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Research Article

IN-VITRO ANTIFUNGAL ACTIVITY OF *DISTEMONANTHUS BENTHAMIANUS* STEM

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ABSTRACT

The antifungal property of the hexane and methanol extracts of *Distemonanthus benthamianus* stem was carried out using agar well diffusion method against clinical strains of *Candida albicans, C. valida, C. tropicalis, C. krusei, C. pseudotropicalis, C. glabrata,* the following dermatophytes: *Trichophyton rubrum, T. interdigitalis, T. tonsurans, Epidermophyton floccosum,* and the standard strains of *C. albicans* (ATCC 90029), *C. krusei* (ATCC 6825) and *C. parapsilosis* (ATCC 22011). The methanol extract exhibited significant antifungal activity against most of the tested fungi species/isolates with zones of inhibition between 13-20mm at the tested concentration. The preliminary phytochemical analysis revealed the presence of only saponins, tannins, traces of terpenoids, flavonoids and alkaloids. The MIC and the MFC of the extract against the sensitive organisms was determined and was in the range of 0.0625-2.00mg/ml. The incorporation of organic matter (20% serum) had a reduction effect on the value of the MIC and the kinetics study revealed a reduction in the number of viable organisms with increase in contact time between the organisms and the extract.

Keywords*: Distemonanthus Benthamianus,* Antifungal property, Phytochemical, Minimum Inhibitory Concentration, Minimum Fungicidal concentRation.

INTRODUCTION

In recent times there have been an increase in the development of drug resistant strains of clinical important microorganisms which has led to the development of multi-drug resistant pathogens[1] .

Plants that are traditionally used in the treatment of bacterial and fungal infections or related ailments could be a good source for new, safe and biodegradable drugs and could offer potential lead towards development of novel compounds that are active against pathogenic microbes. This will go a long way to help in combating the development of multiple drug resistant bacteria and fungi, the high cost of antimicrobial drugs and the re-emergence of deadly diseases such as tuberculosis especially in developing countries.

Distemonanthus benthamianus is one of the perennial trees of the evergreen, semi-deciduous and secondary forest of West Africa tropics mainly in the Cameroon, Ghana and Nigeria. It belongs to the family Leguminosae. It is commonly known as 'Anyan' in Yoruba language. It grows up to 40m high or more with trunk of 1.20m or slightly smaller. There are approximately 2 species in the genus (*D. benthamianus* and *D. laxus*). The plant is rich in flavonoids compounds such as Oxyayanin A, Oxyayanin B, Ayanin and distemonanthin[2]. Malane *et al* isolated from the heartwood of *D. benthamianus* a new flavonolignan2,3-trans-2-(4-hydroxy-2,3-dimethoxyphenyl)-9-(5-hydroxy-2-methoxyphenyl)-2-hydroxymethyl-2,3-dihydro-7H-1,4-dioxinol [2,3-h] chromen-7-one with a rare 1,2,3,4-tetrasubstituted D-ring[3]. The bark of the tree is used traditionally for blood disorders; cutaneous, subcutaneous, parasitic infections, as a laxative and the dye is used as a pain killer.

The components of the plant (flavonoids compounds: oxyayanin A and B, Ayanin and Destemonanthin) have been implicated in antitumour activity, antioxidative activity, anti-adrenergic activity and in contact dermatitis respectively[4]. *D. benthamianus* is used in traditional Africa medicine to treat bacterial, fungal and viral infections[2] and it is used as chewing stick for oro-dental hygiene[5,6]. Extracts from the stem bark of the plant exhibit significant bactericidal effect on *Bacteroides gingivalis* and *Streptococcus mutans* which are implicated in oro-dental infections[4]. In view of the importance of *D. benthamianus* in ethnobothany as health remedy and also as a result of its use as a chewing stick locally, we set out to investigate the antifungal activity of the stem extract against some oral fungi pathogens and some dermatophytes, since there is no much report on its antifungal activity and also to determine the phytoconstituents that may be present in the stem of the plant.

MATERIALS AND METHODS

Plant collection

The stems of *D. benthamianus* were collected from Iganga, Ibarapa L.G.A of Oyo state. The collected plant was identified and authenticated in the Forest Research Institute of Nigeria (FRIN) Jericho Ibadan where voucher specimen was kept (FHI 108278). The stems were oven dried at 50^{0}C and pulverized.

Microorganism

The fungi species used were *Candida albicans, C. valida, C. tropicalis, C. krusei, C. pseudotropicalis, C. glabrata, Trichophyton rubrum, T. interdigitalis, T. tonsurans* and *Epidermophyton floccosum.* The *Candida* species were isolated and identified to species level using Chrome agar. While the dermatophytes were identified to species level with Lactophenol cotton blue preparation. The plant extract was also tested against the following standard strains of *Candida* from the laboratory stuck culture of the department of Pharmaceutical Microbiology, University of Ibadan Nigeria: *Candida albicans* ATCC 90029, *Candida parapsilosis* ATCC 22011, and *Candida krusei* ATCC 6825

Extract preparation

A 170g of the dried pulverized stems of *D. benthamianus* was extracted with Soxhlet apparatus using hexane and methanol in succession for six hours. The solvents were recovered and the extracts evaporated to dryness in a water bath and different residues of the plant extracted were resuspended in the solvent to a concentration of 2mg/ml and stored at 4^{0}C in the refrigerator until used.

Media

Sabouraud dextrose agar pH 7.3±0.2 (ANTEC), Chrome agar pH 6.1 ± 0.2 at 25ºC and Tryptone soy broth pH 7.3±0.2 (OXOID) were used in this study

Preliminary phytochemical studies

Preliminary phytochemical studies were carried out for the presence of saponins, tannins, flavonoids, anthraquinones, cardenolides, terpenoids and alkaloids using the method adopted in similar surveys[7].

Antifungal activity assay

The antifungal assay was carried out using agar-well diffusion

method[8]. An overnight broth culture of the test fungal isolates in Tryptone soy broth was standardized to 0.5 McFarLand standards (10^6 CFU/ml). Fungal carpeted plates were prepared by spreading 200µl of the standardized cell suspensions on Sabouraud dextrose agar plate and allowed to dry. With sterile cork borer, holes of 7mm were bored on the surface of the seeded plates and approximately 100µl of the crude extracts at concentration of 2.00mg/ml were introduced into the wells, allowed to stand at room temperature for about 2hrs to allow diffusion of the extract into the agar. The effect of the crude extract was compared with that of standard drug (ketoconazole) in separate hole and since the hexane and methanol extracts were re-suspended in the solvents before being tested, the solvents were included in separate holes in each plate as a solvent control beside the ketoconazole. The plates were incubated at 25°C for 48hours after which the plates were observed for zones of inhibition.

Determination of the minimum inhibitory concentration (MIC)

The MIC of the extract was determined using agar dilution method[9]. A 19 ml of a sterile molten tryptone soy agar maintained at 45^{0}C was added to 1 ml volume of the dissolved extract of the following concentration 2.00 mg/m1, 1.00 mg/m1, 0.50 mg/m1, 0.25 mg/m1, 0.125 mg/m1, 0.0625 mg/m1 and 0.03125mg/ml. They were properly mixed for even distribution of the extract within the agar medium. The mixtures were poured and allowed to set. The plates were then dried at 37^{0}C for 30mins. A 100µl of the cell suspensions were inoculated to each concentration of the solidified agar-extract mixture in duplicates. Controls were prepared by inoculating plates without the extracts with the cell suspensions. The plates were then examined for the presence of colonies after the incubation period of 48h at 25^{0}C. The least concentration that gave no visible colonies was taken as the minimum inhibitory concentration of the extract for the particular dilution of the organism.

Determination of the Minimum fungicidal concentration (MFC)

The MFC of the plant extract was determined by the method of Aibinu *et al*.[10]. To a 0.5ml. extract at different concentration as used in the MIC assay that showed no visible growth on the agar plates, was added 0.5 ml. of test organism in tubes. These were incubated at 25^{0}C for 48hrs. Samples were streaked out from the tubes on to tryptone soy agar plates to determine the minimum concentration of the extract required to kill the organisms. These concentrations were indicated by failure of the organisms to grow on transfer to these media plates. The lowest concentration that prevented fungal growth after 48hrs of incubation was recorded as the minimum fungicidal concentration (MFC). All tests were performed in duplicates to ensure accuracy. Agar plates without extract and another agar plates without any inoculated organism were also incubated serving as positive and negative control plates respectively.

The effect of organic matter was on MFC

This was determined using the method of Perl *et al*.[11]. To 2ml of the test extract 0.5ml of human serum was added and mixed. The mixture was introduced into 17.5ml molten Tryptone Soy Agar to give 20% ($^v/_v$) of serum. The mixture was thoroughly mixed, poured and allowed to set. A 100µl of the cell suspension (diluted to 10^6 CFU/ml) were spread on the agar surface after drying at 37ºC for 30 minutes. After which the plates were incubated at 25ºC for 48hrs. The least concentration that gave no visible colonies was taken as the minimum inhibitory concentration of the extract for the particular dilution of the organism.

The fungicidal activity/kinetic study of the plant extract

This was determined using the viable counting technique. A 0.5 ml. of each culture was subculture into a warm (37 ^{0}C) 4.5 ml. Tryptone Soy Broth and incubated for 90 minutes using a Gallenkamp orbital incubator to give a logarithmic phase culture. A 0.1 ml of the logarithmic phase culture was then inoculated into a 4.9 ml. of tryptone soy broth containing the MIC/MFC concentrations of the tested compound to give 1 in 50 dilution of the culture (equivalent to approximately 1x 10^7 colony forming units) and 4.9ml TSB inoculated with only the test organism to serve as the positive control. A 1ml of the test sample (extract culture mixture and the control) were withdrawn immediately, diluted out in normal saline and two drops of each dilution plated into an oven dried tryptone soy agar to give culture count at 0 minutes. Samples were taken at an interval of 30, 60, 120, 180, 240 and 360 minutes respectively. The procedure was carried out in duplicates to ensure reproducibility. Plates were incubated at 25 ^{0}C for 48-72h before counting the colonies. The positive control plates were also incubated. The number of colony forming unit were counted after the period of incubation. The numbers of surviving bacterial cells per ml were calculated by taking into consideration the dilution factor and the volume of the inoculums. All the procedure was repeated for MFC 2 x MBC/MFC and 4 x MBC/MFC respectively. The percentage survival of the organism was calculated for each of the time intervals at various concentrations and the control. A graph of percentage survivors of the organisms against contact time in minutes was plotted on a semi-logarithm graph.

RESULTS

The 170g pulverized stem of *D. benthamianus* yielded 13.33g of extract with methanol and 3.33g of extract with hexane. The preliminary phytochemical study revealed the presence of saponins, tannins, traces of terpenoids, flavonoids and alkaloids in the stem of the plant. The hexane extract of the plant exhibited insignificant activity while The methanol extract exhibited significant antifungal activity against most of the tested fungi species/isolates with zones of inhibition between 13-20mm at the tested concentration . All the strains of *Candida* and the dermatophytes tested were sensitive to the extract except one strain of C.*krusei* and C. *tropicalis*. Most of the organisms were resistant to the activity of ketoconazole as shown in the table 1. The MIC extract against the clinical isolates ranged from 0.0625-0.500mg/mL. while the MFC ranged from 0.50 to 2.00mg/mL

Table 1: The diameter zones of inhibition, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the extract against the fungal species

Organisms	Inhibition zone (mm)	Inhibition zone of STD (mm)	MIC (mg/ml)	MFC (mg/ml)
C. C.albicans PHM 1008	15±2.00	12±0.50	0.50	2.00
C. C. albicans PHM 0309	18±0.50	16±0.50	0.25	1.00
C. C. krusei PHM 1108	13±2.00	-	0.50	2.00
C. C. krusei PHM 0309	-	-	-	-
C. C. glabrata PHM 1208	20±2.00	25±0.50	0.25	1.00
C. glabrata PHM 0409	15±0.00	23±2.00	0.0625	0.50
C. C. tropicalis PHM 1308	-	-	-	-
C. C. tropicalis PHM 0509	12±0.50	-	0.25	1.00
C. C. pseudotropicalis PHM 1408	14±0.00	-	0.50	1.00
C. C. valida PHM 1508	15±0.50	-	0.50	1.00
T. rubrum PHM 1608	17±1.00	-	0.125	1.00
T. T. interdigitalis PHM 1708	15±1.00	12±1.00	0.125	0.25
T T. tonsurans PHM 1808	17±0.00	20±0.50	0.125	1.00
E. E. floccosum PHM 1908	18±0.50	-	0.125	0.25

Key: - = Not active/resistant; STD = Standard drug (ketoconazole); PHM = Pharmaceutical Microbiology, Ibadan.

The diameter zones of inhibition of the extract and the control drug, the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of the extract against standard strains of *Candida* species are presented in Table 2 below. All the standard strains were sensitive to the activity of the extract with lower MIC of 0.0625 mg/mL and MFC of 0.125mg/mL. The standard strains were all resistant to the activity of ketoconazole.

The investigation of the effect of organic matter (serum) on the activity of the extract against some of the test organisms showed a little increase in the value of the MIC as only the value of the extract against *C. glabrata* changed from 0.25mg/ml to 1.00mg/ml (Table 3).

Table 2: The diameter zones of inhibition, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the extract against the standard strains of *Candida*

Organisms	Inhibition zone (mm)	Inhibition zone of STD (mm)	MIC (mg/ml)	MFC (mg/ml)
C. C. albicans ATCC 90029	15±2.00	-	0.0625	0.125
C. C. krusei ATCC 6825	15±0.00	-	0.0625	0.125
C. C. parapsilosis ATCC 22011	15±1.00	-	0.0625	0.125

Key: - = Not active/resistant; STD = Standard drug (ketoconazole)

Table 3: Minimum Inhibitory Concentration (MIC) of the extract against some *Candida spp*. in the presence of Serum

	Concentration (mg/ml)					
Distemonanthus	*C. valida*	*C. pseudotropicalios*	*C. tropicalis*	*C. glabrata*	*C. krusei*	*C. albicans*
D. benthamianus	PHM 1508	PHM 1408	PHM 1308	PHM 1208	PHM 1108	PHM 1008
	0.50, 0.50*	0.50, 0.50*	-	0.25, 1.00*	0.50, 0.50*	0.50, 0.50*

- = Not active/ resistant; PHM = Pharmaceutical Microbiology, Ibadan; * = MIC in the presence of serum.

Figures 1 and 2 show the kinetics fungicidal activities of the plant extract against *C. glabrata* PHM 1208 and *E. floccosum* PHM 1908 which revealed a reduction in the number of viable organism with increase in time of contact between the extract and the organisms.

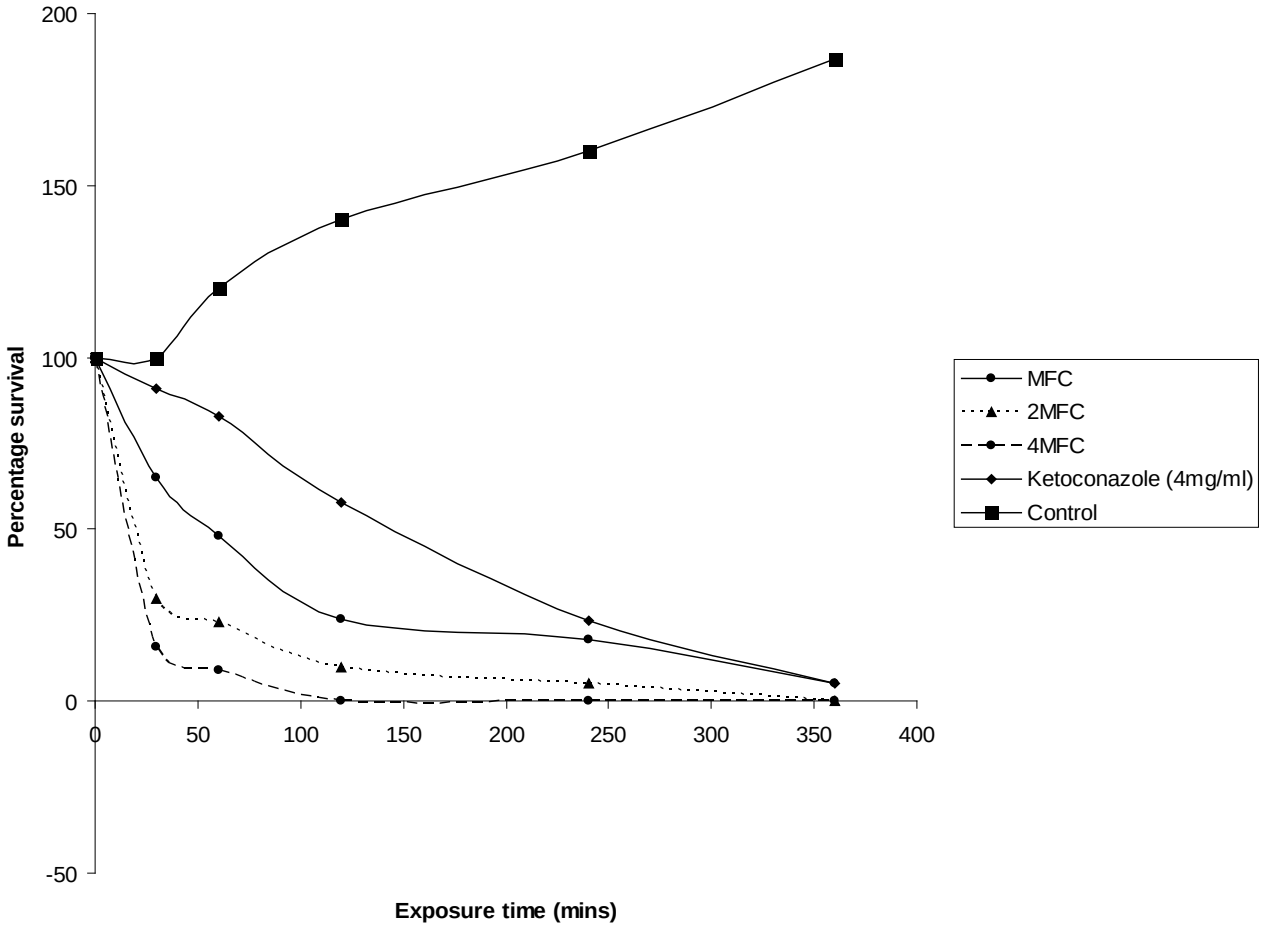

Fig. 1: Kinetics of fungicidal activity of *D. benthamiansus* and ketoconazole (4mg/ml) against *C. glabrata* PHM 1208

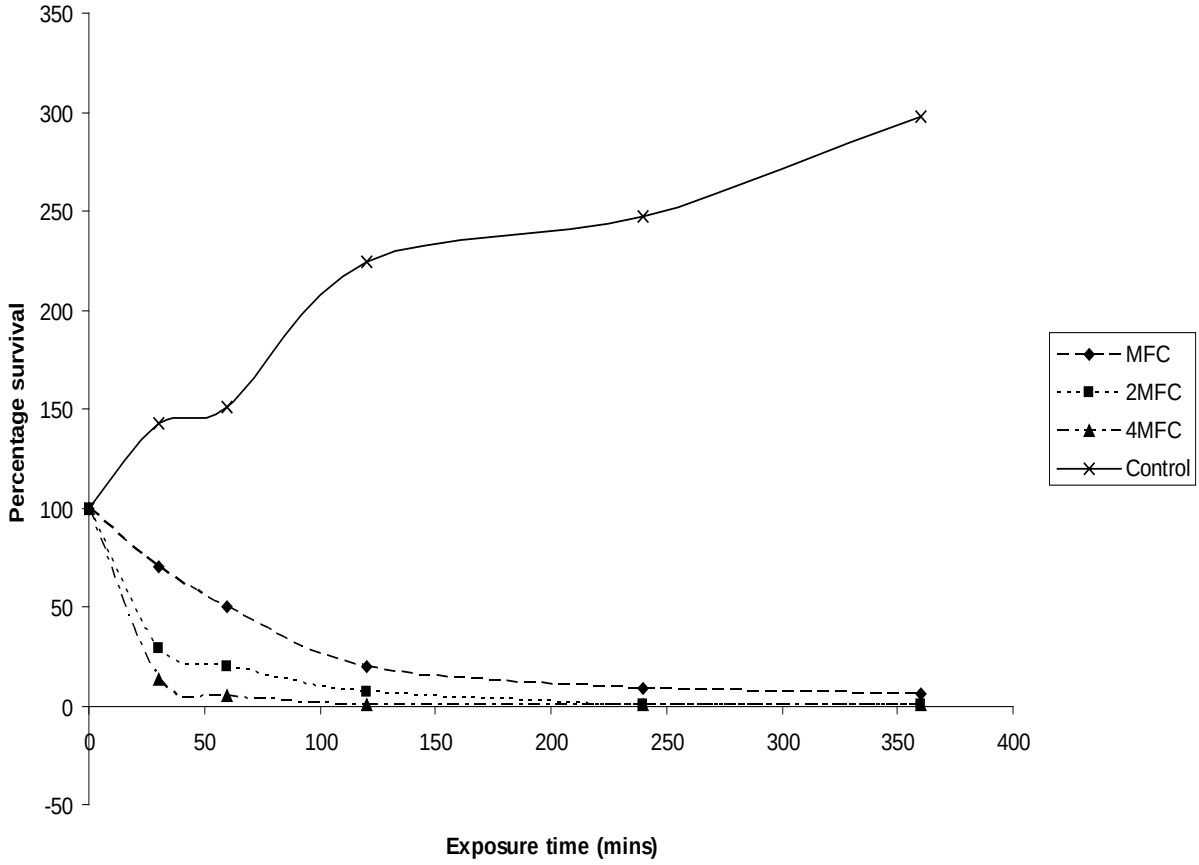

Fig. 2: Kinetics of Fungicidal activity of *D. benthamianus* against *E. floccosum* PHM 1908

DISCUSSION

Among the HIV/AIDS patient opportunistic infection is candidiasis and other skin infections caused by dermatophytes[12] and the methanol extract of *D. benthamianus* was active against almost all the isolates (*Candida spp*, Dermatophytes spp and *Candida* standard strains). Among the seventeen isolates of *Candida* (standard and clinical strains) and the dermatophytes tested only two isolates/strains were resistant to the extract. This finding corresponds to the report of Aiyegoro *et al.,*[4] who has reported the antibacterial activity of crude extract of stem bark of *D. benthamianus* against bacterial isolates implicated in oro-dental infections. The effectiveness of the extract of this plant may be attributed to the presence of the secondary metabolites present in the plant as unveiled by the phytochemical screening (alkaloids, flavonoids, traces of terpenoids, saponins and tannins) which are known antimicrobial agents present in plant. Adekunle and Odukoya[13] have reported the antifungal activity of *D. benthamianus,* which agrees with the results obtained in this study. The use of the plant for the treatment of bacterial, fungal and viral infections has also been reported[2]. It is also of interest to note the activity of the extract compared to that of Ketokonazole. This is an indication that this plant if well harnessed and used as medicine can compete favorably with some of the existing antifungal drugs in the treatment of Candida and Dermatophyte infections.

CONCLUSION

The antifungal activities of the extract of *D. benthamianus* stem as have discovered in this research against species of *Candida* and dermatophytes shows that the plant can be used in the treatment of infections caused by the above mentioned organisms and this forms a basis for the local use of the stem of this plant as chewing stick and in the treatment of various types of infections.

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