In- vitro evaluation of antifungal effects of hydro alcoholic extract of *Euphorbia zeylanicum.*

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ABSTRACT

This study was to evaluate extracts of Euphorbia zeylanicuma for anti-fungal activity. cactus-like plant of the family euphorbiaceae usually referred to as shend by the people of south-West sangli district. The stem-bark and latex of this plant were extracted using 50%-methanol, water and absolute alcohol. Antifungal result of the extracts was evaluated using agar, and macro-broth dilution strategies as well as the Time kill assay. Strains of Rhizopus ,Aspegillus, Rodotorula, Mucor, Basidiobolus, Geotricum, Trichophyton, Microsporum, Epidermophyton and candida species were used as test fungi for the study. From extraction of the stem-bark the yield is 18%,15% and 25% respectively for absolute methanol and water and 50% methanol.The latex yielded around 13%, 12% and 15% for said absolute methanol water and 50% methanol extracts respectively. On Sabouraud dextrose agar plates growth inhibition test was performed for a period of 7-14 or many days. The range of MIC was found to be 0.39-50.0 mg/ml for extracts from the stem bark and for the latex extracts it was found to be 1.95- 50.0. There was a significant distiction in the growth inhibition by using 50% methanol extracts of the stem-bark along with latex (P=0.5) with significant value of 5.361 and 7.1086 respectively. *Candida albicans* was found to be the most susceptible of the yeasts tested (MIC_{90} 0.313 mg/ml). The plant extracts showed broad spectrum of activity against a fungi that was tested. Results authenticiated the ethno-medicinal applications of *Euphorbia zeylanicum* for the treatment of skin infections.

Keywords: Evaluation; Euphorbia zeylanicum extracts; antifungal effects;

Introduction

The diseases caused by multidrug resistant fungal pathogens(e. g dermatophytes like *Trichophyton, Microsporum* and *Epidermophyton* as well as opportunistic fungi like *Aspergillus species, Candida albicans*, *Mucor*and *Rhizopus* species) are becoming the world`s leading cause of health complications if not death, especially in immunosuppressed patients [1]. Though fungal diseases (especially superficial and systemic) appear to be rare, they are not easily treated when contacted and *thus* most often, some cases of HIV AIDs, Hepatitis B, Cancer etc. and other immunosuppressive diseases become complicated by some of these fungal infections[2].The treatment of immunocompromised, AIDS and cancer patients becomes sometimes difficult due to this problem [3]. This is even worst in under developed countries like Nigeria, where healthcare facilities are not easily assessable by the vast majority of the population. A greater part of the population in the interior areas of these countries therefore, rely on plant sources for treatment of skin diseases and other mycotic infections. The investigation of these plants used in folklore medicine for skin infections could invariably be a source of the antimycotic agents urgently needed in less developed world today. Some plants of the genus *Euphorbia*, have been used in folklore medicine from creation till today e. g *Euphorbia kamerunica* and other plants belonging to the family *Euphorbiaceace* have been used to treat skin infections such as ulcers, warts, cancers, tumors [4,2]. *E. tirucalli* has been used for treatment of swelling, asthma, cough, skin problems and rheumatism. Also its latex is used as treatment for sexual impotence, warts, toothache, hemorrhoids, and snake bites. *Euphorbia granulate* has also shown inhibitory activity against Human immunodeficiency virus (HIV-1) protease [5].This study therefore seeks to explore the largely unexplored rich natural constituents of *Euphorbia zeylanicum*, with the aim of evaluating the antimycotic activity. This plant *Euphorbia zeylanicum is* an erect, eight-angled branched tree with deep angles edged with a border of closely packed paired spines (about 1cm long) [6].

Materials and Methods

Collection of Plant Materials

The plant parts used for this study were *Euphobia zeylanicum* stem bark and the latex. These were collected from south West region of sangli District. In the Department of Botany, Sangli District the plant was authenticated and the voucher specimen also deposited ai Dept of Botany Y.C. College Karad. The latex or sap of *Euphorbia zeylanicum* was collected by cutting open, parts of the bark on the stem and branches. A container was connected to the bottom of the opening from which the latex dripped into the container. It was then allowed to dry in the water bath at 56°C and stored in a close capped bottle pending its use. The plant stem-bark was rinsed thoroughly in running tap water, cut into tiny pieces and air dried in the dark. The dried material was then ground to powder in a mortar, weighed and stored in plastic bags in the dark.

Extraction of plant materials

Approximately 400 ml of solvent (absolute methanol, water or 50% methanol in water) in a 1 L conical flask was used to soak a 100 g weight of powdered plant material and then covered with cotton wool plugs. After vigorous shaking, the flask was intermittently shaken for 24 hours leaving it in a water bath maintained at 40°C between the intervals of shaking. Three layers of clean muslin cloth were first of all used to filter the mixture before passing it through Whatman no 1 filter-paper. The filtrates were evaporated to dryness in a water bath at 56°Cand the percentage yields of the crude extract determined [7].

Test Organisms

Trichophyton mentagrophytes, Trichophyton tonsurans, Trichophytonviolaceum, Microsporum gypseum, Microsporumcanis, Epidermophyton floccosum, Candida albicans, species of Aspergillus, Basidiobolus, Rodotorulla, Mucor, Rhizopus and Geotricum, all collected from the Microbiology laboratory of the Y. C. College Karad, were the fungi used for the study. They were cultured on Sabouraud Dextrose Agar plates at 25-35°C for 48 hours or more and the

resultant pure mature colonies were sub-cultured on Sabouraud Dextrose Agar slants and stored as stock cultures.

Fungal stock cultures were sub cultured on Saboura ud Dextrose Agar and incubated at 25-35°C for 7 to 14 days. The matured fungal growths were covered with 2ml of distilled water and gently probed with a sterile loop or the tip of a Pasteur pipette. The resulting suspensions were transferred to sterile test tubes and allowed to settle for about 3-5 minutes. The resultant supernatant suspensions were drained into sterile bottles. The spores or yeast cells (colony forming units (CFU)) in the suspensions were counted using a haemocytometer and the suspensions were then diluted with Sabouraud dextrose broth to correspond to the final standard inoculate suspension (spores or yeast cells) of approximately 1 x 10^5 colony forming unit per ml[8].

Susceptibility Testing of Fungi by Pour-plate Method

The susceptibility testing of fungi was done using pour-plate method of[10]. A 2.0 ml amount of each reconstituted plant extract at the concentration of 1000 mg/ml was pipetted into sterile glass test tube containing 18mls of molten Sabouraud Dextrose Agar (at about 45°C). The mixtures were swirled carefully for the contents and agar to be thoroughly mixed. Then 100µl of the standard fungal inoculate were seeded onto each of the tubes. Again they were thoroughly mixed and poured into each of the plates and allowed to set. They were then incubated at about 25-35°C. A sterile plate without the extract served as the positive control for growth while another plate containing 2.0 ml of 16 µg/ml Miconazole as the negative control. As soon as growth was observed at the positive control plates the test plates were checked for growth daily and the period of inhibition of growth was recorded in days.

Macro-Broth Dilution Method for determination of Minimum Inhibitory Concentration (MIC) of the Plant Extracts on the Fungi

A two-fold serial dilution of the plant extract was carried out in tubes of Saboraud Dextrose broth to obtain dilutions ranging from 200 mg/ml down to 0.39 mg/ml. The 11th tube, which was used as positive growth control culture, did not contain any extract. The control

antimycotic agent, Miconazole, was similarly serially diluted but to attain concentrations ranging from 0.125-128µg/ml. Each dilution was seeded with 100µl of the standardized suspensions of the test fungal spores and incubated at 25-35°C for ≥48 hours. The lowest dilutions without visible growth in the tube cultures, compared to the positive and negative controls were considered as the MICs. The tests were carried out in quadruplets and the means of the MICs calculated[8].

Determination of Minimum Concentration at which 90% Fungal Growth Inhibition was Observed (MIC_{90}) from Broth Dilution Tubes

A 100µl volume of the fungal test suspensions from all the tubes showing no visible fungal growth was sub-cultured on Sabouraud Dextrose agar plates and incubated at 25-35°C. Positive growth control plates were also included and checked simultaneously in comparison with the test plates for fungal growth. The Minimum Fungicidal Concentration (MFC) was considered as the minimum concentration of the test substances that yielded 99%-100% visible growth inhibition on sub-culture of 100 µl of serial dilutions and incubation at 35°C for more than 48hours. Any dilution showing 90% growth inhibition was recorded as MIC_{90} [8].

Statistical Analysis:

The Randomised Complete Block Design (Two-way analysis of variance) was used to analyze the data obtained in the study. The means that were significantly different were separated using Duncan's New Multiple Range Test.

Results

Antifungal activities of *Euphorbia zeylanicum* Extracts

The results of the antifungal activities of 100mg of *E. zeylanicum* stem- bark showed that the extract inhibited the growth of all the fungi tested. However, the 50% methanol extract showed better activity.

The MICs and MIC_{90}s (mg/ml) of 50% Methanol Extract of Plants on the Fungi

The MICs and MIC_{90}s were evaluated using the 50% methanol extracts of *E. zeylanicum* stem-bark. was more active on the yeasts, with *Candida albicans* showing MIC value of 0.39 mg/ml and MIC_{90} of 1.56 mg/ml, followed by *Rhodotorula* species (MIC 0.49 mg/ml, MIC_{90} 3.13 mg/m) and *B. haptosporus* (MIC 0.59mg/ml, MIC_{90} 3.13 mg/ml). *E. floccosum* was the most susceptible of the dermatophytes with MIC of 0.98 mg/ml and MIC_{90} of 1.56mg/ml. This was closely followed by *Trichophyton rubrum* (MIC 0.1.95 mg/ml, MIC_{90} 3.13mg/ml), *M. canis* (MIC 3.9 mg/ml, MIC_{90} 3.13 mg/ml) and *T mentagrophytes* (MIC 0.39 mg/ml, MIC_{90} 6.25 mg/ml) respectively. The least susceptible of the fungi were *Microsporum gypseum* (MIC 25 mg/ml, MIC_{90} 25 mg/ml) and *T. tonsurans* (MIC 12 mg/ml, MIC_{90} 25 mg/ml) respectively (Table 2). Extracts of E. *zeylanicum* latex showed its lowest MIC (1.95 mg/ml) on, *B. haptosporus* and *E. floccosum* respectively and least MIC_{90} (3.13 mg/ml) on *E. floccosum* and *C. albicans* respectively).The least susceptible of the fungi tested to this extract was *Microsporum gypseum* (MIC /MIC_{90} of 25 mg/ml) (Table 2). For the control drug (Miconazole), *C. albicans* (MIC 0.313 µg/ml, MIC_{90} of 0.5 µg/ml), and *T. rubrum* (MIC 0.375 µg/ml, MIC_{90} of 0.5 µg/ml), were the most susceptible fungi while the least susceptible of the fungi was *B. haptosporus* with MIC of 1.25 µg/ml, and MIC_{90} of 4 µg/ml (Table 1).

Table1. MICs and MIC_{90}s of plant extracts (mg/ml) on some pathogenic fungi.

Fungal species	*E. zeylanicum** Stem-bark Extract		*E. zeylanicum* latex Extract		Miconazole
	MIC±SD (mg/ml)	MIC_{90} (mg/ml)	MIC±SD (mg/ml)	MIC_{90} (mg/ml)	MIC±SD (µg/m
C. albicans	0.39±0.00*	1.56	2.73±0.78	3.13	0.313±0.13
Rhodotorulla species	0.49±0.19	3.13	2.34±0.90	6.25	0.75±0.29
B. haptosporus	0.59±0.23	3.13	1.95±0.78	6.25	1.25±0.50
E. floccosum	0.98±0.39*	3.13	1.95±0.78	3.13	0.313±0.13
T. mentagrophytes	3.9±1.6 0	6.25	6.25±0.00	12.5	0.189±0.07
T. rubrum	1.95±1.36	3.13	3.91±1.56	12.5	0.375±0.14
T. violaceum	3.91±1.56	6.25	5.476±1.56	12.5	0.50±0.00
M. canis	3.91±2.70	3.13	2.73±2.3	6.25	0.25±0.00
T. tonsurans	12.5±0.00	6.25	18.7±57.20	25	0.25±0.00
M. gypseum	25.0±0.00	25	25.0±0.00	25	0.75±0.29
Aspergillusfavus	12.5±0.00	25	15.63±6.30	25	0.25±0.00
Aspergillus Niger	6.25±0.78	25	12.5±0.00	25	0.25±0.00
Aspergillusfumigatus	6.25±0.59	25	12.5±0.00	25	0.25±0.00
Rhyzopusnigrcans	12.5±0.78	25	25.0±0.39	50	0.189±0.07
Mucor species	25.0±0.39	50	25.0±0.39	50	0.375±0.14
Geotricum species	50.0±0.195	100	50.0±0.195	100	0.75±0.29
P = .05					

Discussion

The study of antifungal activity of crude extracts (absolute methanol, water and 50% methanol) of *Euphorbia zeylanicum* by agar dilution and macrobroth dilution method showed that the extracts inhibited the growth of the test organisms. In the agar dilution, there was no significant difference between the activity of the stem-bark and that of the latex extracts. Also there was no significant difference between the activity of the absolute methanol and the aqueous extracts even though the methanol extract were more active when compared with the aqueous extract. The slight difference in the activity might have been probably due to the solubility of the chemical and bioactive constituents in the extracting solvents (methanol and water). [1] Reported the significant antifungal activity of methanol and aqueous extracts of *Euphorbia tirucalli* with mean inhibition zone of 15.33±0.88 mm and 17.33±0.33 mm respectively for *C. albicans* (ATCC9002) and *A. niger* 14.67±0.67 and 16.33±0.33 for methanol and aqueous respectively. The antifungal activity of methanol and aqueous extracts of other *Euphorbia* species have also been reported by other researchers [9-15]. The 50% methanol extracts of the two plant parts (stem-bark and latex) showed significantly higher antifungal activity than the absolute methanol and aqueous plant extracts. In the macrobroth dilution method the results showed that at a significant value of alpha=.01 the 50% methanol extract of the stem-bark was more active than the 50% methanol extract of the latex. They were significant at mean values of 5.361 for stem-bark extract and 7.109 for the latex extract. Similar observations as these have also been reported with other *Euphorbia* species extracts by other researchers [16]. It has been observed that organic solvents extract better than water [13]. However a mixture of the two solvents yielded better results in this study.

Conclusion

From this experiment crude extracts of *Euphorbia zeylanicum* plant were efficacious against some dermatophytes, yeasts and opportunistic pathogens which support its ethno medicinal uses as a broad spectrum herbal remedy. Further research on the fractions of these plant extracts to reveal the actual bioactive compounds will be of great value. This plant might thereafter be used as treatment for infections caused by these fungi.

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