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Antifungal activity of *Tabernaemontana stapfiana* Britten (Apocynaceae) organic extracts

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ABSTRACT

The methanol and successive (hexane, dichlroromethane, ethyl acetate and methanol) extracts of leaves, fruits, root and stem barks of *Tabernaemontana stapfiana* Britten were investigated for antifungal activity against four fungi; *Candida albicans, Cryptococcus neoformans, Microsporum gypseum* and *Trichophyton mentagrophytes*. The extracts of the different plant parts showed varied activities against the tested fungi with the stem and root barks showing significant antifungal activities. The minimum inhibitory concentrations (MICs) of methanol and sequential extracts ranged between 31.25 and 8000 µg/ml while the minimum fungicidal concentrations (MFCs) ranged between 125 and 8000 µg/ml. Among the test microorganisms, *Candida albicans* was the most susceptible with the lowest MIC of 31.25 µg/ml followed by *Cryptococcus neoformans* with an MIC of 62.25 µg/ml. These results show that it is possible to find antifungal compounds in the extracts of *T. stapfiana*.

Keywords: *Tabernaemontana stapfiana* Britten; Apocynaceae; Antifungal activity; Minimum inhibitory concentration; Minimum fungicidal concentration.

INTRODUCTION

Emergence of HIV/AIDS has led to increasing incidences of fungal infections. Although *Candida albicans* infections remain the major species isolated from clinical samples in HIV patients, other infections such as those caused by *Cryptococcus neoformans, Microsporum gypseum* and *Trichophyton menthagrophytes* are on the rise (1). Some of these fungal infections are showing resistance to common antifungals such as fluconazole and amphotericin B (2, 3). Others like griseofulvin and most of the azoles have adverse side effects such as gastrointestinal disturbances, hepatotoxicity and leucopeania (4). It is therefore important to search for novel, more effective and less toxic agents for the management of fungal infections, especially the HIV/AIDS related opportunists.

Plants and their extractives have been used all over the world since antiquity in folk medicine (5). This use has been supported by the isolation of active antifungal compounds from plants (6). Medicinal plants are widely used in Kenya in controlling various ailments including fungal infections and *Tabernaemontana stapfiana* Britten (Apocinaceae) is one of the commonly used medicinal plants (7). This species grows up to 21 m high and is

usually found in disturbed forests (8). The roots and stem barks are used by the Keiyo community in Rift Valley for management of abdominal problems, skin infections, sexually transmitted infections and upper respiratory tract infections. There has been no antifungal study on *T. stapfiana*. In this study, *T. stapfiana* methanol and sequential (hexane, dichloromethane, ethyl acetate and methanol) extracts were assayed for antifungal activity.

MATERIALS AND METHODS

Plant material

Plant samples were collected from Kaptagat Forest in Keiyo district about 40 Km East of Eldoret town, in Kenya. The samples were authenticated by Mr. Karimi, a taxonomist at Kenyatta University, Kenya. The leaves, fruit, root and stem barks from mature plants were collected in March 2006 and floral reference parts (Reference number ER/001/06) deposited at the Kenyatta University herbarium. The plant samples were dried in the shade, and ground into powdered material using a grinding mill (Christy and Norris Ltd, England). Powdered plant material were hermetically sealed in polythene bags and stored in a refrigerator at 4°C in the dark until the time of extraction.

Methanol extraction

Powdered plant parts leaves, stem bark, root bark and fruits', (100 g), were extracted using cold extraction method by soaking in methanol for 72 h in the dark in an Erlenmeyer flask, covered with aluminium foil with frequent swirling. The extracts were then filtered using Whatman filter paper No 1, and the filtrate evaporated under reduced pressure. The final extracts were stored in labeled screw-capped bottles in a deep freezer at -20° C.

Successive extraction

Powdered root and stem barks (100 g) of *T. stapfiana* were extracted using cold extraction method by successively extracting three times with solvents of increasing polarity starting with hexane, dichloromethane, ethyl acetate and methanol for 48 h each, with occasional swirling to ensure thorough extraction. The filtrates were concentrated at a temperature of 45°C under reduced pressure to obtain gummy extracts.

Bioassay culture strains

The microorganisms used in the bioassay tests were obtained from Kenya Medical Research Institute (KEMRI). These included reference strains and local clinical isolates. The microorganisms that were used included: two yeasts; *Candida albicans* (ATCC 90028) and *Cryptococcus neoformans* (ATCC 66031) and two clinical isolates of dermatophytes; *Trichophyton mentagrophytes* (KMR 100) and *Microsporum gypseum* (KMR 101). The test strains were maintained on Potato Dextrose Agar (PDA) (Becton Dickinson, BBL, Heidelberg, Germany) at 4°C. These fungi were subjected to two successive transfers in broth and tested for purity and viability using biochemical and morphological characteristics before use as described by Elgayyar et al., (9). The 1.0 McFarland standard was prepared by mixing 0.1 ml of 1.175% barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$), with 9.9 ml of 1% sulfuric acid (H_2SO_4) (10).

Antifungal disk assay

Antifungal activities of plant extracts were investigated using the Baur and Kerby disk diffusion method as described in Clinical and Laboratory Standards Institute (CLSI), 2002 (11). The PDA broth containing 24 h broth culture diluted to match 1.0 MacFarlands standard, 10^8 Colony Forming Units (CFU)/ml for yeasts and 10^8 spores/ml for filamentous fungi, were spread aseptically on the solid PDA plates using sterile L-shaped glass rod (10). Disks (6.00 mm diameter) impregnated with 10 µl of each extracts at a concentration of 200 mg/ml of solvent used in extraction and air dried, were then placed on the inoculated plates. The controls had 10 µl of each extract solvent on the disc without the extract but evaporated to dryness. Discs containing fluconazole 25 µg (Pfizer, New York, N.Y.), were used as standard controls. Both test and control discs were run parallel. All of the plates were incubated at 37 °C for 48 h for yeast and at 25 °C for 5 days for filamentous fungi. The zones of growth inhibition around the disks were measured after incubation.

The sensitivity of the microorganisms to the plant extracts was determined by measuring the diameters of inhibition zones. Values < 9 mm were considered as not active against microorganisms. All of the experiments were performed in triplicate. The results are reported as the average of the 3 experiments.

Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC)

The MICs were determined by broth dilution methods (12). Two-fold serial dilutions (1.95 to 8000.00 µg/ml) of the extracts were prepared in PDA broth. The diluted extracts were then inoculated with 0.1 ml of approximately 10^8 CFU/ml of fungal suspension. The inoculated tubes were then incubated at either 37 °C for 48 h for yeast or

25 °C for 5 days for moulds. The MICs were taken as the average of the first tubes showing no visible growth of the micro-organisms (13). Each assay was performed in triplicate.

The MICs to fluconazole powder (Pfizer, New Tork) was used as a standard and quality control as the range of MIC is known. Sterility of the media was controlled by use of broth only in a negative control tube, and growth of the organism was checked by broth plus microoganism in question in a positive control tube. All the controls were run parallel to the tests.

The MFC were determined by sub-culturing on PDA plates 0.1 ml of all the tubes showing no growth in PDA broth. They were incubated as already described previously for MIC cultures. After incubation, the first plates showing no growth were recorded as the MFCs (14).

RESULTS

Activity of Methanolic extracts of T. stapfiana using disk diffusion method

The root and stem bark extracts showed activity against *Candida albicans* (10 mm), *Cryptococcus neoformans* (10 mm) and *Trichophyton mentagrophytes* (10 mm) (Table 1). There was no antifungal activity exhibited by the leaf and fruit methanol extracts and there was no further studies done on them. The controls using solvents exhibited no activity.

Disk diffucsion test for Sequential extracts

The successive extracts of the root and stem barks against the test fungi showed an increased activity compared to methanol extracts. The ethyl acetate extracts of the root exhibited inhibition zones of 19 and 18 mm against *C. albicans* and *C. neoformans,* respectively (Table 2). The ethyl acetate stem bark extract exhibited activity with an inhibition zone size of 18 mm against both test yeasts (Table 3). There was a 20 mm zone of inhibition for methanol sequential extract of the root and stem barks against *C. albicans* and also 17 mm (stem bark) and 18 mm (root bark) zones of inhibition against *C. neoformans*. The zone of inhibition of ethyl acetate sequential extracts of the root bark against *T. mentagrophytes* was 14 mm. The *M. gypseum* was sensitive to ethyl acetate extract with the root bark giving 12 mm zone of inhibition while the stem bark did not show any activity. The stem bark methanol portions had activity against *M. gypseum* with a 9 mm zone diameter but the root bark showed no activity. The hexane and dichloromethane portions of both the root and stem bark exhibited no antifungal activity. The controls using solvents exhibited no activity.

Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC)

The methanol extracts of both root and stem barks showed strong MICs against *C. albicans* and *C. neoformans* with MICs of 62.5 and 125 µg/ml, respectively (Table 1). There was also strong fungicidal activity against the test yeasts with both parts showing MFCs of 250 µg/ml. Weak activities were demonstrated against the dermatophytes tested, with the root and stem bark methanol extracts showing an MIC of 1000 µg/ml against *T. mentagrophytes*. The MFCs were 4000 µg/ml (root) and 8000 µg/ml (stem) against *T. mentagrophytes*.

Table 1 Antifungal activity of *Tabernaemontana stapfiana* Britten methanol extracts

Microorganism	Root bark extract			Stem bark extract			Cont	Flu	
	Zone (mm)	MIC µg/ml	MFC µg/ml	Zone (mm)	MIC µg/ml	MFC µg/ml	Zone (mm)	Zone (mm)	MIC µg/ml
C. albicans (ATCC 90028)	9	62.50	250	9	62.50	250	6	19	0.125
C. neoformans (ATCC 66031)	10	125	250	10	125	250	6	18	4
T. mentagrophytes (L)	10	1000	4000	10	1000	8000	6	15	4
M. gypseum (L)	8	nd	nd	8	nd	nd	6	16	8

Key; L = clinical local isolate, nd = not done, MIC = minimum inhibitory concentrations, MFC = minimum fungicidal concentrations, Cont = control disc, Flu = fluconazole and ATCC = American Type Culture Collection

Table 2 Antifungal activity of the root sequential extracts of *Tabernaemontana stapfiana*

Microogaism	Cont	EtOAc			Cont	MeOH		
	Zone (mm)	Zone (mm)	MIC µg/ml	MFC µg/ml	Zone (mm)	Zone (mm)	MIC µg/ml	MFC µg/ml
C. albicans (ATCC 90028)	6	19	31.25	500	6	20	31.25	125
C. neoformans (ATCC 66031)	6	18	62.50	125	6	18	62.50	125
T. mentagrophytes (L)	6	14	500	1000	6	10	1000	2000
M. gypseum (L)	6	12	1000	2000	6	6	nd	nd

Key; L = clinical local isolate, EtOAc = ethyl acetate and MeOH = methanol, nd = not done, MIC = minimum inhibitory concentrations, MFC = minimum fungicidal concentrations, Cont = control disc and ATCC = American Type Culture Collection

Table 3 Antifungal activity of the sequential extracts of *Tabernaemontana stapfiana* stems

		EtOAc			Cont	MeOH		
Microogaism	Cont	Zone (mm)	MIC µg/ml	MFC µg/ml	Zone (mm)	Zone (mm)	MIC µg/ml	MFC µg/ml
C. albicans (ATCC 90028)	6	18	31.25	500	6	20	31.25	250
C. neoformans (ATCC 66031)	6	18	62.50	250	6	17	62.50	250
T. mentagrophytes (L)	6	13	500	1000	6	9	1000	4000
M. gypseum (L)	6	11	1000	2000	6	6	nd	nd

Key; L = clinical local isolate, nd = not done, MIC = minimum inhibitory concentrations, MFC = minimum fungicidal concentrations, Cont = control disc, Flu = fluconazole and ATCC = American Type Culture Collection

The MICs and MFCs of the methanolic sequential extracts of the root bark against *C. albicans* were 31.25 and 125 µg/ml, respectively while that for the stem bark were 31.25 and 250 µg/ml, respectively (Tables 2, 3). The MICs of ethyl acetate extracts of both the stem and root barks were 31.25 µg/ml and MFCs of 500 µg/ml against *C. albicans*. The MICs of methanol extracts of both the root and stem barks against *C. neoformans* was 62.5 µg/ml while the MFCs were 125 µg/ml for the root and 250 µg/ml for the stem. The MICs of ethyl acetate extracts of both the root and the stem barks against *T. mentagrophytes* were 500 µg/ml, while the MFCs were 1000 µg/ml. For the methanolic extract of the stem bark, the MIC was 1000 µg/ml and MFC, 4000 µg/ml against *M. gypseum*.

DISCUSSION

Medicinal plants constitute an effective source of both traditional and modern medicine. World Health Organization (WHO) encourages countries to examine traditional medicine with a view to identifying and exploiting aspects that provide safe and effective remedies for ailments (15). The results obtained from this study support the WHO recommendation as it provides scientific evidence that the root and stem bark extracts of *T. stapfiana* have antifungal properties. The sequential extracts showed a better antifungal activity as compared to methanol extracts. The root and stem bark methanol extracts that had MIC of 62.5 µg/ml and MFC of 250 µg/ml against *C. albicans* got lowered to MIC of 31.25 µg/ml and MFC of 125 µg/ml (root bark) and MIC of 31.25 µg/ml and MFC of 250 µg/ml (stem bark) in the methanolic extract of the sequential extracts. This activity is fungicidal as the ratios of MFCs to MICs are less than 4 (16) .

Phytotchemical screening of *T stapfiana* documented before, indicate presence of plant metabolites well known for antimicrobial activity such as alkaloids, flavonoids, coumarins and saponins (17). The alkaloids and saponins have been demonstrated to have a powerful anti-fungal effects and are some of a number of natural substances tested against pathogens by functional medicine labs (18,19). The coumarins have been shown to inhibit *Candida albicans in vitro* (20) while flavonoids on the other hand have been found *in vitro* to be effective antimicrobial substance against a wide range of microorganisms including fungi (21). Therefore, the antifungal activity of *T stapfiana* could be associated with any of these classes of compounds present. The activity could also be due to synergism or additive activity of two or more of the compounds present in the extracts (22).

The antifungal demonstrated *in vitro,* supports ethnobotanic uses of *T. stapfiana* among the Keiyo community. Extracts from *T. stapfiana* is traditionally used for dermatological problems of fungal nature (22) and this is confirmed in the present study. These results correlate fairly well with the literature on other *Tabernaemontana* species as shown by previous studies that indicated extracts from *T. cymosa* and *T. pachysiphon* have significant antifungal activities (23, 24). This shows the potential of members of the genus *Tabernaemontana* in antifungal therapy.

CONCLUSION

These antifungal studies suggest that *T. stapfiana* could be a rich source of antifungal agents. The present results further show that there is some merit in the use of this plant in traditional medicine. However, further studies with multiple strains of test fungi are recommended to confirm the utility of this plant extracts as alternative medicine in the war against fungal infections.

The fact that current dermatophytosis therapy is associated with considerable adverse effects in some patients, make these plant-derived compounds the subject of future research. This calls for further investigations on the isolation of the active principle(s) which is currently underway in our research laboratories and will be reported at a later stage.

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