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Antifungal activity of triterpenoid isolated from *Azima tetracantha* leaves

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Abstract: The present study was designed to evaluate the antifungal activity of *Azima tetracantha* extracts and isolated compound (friedelin) against fungi. Antifungal activity was carried out using broth microdilution method and fractions were collected using (silica gel) column chromatography. The antifungal activity of *Azima tetracantha* crude extracts and isolated compound (friedelin) were evaluated using the micro dilution method. Hexane extract showed some antifungal activity. The compound also exhibited antifungal activity against tested fungi. The lowest MIC against *Trichophyton rubrum* (296) was 62.5 µg/ml and the MIC for *Curvularia lunata* was 62.5 µg/ml. These results suggest that Friedelin is a promising antifungal agent.

Key words: antibacterial, antifungal activity, *Azima tetracantha*, friedelin, Dermatophytes, Minimum Inhibitory Concentration, MIC

Introduction

Plants are still widely used for ethno medicine around the world and phytomedicines derived from plants have shown great promise in the treatment of intractable infectious diseases including those caused by opportunistic pathogens [1]. Microorganisms have been developing resistance to many antibiotics due to the indiscriminate use of antimicrobial drugs, increasing clinical problems in the treatment of infections. For example, the effectiveness of an antibiotic, ketoconazole, showed poor response in immuno-suppressed patients and in the treatment of meningitis [2].

Many of the plants used today were known to the people of ancient cultures throughout the world and they were valued for their preservative and medicinal powers. Scientific experiments on the antimicrobial properties of plants and their components have been documented in the late 19th century [3]. Plants have been shown to contain low molecular weight compounds which inhibit the growth of fungi *in vitro*.

Azima tetracantha (Salvadoraceae) is known as 'Mulsangu' in Tamil and 'Kundali' in Sanskrit, respectively. Its root, root bark and leaves are used with food as a remedy for rheumatism [4]. It is a powerful diuretic given in rheumatism, dropsy, dyspepsia and chronic diarrhoea and as a stimulant tonic after confinement [5]. *Azima tetracantha* as efficient acute phase anti-inflammatory drug is traditionally used by Indian medical practitioners [6]. *Azima tetracantha* is used to treat cough, phthisis, asthma, small pox and diarrhea. The decoction of the stem bark is considered astringent, expectorant and antiperiodic [7-9].

Its leaves were found to possess azimine, azecarpin, carpine and isorhamnitine-3-O-rutinoside [10,11]. Friedelin, lupeol, glutinol and β-sitosterol have been isolated from the leaves of *A. tetracantha* [12]. Recently some novel fatty acids were isolated from seeds of this plant [13] .Plant extracts and essential oils show antifungal activity against wide range of fungi [14]. The current study was focused on the antifungal activity of hexane, ethyl acetate, methanol extracts and isolated compound (Friedelin) from *Azima tetracantha* leaves against dermatophytes responsible for infection of keratinized tissue and *Candida albicans*.

Materials and methods

Plant collection. *A. tetracantha* leaves were collected in the month of February from Kalvarayan hills, (altitude, 200 m), Tamil Nadu,

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South India. The plant was identified by authenticated botanist. A voucher specimen (ERI-569) has been deposited in Entomology Research Institute, Loyola College, Chennai, India.

Extraction and isolation. Leaves of *Azima tetracantha* were dried at room temperature and powdered by using electric blender (500 g). The dried powder was extracted with hexane by cold percolation for a period of 42 hours. The solvent was removed under reduced pressure using rotary evaporator; it gave 40 g of crude extract. This residue was dark black while subsequent extractions with more polar solvents (ethyl acetate and methanol) gave considerably dark brown residues. Hexane extract (20 g) was subjected to an initial separation on silica gel (acme's 100-200) using a short glass column (7.4 cm) and eluted with n-hexane and ethylacetate. Twenty five fractions were collected each having 100 ml. The fraction 13 and 14 were eluted by using hexane/ethyl acetate (95:5); it yielded 1.20 g of white powder. This showed three spots on TLC (Thin layer Chromatography); this was further fractionated using small silica gel column (3.2 cm). Initially 25 ml fractions (1-25) were collected and subsequently increased to 50 ml (25-44). Fraction 18 gave 90 mg of needle like substance which was separated using hexane-ethyl acetate (90:10) as eluents.

Instruments. The structure of the isolated compound was identified by nuclear magnetic resonance (AL-300 JEOL) using, ^{1}H-NMR (300 MHz), ^{13}C-NMR (75.45 MHz) analysis. IR spectrum was recorded in Shimadzu by KBr pellet method. Mass spectrometric analysis was conducted at the Mass Spectrometry, Shimadzu with temperature of EI method, Piramal Healthcare Ltd, Chennai, India.

Preparation of sample for testing. Crude extracts and Friedelin were dissolved in 5% dimethyl sulfoxide (DMSO). For minimum inhibitory concentration (MIC) tests, extracts and compound first diluted to the highest concentration and then serial twofold dilutions were performed in a concentration range from 16.2 µg/ml to 1000 µg/ml for extract and 3.9 µg/ml to 250 µg/ml for compound in 96 well titrate plate containing Mueller-Hinton Broth. Pre-experimental procedures demonstrated that final DMSO concentration below 5% did not inhibit microorganism growth.

Microorganism. Fungi, *Trichophyton rubrum* MTCC 296, *T. rubrum* 57/01, *T.mentagrophytes* 66/01, *T. simii* 110/02, *Epidermophyton floccosum* 73/01, *Scopulariopsis sp*. 101/01 *Aspergillus niger* MTCC 1344, *Botyritis cinerea, Curvularia lunata* 46/01 and *Candida albicans* MTCC 227 were used for the experiment. All the cultures were obtained from Madras Medical College, Chennai, India.

Preparation of inoculums. The filamentous fungi were grown on Sabouraud Dextrose Agar (SDA) slants at 28°C for 10 days and the spores were collected using sterile doubled distilled water and homogenized. Yeast was grown on Sabouraud Dextrose Broth (SDB) at 28°C for 48 h.

Antifungal assay. The antifungal assay was performed according to standard Broth microdilution method [15]. MHB (Mueller Hinton Broth) was prepared and sterilized by autoclaving at 121°C, 15 lbs for 15 minutes. The required concentrations of the extract (15.62, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml) and compound (3.90, 7.81, 15.62, 31.25, 62.5, 125 and 250 µg/ml) were added to the 96 well micro titer plate containing 0.1 ml broth. 5 µl of log phase culture was introduced into the respective wells and the final inoculum size was 1×10^{4} cfu/ml of fungal spore. The plate was covered and incubated at 27°C for 72-98 hours for fungi. Appropriate controls included solvent controls that were used to dissolve the test compound, broth alone and the standard antibiotics: ketoconazole and fluconazole. Three replications were maintained. After incubation period the MIC for fungi was defined as the lowest drug concentrations that inhibited visible growth of the fungi in 96 well plates. The results were evaluated by comparing them with control wells.

Results and discussion

Traditionally, *Azima tetracantha* has been used to treat many diseases. Hexane, ethyl acetate and methanol extracts were tested against fungi. Hexane extract showed some activity against tested fungi. The results are presented in table 1. Hexane extract was inhibiting the growth of *Trichophyton rubrum* 296, *T. simii*, *E. floccosum*, *A. niger* and *Curvularia lunata* at 62.5 µg/ml. Growth of *T. rubrum* 57/01 and *Magnethopora* sp were moderately inhibited at 250 µg/ml. However it was ineffective against bacteria. Ethyl acetate extract inhibited the growth of *T. rubrum* 296 and *A. niger* at 62.5 µg/ml. The lowest MIC values observed in hexane extract against *T. simii*, *T. rubrum* 296, *E. floccosum*, *A. niger* and *C. lunata* was 62.5µg/ml. Methanol extract inhibited the growth of *T. rubrum* 57/01 at 1000 µg/ml, *E. floccosum* at 500 µg/ml and *A. niger* at 500 µg/ml. All the three extracts were not inhibiting the growth of *Scopulariopsis sp.* and *C. albicans* (Table 1).

Based on the screening results we selected the hexane extract for fractionation. Totally 25 fractions were collected and screened against fungi (Data were not shown). Further the active fraction was re-chromatographed and a compound was obtained from fraction 18. The isolated compound was crystallized using hexane: ethyl acetate mixture; it was colorless needle (yield 90 mg, m.p 263-265°C). It answered Naller's test (Thionyl chloride gave pink colour). The isolated compound was confirmed as Friedelin by comparison with authentic sample (m.p, mm.p, super imposable IR and ^{1}H NMR, ^{13}C-NMR and MASS spectral data) and our results compared satisfactorily with those reported in the literature [16]. The compound inhibited the growth of fungi such as *T. rubrum* 296 (62.5 µg/ml), *C. lunata* (62.5 µg/ml), *T. simii* (125 µg/ml) *E. floccosum* (125 µg/ml) *A. niger* (125 µg/ml) and *Magnethophora* sp (125 µg/ml) (Table 1). The lowest MIC values were observed against *C. lunata* and *T. simii* at 62.5 µg/ml.

Vasanth *et al* [17] reported that friedelin isolated from hexane extract of *Notonia grandiflora* showed activity against *Proteus mirabilis* at 1000 ppm concentration.

Friedelin isolated from methanolic extract of *Vismia laurentii*. Fridelin was found to be the most active compound. The lowest minimum inhibition concentration (MIC) values as obtained by the micro-dilution assays were 19.53 and 1.22 µg/ml for the crude extracts and purified compounds, respectively [18].

Friedelin was also tested against bacteria but did

Table 1. Antifungal activity of *Azima tetracantha* crude extracts and isolated compound friedelin. (-) – no activity, MIC of <100 μg/ml – good activity, MIC of 100 to 500 μg/ml – moderate activity, MIC of >1000 μg/ml – inactive.

Microorganism tested	Crude extracts [μg/ml]			Friedelin [μg/ml]	Antibiotic MIC [μg/ml]	
	hexane	ethyl acetate	methanol		fluconazole	ketoconazole
T.mentagrophytes	1000	1000	>1000	>250	25	<12.5
T. simii	62.5	>1000	>1000	125	<12.5	<12.5
*Trichophyton rubrum*296	62.5	62.5	>1000	62.5	<12.5	<12.5
T. rubrum 57/01	250	>1000	1000	>250	25	<12.5
Epidermophyton floccosum	62.5	>1000	500	125	12.5	<12.5
Scopulariopsis sp.	>1000	>1000	>1000	>250	<12.5	<12.5
Aspergillus niger	62.5	62.5	500	125	100	<12.5
Curvularia lunata	62.5	500	>1000	62.5	<12.5	<12.5
Magnethophora sp.	250	>1000	>1000	125	-	-
Candida albicans	>1000	>1000	>1000	>250	>100	25

not show activity (data not shown). Pretto *et al*. [19] reported that friedelin was isolated from *Calophyllum brasiliense* and tested against Gram-positive and Gram-negative bacteria at 1000 μg/ml. The compound was ineffective.

A thorough analysis of the results indicated that among the extracts of *Azima tetracantha* only the hexane extract showed some antifungal activity against tested fungi. A pure compound identified as Friedelin was isolated from the active hexane extract which exhibited antifungal activity. The compound and extracts possessed good antifungal activity *in vitro* and can be considered as potential candidate drug in the treatment of infectious diseases caused by pathogenic fungi.

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