentagrophyte	Methanolic	-	-	-	-	±	+	2.4
	Chloroform	-	-	-	-	±	+	2.4
	Aqueous	+	+	+	+	+	+	NA
Trichophyton Terrestre	Methanolic	-	-	-	-	-	±	1.2
	Chloroform	-	-	-	-	±	+	2.4
	Aqueous	+	+	+	+	+	+	NA
Trichophyton verrucosum	Methanolic	-	-	-	-	±	+	2.4
	Chloroform	-	-	-	-	±	+	2.4
	Aqueous	+	+	+	+	+	+	NA
Microsporum canis	Methanolic	-	-	-	-	±	+	2.4
	Chloroform	-	-	-	-	-	±	1.2
	Aqueous	+	+	+	+	+	+	NA

Key: NA = No Activity

- = No Turbidity + = Turbidity, ± = Slight Turbidity

Table 5: MFC Based on Observation of Growth on the Plate

4665

Test organisms	**Extracts**	**Extracts Concentration (μg/ml)**				**MFC (μg/ml)**
		19.5	**9.8**	**4.9**	**2.4**	
Trichophyton mentagrophyte	Methanolic	-	-	-	+	4.9
	Chloroform	-	-	-	+	4.9
	Aqueous	+	+	+	+	NA
Trichophyton Terrestre	Methanolic	-	-	-	+	4.9
	Chloroform	-	-	-	+	4.9
	Aqueous	+	+	+	+	NA
Trichophyton verrucosum	Methanolic	-	-	-	+	4.9
	Chloroform	-	-	-	+	4.9
	Aqueous	+	+	+	+	NA
Microsporum canis	Methanolic	-	-	-	+	4.9
	Chloroform	-	-	-	+	4.9
	Aqueous	+	+	+	+	NA

Key: NA = MFC greater than the highest concentration used

- = No Growth + = Growth

Table 6: Retentive factors of the various fractions of the extracts

Plant extract	Retardation		
	RF_1	RF_2	RF_3
Methanolic extract	0.17	0.67	0.89
Anthraquinone	0.22	0.67	0.89

Key: RF_1 = Fraction -1
RF_2 = Fraction -2
RF_3 = Fraction -3

Table 7: Brine Shrimp Lethality Result

Plant Extracts	Concentration (μg/ml)	No of survivor Nuplii after 24hrs			Total No. of Survivor	% Mortality	Mean Mortality	LC_{50}
		T_1	T_2	T_3				
Methanolic	10	10	10	10	30	0	0	
Extract	100	7	8	9	24	20	2	919.3
	1000	6	5	4	15	50	5	
Chloroform	10	10	10	10	30	0	0	
Extract	100	5	4	6	15	50	5	370
	1000	0	0	0	0	100	10	

CONCLUSION

The result of this study showed that, the methanolic and anthraquinone stem bark extracts of *Khaya senegalensis* significantly inhibited the growth of *T. mentagrophyte, T. terrestre, T. verrucosum* and *M. canis* due to the presence of some secondary metabolites (such as, alkaloids, glycoside, tannins, saponins, flavonoids, steroids and anthraquinone). The bioactive compounds were found to be carboxylic acid esters as shown by the GCMs results. The brine shrimp lethality test of the extracts showed that the extracts are non-toxic to human which suggest that the extract could be reputed as candidate for development of an alternative source of drug for the treatment of dermatophyte infections.

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