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Antifungal Activity against Onychomycosis causative fungi and Brine shrimp lethality of a Tanzanian ornamental plant *Euphorbia cotinifolia* L. (Euphorbiaceae)

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Abstract

Onychomycosis is a prevalent superficial fungal nail infection; although not a life-threatening disease it can significantly reduce patients' quality of life. It is difficult to diagnose and treat, also antifungal therapy is associated with 20-25% treatment failure leading to poor prognosis. Adverse effects and affordability, especially for the new antifungal agents, is another concern.

The current study determined the antifungal activity and brine shrimp lethality of the latex, methanolic leaf and stem bark extracts of *Euphorbia cotinifolia* L, an ornamental plant, the latex of which, is popularly used in Dar es Salaam and Kilimanjaro regions, in Tanzania, to treat fungal infections of the nails. Disc diffusion and broth microdilution methods were used for antifungal screening and determination of minimum inhibitory concentrations (MICs), respectively and brine shrimp lethality was determined as explained in our previous study. The extracts inhibited *Trichophyton mentagrophytes* and *Aspergillus niger* with inhibition zones of 17 and 15 mm, respectively, for leaves and 9 mm for both fungi in the case of stem bark extracts, the leaf extract also inhibited *Trichophyton rubrum* (12 mm), the MICs ranged from 2.5- 5 mg/ml. All tested samples were inactive against *Candida albicans* while all tested fungi were not inhibited by fluconazole and the latex. The extracts exhibited a strong activity in the brine shrimp lethality test with LC_{50} and LC_{90} of 13.30 and 55.90µg/ml (leaves) and 14.02 and 63.08µg/ml (stem bark) compared to cyclophosphamide (16.36 and 61.02 µg/ml). Further studies are recommended so as to isolate active compounds, explore the anticancer potential of the plant by using various cell lines and also verify further the activity of the latex collected during different seasons and at different times of the day.

Key Words: *E. cotinifolia*, *Trichophyton rubrum*, *T. mentagrophytes*, *Aspergillus niger*, antifungal activity

INTRODUCTION

Onychomycosis is a prevalent superficial fungal nail infection which affects the entire nail plate and often the nail bed and its presentation varies depending on the causative agent [1-3]**.** Toe nails are affected more than fingernails and also the disease is more frequent in males than females [4, 5]**.** Onychomycosis is the most common nail disorder in adults and accounts for 33% of all skin fungal infections and 50% of all nail diseases [6]. It has been documented that the disease affects 10-30% of the global population [7]. Unfortunately, the incidence and prevalence of onychomycosis in Tanzania is not documented. However, in Western countries the disease is reported to be prevalent in approximately 10% of the general population, 20% of the population aged >60 years, up to 50% of people aged >70 years and up to one-third of diabetic individuals [3]**.** Other studies indicated that the indicated prevalence is over-estimated and in one study a prevalence of 4.3 % was obtained for Europe and North America [8]**.**

Although onychomycosis is not a life-threatening disease, it can significantly reduce patients' quality of life. It is associated with a negative impact with regard to patients' physical, social and emotional well-being [9, 10]**.** A study done in Poland found out that females and patients with better education and living in towns were the most affected emotionally and socially [9].

Dermatophytes including various species of *Trichophyton* for example *T. rubrum,* and *T. mentagrophytes*, and *Epidermophyton* species such as *E. floccosum* are the most causative agents worldwide though other infections may be due to other fungi; yeasts (*Candida. guilliermondii*, *C. albicans* and *Trichosporon species*) and non dermatophyte molds including *Aspergilus* species, *Scopulariopsis brevicaularis* are less common [5, 11-14]. However, 90% of onychomycosis cases are due to *T. rubrum* [2, 15]. A study done at the Department of Dermatology of the Leipzig University, reported an encounter of 22% of mixed infections [14]. Dermatophytes are ubiquitous and hence infections can be acquired from infected humans and animals and also from the soil, however, there have been reports of infection originating from swimming pools [14, 16].

Onychomycosis is not easy to diagnose and manage and if not well managed it may lead to other complications such as infectious lesions in other parts of the body, cellulitis, which may further badly affect the limbs in those with diabetes or peripheral vascular disease [2, 3]. Despite the fact that these complications are rare, they are grave when they occur.

Management of the disease is based on both topical and systemic antifungals. The commonly used systemic antifungals include terbinafine, itraconazole, griseofulvin and other new triazoles. The topical preparations involve either an antifungal agent (polyene, imidazole, allylamine), or preparations containing an antifungal, antiseptic and sometimes a keratolytic agent such benzoic acid, benzyl peroxide, salicylic acid or undecenoate [2]. However,

topical medications are recommended only for mild to moderate cases due to their poor access to the infected site. Other management options include usage of new nail enhancers, and physical and/or chemical removal of the diseased nails [1, 4, 7, 13].

The use of antifungal therapy is associated with 20-25% treatment failure and/ or relapse [7]. However, in one study a higher treatment failure rate of 25–40% was noted whereby clinical indicators for poor prognosis included the development of residual foci of subungual fungal growth, onycholysis and severe disease [1]. Furthermore, adverse effects such as skin rashes, menstrual disorder, gastrointestinal disorders, visual and taste disturbances, reverse elevation of liver enzymes levels and headache are other negative aspects of oral therapies [17].

For developing countries like Tanzania another concern is affordability especially for the new antifungal agents and techniques of which the cost may be prohibitive. In view of this, extracts of *Euphorbia cotinifolia* were investigated for the activity against fungi associated with onychomycosis aiming at obtaining a cheap and effective alternative agent for onychomycosis.

Euphorbia cotinifolia belongs to Euphorbiaceae, a family comprising of over 300 genera and 8,000 species. *Euphorbia* is the largest genus of Euphorbiaceae and some of its species are medicinally important [18-20]. For instance Dysenteral®, an extract from *Euphorbia hirta* is used in the treatment of diarrheal diseases [21] while Radix,an extract from *Euphorbia kansui* roots is used as a purgative [22]. The latex of *Euphorbia* species has also been used by tribes from the Colombian Amazonia, both as fish poisons and arrow poisons to kill large animals, to treat ulcers, cancers, tumors, warts, and other diseases [19, 23]. Some of the biological activities of the genus include antibacterial [19, 24-27], antifungal [27, 28], cytotoxic [23, 30], piscicidal [31] and antioxidant activities [25].

The latex of *E. cotinifolia* a plant used for ornamental purposes has gained popularity in Dar es salaam and Kilimanjaro regions in Tanzania for the treatment of chronic fungal infection of the nails referred to as of onychomycosis. The latex is usually applied to the affected nail which eventually leads to destruction of the nail and growth a completely new nail a process which is usually accompanied by pain. The latex is similarly used in Venezuela and it is also reported to be used for the treatment of warts and condyloma in Brazil [32, 33]. Leaves of the plant have been reported to be previously employed as poison for catching fish by Southern American Indians [31]. Various extracts of the stem and leaves of *E. cotinifolia* were reported to exhibit antiviral and cytotoxic activities [18, 23]. The methanolic extract of *E. cotinifolia,* was reported to exhibit marked piscicidal activity against killifish [31]. In an investigation on crude drugs used to treat diarrhea in Kinshasa, Democratic Republic of Congo, *E. cotinifolia* was found to exhibit weak and moderate activity against *Salmonella enteritis* and *Staphylococcus aureus,* respectively [24]. Methanol and ethyl acetate extracts were reported active against a number of phytopathogenic bacteria exhibiting MICs ranges of 0.3-1.3 and 0.3-0.8 mg/ml, respectively [19]. The methanol and ethyl acetate leaf extracts of *E. cotinifolia* exhibited a significant activity against *Bacillus subtilis* and *Enterobacter aerogenes*, with a maximum inhibition zone of 17.25 mm and MICs for different bacteria ranged from 0.3–1.25 mg/ml. Different extracts of leaves, stem and fruits of *E. cotinifolia* were reported to contain various phytochemicals including; aminoacids, anthraquinones, glycosides, saponins, steroids, polyphenol and tannins, triterpenoids and flavonoids [25, 34].

The aqueous extract of different plant parts of *E. cotinifolia*, were found active against a spectrum of microorganisms including *Candida* species [35]. The latex has been reported to exhibit caseinolytic activity [36]. Different extracts of the plant growing in Venezuela showed no activity on the tested Gram positive and Gram negative bacteria [32].

The current study investigated the antifungal activity and brine shrimp lethality of the methanolic leaf and stem bark extracts of *E. cotinifolia*.

Fig 1: *E. cotinifolia* (Euphorbiaceae)

MATERIALS AND METHODS

Plant Collection and authentication

Fresh branches of the plant *Euphorbia cotinifolia* L. (Fig. 1) were collected from plants cultivated for ornamental purposes in a flower garden at Tabata Kisukuru, Dar es Salaam, Tanzania. Identification of the plants was done by a botanist at the herbarium of the Department of Botany, University of Dar es Salaam, where voucher specimens were deposited.

Preparation of the extracts

The leaves and tender stems were separated and individually crashed using a large motor and pestle. The crashed plant materials were exhaustively extracted with methanol, by repeated maceration at room temperature. The extracts were dried *in vacuo* at a temperature below 50^{o}C using Buchi rotary evaporator. The dried extracts were stored in a refrigerator until required for antifungal screening.

Screening for antifungal activity

Test fungi

Test fungi included two dermatophytes (*Trichophyton rubrum* and *Trichophyton mentagrophytes*), one non-dermatophyte mould (*Aspergillus niger*) all being clinical isolates and one yeast (*Candida albicans* ATCC 90028). These fungi were obtained from the Department of Pharmaceutical Microbiology, School of Pharmacy, MUHAS.

Inocula

Prior to the sensitivity testing the fungi were separately cultured repeatedly on Sabouraud dextrose agar (Oxoid Ltd. UK) in order to ensure viability. From these cultures few colonies were suspended in normal saline and their densities adjusted to 0.5 MacFarland; these were the inocula which were applied evenly using a sterilized swab on the surface of the agar plates during the antifungal screening**.**

Antifungal screening

Screening for antifungal activity was accomplished through disc diffusion method and broth dilution method was used to determine the minimum inhibitory concentrations. Sterile filter paper discs (6 mm, Whatman No.20) were separately impregnated with 10 µL of the extract solutions (500 mg/ml in DMSO) and the latex (10 mg/disc) and allowed to dry. Fluconazole (100 µg/disc) susceptibility test discs and filter paper disc impregnated with 10 µL of DMSO were used as positive and negative controls, respectively. The test disc and positive control discs were placed on the surface of the inoculated plates. The plates were incubated over night at 30^{o}C after which the zones of inhibition were measured.

Determination of Minimum Inhibitory Concentrations (MICs)

Determination of MICs was accomplished through microdilution susceptibility test utilizing a 96 well microtitre plate consisting of twelve columns and eight rows. Test extracts were prepared by dissolving 100 mg into 1 ml DMSO and diluted with 4 ml of distilled water to make a concentration of 20 mg/ml. Double and normal strength Sabouraud Dextrose Broth (Himedia Laboratories, Mumbai, India) were prepared according to the manufacturer's instruction. Fifty (50) µL of the prepared double strength media were placed in each well of the first row of the microtitre plate whereas the normal strength media were placed in the rest of the wells. Fifty (50) µL of various test samples were introduced into the first row of the microtitre plate and mixed well, followed by a two-fold dilution down the column. Test microorganisms (50 µL) of appropriate density, were subsequently added to each well to attain a final density of 0.5 MacFarland. The adjusted final concentrations of the various extracts test samples ranged from 5 mg/ml to 3.9 µg/ml) and DMSO (5% v/v) was used as the negative control. The plates were then incubated for 24 hours at 30ºC after which they were removed from the incubator and 40 µL of 0.2 mg/ml *p*-iodonitrotetrazolium (INT) chloride dye was added, followed by further incubation of the plates for 30 minutes. The lowest concentrations which showed no purple colour (indicating absence of growth) were taken as the minimum inhibitory concentrations (MICs).

Brine shrimps lethality test

Extracts of the leaves and stem bark of *E. cotinifolia* L were also subjected to brine shrimps lethality test as previously described [37]. Brine shrimp eggs were kindly provided by the Insititute of Traditional Medicine, MUHAS. The eggs were hatched in a shallow glass container, containing artificial brine containing 9.5 g artificial sea salt in 250 ml distilled water. The pH of the solution was adjusted to 7. The container was covered with aluminium foil (80%) and partially illuminated with a lamp. Hatching took about 72 h, after which the shrimps were ready for tests.

Stock solutions of the leaf and stem bark extracts were prepared by dissolving 10 mg of accurately weighed extracts in 1ml of DMSO. Different dilutions of the test extracts with concentrations ranging from 60 to 2 µg/ml were prepared by adding stock solutions ranging from 30 µl -1µl in vials containing almost 5mls of artificial sea water. Ten (10) actively swimming shrimps were finally added to the vials and volume adjusted to 5mls by adding artificial sea water. The negative control vial contained brine shrimps and DMSO 0.6%v/v in artificial sea water. The vials were loosely covered with aluminum foil and kept at room temperature (29 ºC) for 24 h.

The number of dead shrimps was determined by counting completely motionless shrimps found at the bottom of the tubes. Tests were performed in duplicate and the procedure was repeated twice for each extract. The average percent mortality was determined, after correcting for control mortality by Abbott's formula [38] when necessary. The average percentage mortality was plotted against the logarithm of concentrations using the Fig P computer program. Regression equations obtained from the graphs were used to obtain LC_{50} and the 95% CI values [39].

Table 1: Inhibition zones for the latex and extracts of *Eurphobia cotinifolia*

Fungi	Inhibition zone (mm)				
	Leaves	Barks	Latex	Fluconazole	5%DMSO
T. rubrum	12	6	6	6	6
A. niger	15	9	6	6	6
T. mentagrophytes	17	9	6	6	6
C. albicans	6	6	6	6	6

*Zone of inhibition includes disc size (6 mm)

Table 2: Minimum inhibitory concentrations of leaves and stem bark extracts

Fungus	Minimum inhibitory concentrations (mg/ml).	
	Barks	Leaves
Trichophyton rubrum	5	5
Aspergillus niger	5	2.5
Trichophyton mentagrophytes	2.5	2.5

Table 3: Lethal concentrations of extracts of *Euphorbia cotinifolia* L to 50 and 90% of brine shrimps

Extract	LC_{50} (µg/ml)	95%Confidence interval (µg/ml)	LC_{90} (µg/ml)	95% Confidence interval (µg/ml)
Methanolic leaf extract	13.30	9.49 - 18.63	55.90	47.42 - 64.38
Methanolic stem bark extract	14.02	9.84 - 19.98	63.08	53.75 - 72.35
Cyclophosphamide	16.36	11.86 - 20.31	61.02	52.41 - 69.61

RESULTS AND DISCUSSION

The extract yields for stem barks and leaves were 2.13% w/w and 0.85% w/w respectively. The latex and extracts were screened for antifungal activity using disc diffusion method whereby fluconazole (100µg) susceptibility test discs were used as standard drug and 5% DMSO in water was used as a negative control. The latex, standard drug and negative control displayed no activity against the tested fungi (Table 1). This observation implies that the observed activities were due to the extracts and the tested fungi were not sensitive to the standard drug fluconazole. The latex in the current study was inactive even though previous studies on a related species reported varied activities. For instance, *Euphorbia characias* latex was found to be active against *C. albicans* where it was reported to exhibit activity with 80% MIC of 159 µg/ml and enhanced the activity of ketaconazole by reducing the 80% MIC of ketaconazole from 0.3901 µg/ml to 0.94 µg/ml and 0.183 µg/ml for 31.25 µg/ml and 62.5 µg/ml concentrations of the latex [40]. Also, the methanolic extract of the latex of *E. antiquorum* from Tamil Nadu India was reported to possess a strong antifungal activity against *C. albicans, A. flavus, A. fumigatus* [41], also Vimal and Das [28] reported activity of methanolic extract of the latex of the same species against various species of *Candida* however, unlike in the current study *Aspergillus* species were found resistant to the latex extract of *E. antiquorum*. The literature indicates that most of the active latex was collected in the morning however, in the current study the latex was collected throughout the day which might have contributed to the inactivity of the latex.

Both leaf and stem bark extracts inhibited growth of *T. mentagrophytes* and *A. niger* with inhibition zones of 17 and 15 mm respectively for leaves and 9mm for both fungi in the case of the stem bark extracts (Table 1). However, the growth of *T. rubrum* was inhibited by the leaf extract only (12mm). Both extracts displayed no activity against *C. albicans* (Table 1) However, previous studies on aqueous ethanol extracts of various leaf, stem and roots of Brazilian plant were found active against this yeast. Furthermore various extracts of other *Euphorbia* species were also reported active as displayed by *E. hirta* methanolic leaf extract and ethanolic extracts of various plant parts of; *E. aleppica*, *E. szovitsii*, *E. falcata, E. denticulata*, *E. macroclada*, *E. cheiradenia*, *E. virgata, E. petiolata,* which had inhibition zones ranging from 8.33-25.05 mm for various *Candida* species and dematophytes including *Trichophyton* species and *Epidermophyton* [42, 43].

The minimum inhibitory concentration determined by broth microdilution method for the extracts ranged from 2.5- 5mg/ml and *Trycophyton mentagrophytes* was the most sensitive dermatophyte (Table 2). As explained earlier the positive control displayed no activity on the tested fungi an observation which stresses the importance of search for new effective antimicrobials in this era of global antimicrobial resistance.

The brine shrimp lethality was determined for both leaf and stem bark extracts for the predetermined concentrations ranging from 2µg/ml -60µg/ml. The extracts exhibited a strong activity with LC_{50} and LC_{90} of 13.30 and 55.90µg/ml (leaves) and 14.02 and 63.08µg/ml (stem bark) respectively indicating that both extracts are more cytotoxic when compared to the positive control, cyclophosphamide (16.36 and 61.02 µg/ml) (Table 3). There was not much difference between the leaves and stem bark extracts. This observation correlates well with previous reports regarding cytotoxicity of *E. cotinifolia* and other species in the genus [23, 30, 35, 44]. Since the brine shrimp lethality test is usually correlated to various biological activities including anticancer, larvicidal, antimicrobial activities this means that the plant extracts might be associated with even more biological activities. The potential of the plant as an anticancer could be explored further by using various cancer cell lines.

CONCLUSION

The methanolic extracts of both leaves and stem bark of *Euphorbia cotinifolia* displayed varied antifungal activity against *Trichophyton rubrum*, *T. mentagrophytes* and *Aspergillus niger* but were devoid of activity against *Candida albicans*. However, the latex and standard drug fluconazole were unable to inhibit growth of any of the tested fungi. Further studies are recommended so as to evaluate the plant for anticancer activity using various cancer cell lines and isolate active compounds from the stem bark and leaves of the plant. Also, the latex collected during different seasons and at different times of the day should be studied so as to verify further its inactivity.

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