Evaluation of antidermatophytic activity of -(2-hydroxy-2-methylpropyl)--[2-hydroxy-3-methylbut-2-en-1-yl] polymethylene - the compound isolated from Caesalpinia bonducella (L.) Flem.

K Sagar, G Vidyasagar

Citation

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Abstract

A compound was isolated from ethyl acetate leaf extract of Caesalpinia bonducella (L.) Flem. and identified as -(2-hydroxy-2-methylpropyl)--[2-hydroxy-3-methylbut-2-en-1-yl] polymethylene by UV, IR, NMR, HPLC and GC/MS and evaluated for antifungal efficacy by Agar and broth dilution methods against clinical dermatophytes (viz., Trichophyton tonsurans, Trichophyton mentagrophytes, Trichophyton rubrum, Microsporum gypseum and Epidermophyton floccosum). In agar dilution method, T. tonsurans was inhibited only at 400 g/ml concentration of the compound, whereas all others were inhibited even at low concentrations; optimum pH was 6.0; size of the colonies of the fungi deceased when inoculum size decreased from 10^7 to 10^4 spores/ml with increase with compound concentration from 100 to 400 g/ml. In broth dilution method, mycelial dry weight of all test fungi decreased with the increase in the concentration of the compound; optimum pH was 6.5; decline in biomass of all fungi along with increase in concentration of the compound were observed which were significantly different ($p<.05$). The dermatophytes used in this study are the most common and widespread of this group and since, there is no scientific evidence to support the medical use of -(2-hydroxy-2-methylpropyl)--[2-hydroxy-3-methylbut-2-en-1-yl] polymethylene, further studies are needed in order to elucidate the mechanism(s) of action of these compounds and their derivatives, as well as the antimicrobial activity against other microbial strain in particularly antibiotic resistant dermatophytes.

INTRODUCTION

In recent years, proliferation of new classes of drugs such as, allylamines (e.g terbinafine) and orally active triazoles (e.g. itraconazole), has represented the most noteworthy trend in dermatophytosis therapy (Weitzman and Summerbell, 1995). However, treatment with both itraconazole and terbinafine for prolonged times requires periodic laboratory monitoring of liver function (Zapata et al., 2003). Moreover, these antifungal agents may have drug interactions with other medications (Huang et al., 2004). Griseofulvin, which had been for many years the only antifungal available for the treatment of dermatophytoses is still the longstanding drug of choice for tinea capitis, but there are concerns with resistance and toxicities with this agent (Huang et al., 2004). Taken together, the improved cure rates, reduced adverse effects; decreased drug interactions and lower cost of topical agents make therapy with these drugs a favourable choice with management of superficial fungal infections including dermatophytoses. The spread of drug resistant pathogens is one of the most serious threats to successful treatment of microbial diseases.

In this context, new antifungals of plant derivatives could be useful alternatives for the treatment of dermatophytoses where a topical therapy is required. These plant compounds can have different structures and different action when compared with antimicrobials conventionally used to control the microbial growth and survival they also have a reduced risk of side effects and lower cost (Nascimento et al., 2000). It is thus not surprising that in recent years, attention has turned to natural antimicrobial agents (antibacterials and antifungls), particularly, there is a rapid growth of interest in the use of medicinal plants to cure skin diseases. There are many reports on the antimicrobial activity of the active the compounds which represent various groups including alkaloids, flavonoids, isoflavonoids, tannins, cumarins, glycosides, terpenes, phenylpropannes, organic acids (Nychas, 1996).

Ceasalpinia bonducella (L.) Flem. (Caesalpiniaceae) grows wild in the scrub forests, of the Eastern Himalayas, in Assam and West Bengal. It is also found in the Eastern Ghats in Andhra Pradesh and Madhya Pradesh, commonly known as fever nut. The leaves and seeds of this plant are used as a folk medicine to treat asthma, tumor, inflammation, liver disorders and chronic fever (Nadkarni, 1976). It is found to be very potent as an antiperiodic, antipyretic anti-inflammatory, antidiabetic, antifungal and utilized as febrifuge, antispasmodic, an antirheumatic and mild purgative (Nadkarni, 1976; Satiyavati et al., 1976). Since, there are very few reports on antimicrobial activity of C. bonducella, thus keeping this in view, attempts have been made to isolate the active the compound from the ethyl acetate leaf extract of C. bonducella and evaluate its antifungal efficacy against clinical isolates of dermatophytes.

MATERIALS AND METHODS

AGAR DILUTION METHOD

END POINTS FOR DETERMINING MICS AND MFCS.

The MIC of the compound was prepared viz., 100, 200, 300 and 400 g/ml tested for the dermatophytes was defined as lowest drug concentrations that yielded visible growth of the fungi. The minimal fungicidal concentrations (MFCs) was determining by sub culturing 0.01 ml from each tube with no visible growth onto SDA plate. The plates were incubated at 30oC for 7 days. Afterward, the colonies were counted, and the MFC was defined as the lowest concentration of drug which yielded a negative subculture or less than two colonies (killing of more than 98% of the fungal inoculum).

The test organisms used were clinical isolates namely, Trichophyton tonsurans, Trichophyton rubrum, Trichophyton mentagrophytes, Microsporum gypseum and Epidermophyton floccosum.

ISOLATION OF THE COMPOUND

The leaves of Caesalpinia bonducella were collected in and around Gulbarga University, Gulbarga during the period from June to December, 2004-2006 and was authenticated at Herbarium, Department of Botany, Gulbarga University, Gulbarga under voucher no. HGUG - 208. The plant material was immediately sprayed with alcohol to cease the enzymatic degradation of secondary metabolites and shade dried. After drying, the material was powdered thereof subjected for soxhlet extraction by using ethyl acetate solvent. 30 g of ethyl acetate leaf extract of C. bonducella was chromatographed over silica gel 100-200 mesh on a column of length 52 cm and 6 cm in diameter. Elution was carried out with solvents and solvent mixtures of increasing polarities (0, 10, 20, 30, 40 and 50%). A total of 24 fractions were collected in 250 ml portions. The active 20% (8.0 mg) fraction showed strong antibacterial activity against the test organisms. This fraction was further chromatographed on silica gel column and eluted with hexane: ethyl acetate (20:1). The column fractions were analyzed by thin-layer chromatography (TLC) (silica gel 60 F_{254}, hexane: ethyl acetate, 20:1), and fractions with similar TLC patterns were combined. One fraction showed strong antifungal activity which was finally selected for purification through further TLC it was found that the fraction showed a single spot on the developed TLC plate. After verifying the purity of an isolated active compound, it was identified based on analysis of its UV, IR, NMR, HPLC and MS spectra.

FUNGAL INOCULUM PREPARATION

All the dermatophytes grown on SDA after a week, spores were flooded with 0.85% saline (Ghannoum et al., 2004). After settling of the larger particles, conidia were counted with a haemocytometer and diluted in SDA medium. A tenfold dilution was made. Nine ml of normal saline solution was taken in 5 test tubes. In first test tube, 1 ml of spore suspension was poured by pipette under aseptic conditions. The solution of first test tube was homogenized with the help of pipette and 1 ml of this solution was transferred to second test tube containing 9 ml of normal saline solution. This process was repeated up to 5th test tube. Triplicates of each dilution were maintained. The SDA seeded petriplates were counted in haemocytometer. The average of 3 petriplates was taken in each case. The test inoculum was adjusted to1.5 x 105 spores/ml.

GERMINATION TEST

Different concentrations of the compound were mixed with 20 ml SDA and poured onto petriplates, immediately added 0.1 ml standard spore suspension of the test dermatophytes on the medium and left for solidification. After 24 h of incubation, square samples (10 by 10 mm) cut off from the plates were mounted in lactophenol cotton blue and examined microscopically. A spore was considered to be germinated when the germ tube was a long as it was wide.

EFFECT OF PH

The activity of and the compound against test dermatophytes

on medium with different pH values was determined by the agar dilution method on SDA medium adjusted to a final pH value of 5.6, 6.0, 6.5 and 7.5 with inoculum sizes of 0.01 ml of standard spore suspension.

EFFECT OF INOCULUM SIZE

The activity of and the compound against test dermatophytes with different inoculum sizes was determined by agar dilution method on SDA medium (pH 5.6) with an inoculum size of 0.01 ml of 104, 105, 106 and 107 spores per ml.

BROTH DILUTION METHOD

For antidermatophytic assay in broth, 5 ml of the medium (SDA) was added to each screw-capped test tube and were autoclaved at 121oC for 15 min. Tubes with 5 ml sterile SDA were inoculated with 10, 20, 30 and 40 mg/ml concentrations of the compound in DMF. Tubes were kept in the salutation position overnight for checking the sterility. The next day, the tubes were inoculated with 20 µl of fungal suspension on the salutation position and all the test tubes were incubated at 37oC. After one week, the results were noted. The visible mycelial growth in the test tubes expressed the degree of activity of the compound.

MYCELIAL DRY WEIGHT

Fungal mycelia from the above culture were separated passing through Whatman filter No.1. A known amount of thoroughly washed mycelia was placed on preweighed petriplates and allowed to dry at 60oC for 6 h to reach a constant weight. Fungal growth inhibition was calculated by considering the control' and sample' mycelial dry weights by following the method of Rasooli and Razzaghi Abyaneh (2004).

EFFECT OF PH

The activity of the compound against test dermatophytes in medium with different pH values was determined by the broth dilution method on SD medium adjusted to a final pH value of 5.6, 6.0, 6.5 and 7.5 with inoculum sizes of 0.01 ml of standard spore suspension.

EFFECT OF INOCULUM SIZE

The activity of the compound against test dermatophytes with different inoculum sizes was determined by broth dilution method in SD medium (pH 5.6) with an inoculum size of 0.01 ml of 104,105, 106 and 107 spores per ml.

STATISTICAL ANALYSIS

All data are expressed as mean ± standard errors of the mean (SEM) were analyzed by the analysis of variance by students 't' test. A P value of <0.05 was considered to be significant.

EXPERIMENTAL RESULTS

In IR spectrum of the compound (Fig. 1) the absorption band at 346 cm-1 was observed due to the presence of OH group. The absorption peak at 2920 cm-1 corresponds to the stretching of C-C bond. The peak was observed at 1459 cm-1 due to –C=C– stretching. The peak at 1651 cm-1due –C-O- stretching. In the 1HNMR spectrum (Fig.2) the singlet was observed at 0.85 due to six protons of two methyl groups attached to C-18. Two singlets at 1.5- and 1.7 due to the protons of two methyl groups attached to C-1. The four protons of $–2CH_2-$ groups present at C-3 and C-17 were resonated as singlet at 2.05 and the signals due to of thirteen methylene groups were resonated at 1.1 – 1.4 . The peak due to two –OH groups observed at 5.2 as broad singlet. In the mass spectrum, it showed the molecular ion peak at m/z 339 due to [M-1]+ ion. These data confirm the structure of the compound as -(2-hydroxy-2-methylpropyl)--[2-hydroxy-3-methylbut-2-en-1-yl] polymethylene with the structural formula $C_{22}H_{44}O_2$.

Figure 1

Fig.1: IR spectrum

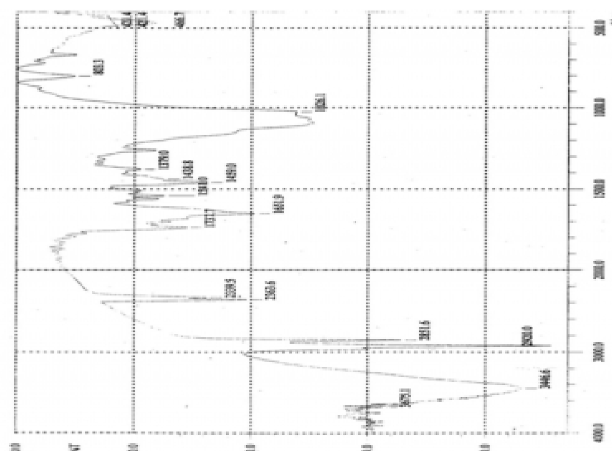


Figure 2

Fig. 2: 13CNMR spectrum Agar dilution method

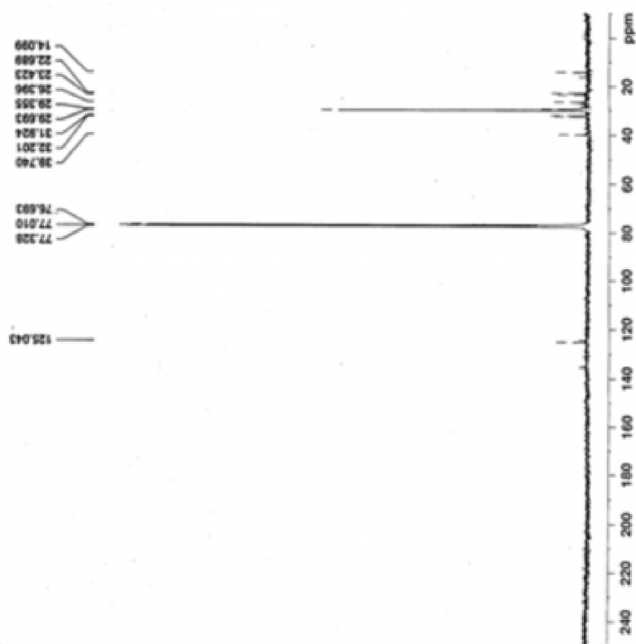
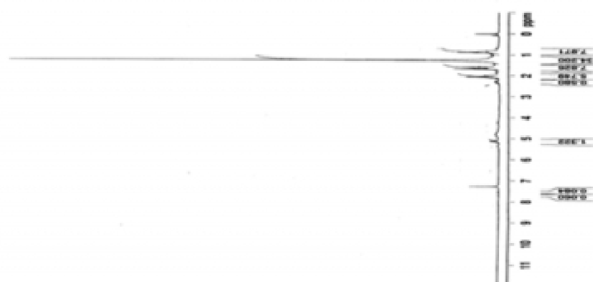


Figure 3

Fig. 3: 1HNMR spectrum



EFFECT ON CONIDIAL GERMINATION

The percent germination of conidia of all the test dermatophytes after one week in the presence of the compound (incorporated into SD Agar medium) was determined by counting the number of germinating spores microscopically. The result is plotted in figure 1.1.

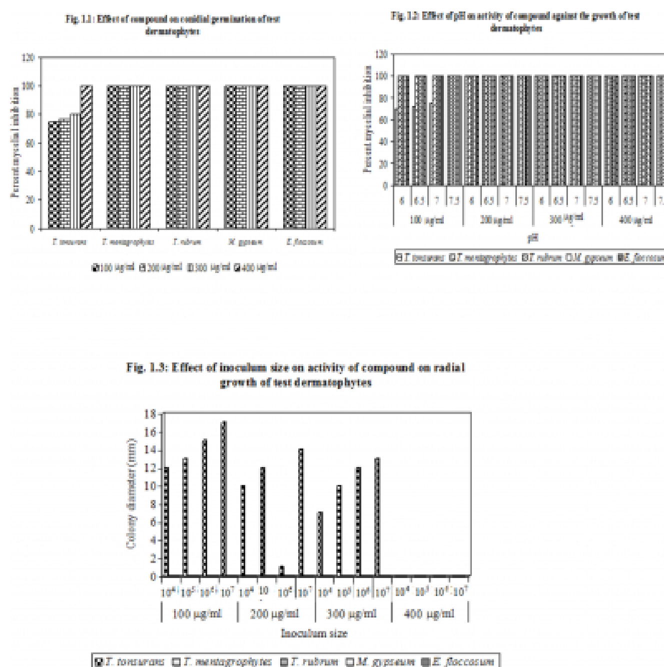
The compound completely inhibited swelling and germination of spores of M. gypseum, T. mentagrophytes, T. rubrum and E. floccosum at concentrations of 100, 200, 300 and 400 μg/ml. But T. tonsurans was not completely inhibited at 100, 200, 300 μg/ml. Whereas, 100% inhibition of T. tonsurans was observed at 400 μg/ml of the compound. The numbers that were germinated decreased with increasing concentrations until a concentration was reached at which no swelling of the spores occurred (400 μg/ml).

After 7 days of incubation, the numbers of spores that germinated at sub inhibitory concentrations of the compound (only of T. tonsurans) increased, and a few germinated at concentrations that had been inhibitory after 7 days.

EFFECT OF PH ON ACTIVITY

The antifungal activity of the compound was assessed at different pH levels viz., 6.0, 6.5, 7.0 and 7.5 with an inoculum consisting of a standard spore suspension fig. 1.2). The compound could potentially inhibit the growth of T. mentagrophytes, T. rubrum, M. gypseum and E. floccosum completely at all the pH tested, whereas more than 70% growth of T. tonsurans was inhibited at pH 6.0 and above that the organism did not grow. M. gypseum proved to be resistant at pH 7.5 where at 100, 200, 300 and 400 μg/ml of the compound could inhibit 10.20±0.43, 9.05±0.57, 8.20±0.43 and 8.40±0.40 percent of mycelial growth of M. gypseum, respectively (Fig. 1.2). The activity of the compound at different pH differed significantly (P<0.05).

Figure 4



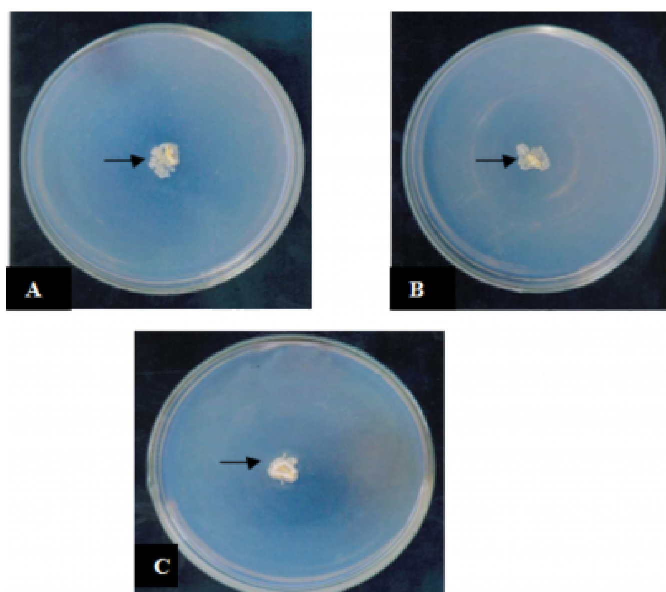
EFFECT OF INOCULUM SIZE ON RADIAL GROWTH OF COLONY

The effect of inoculum size on radial growth of colonies of test dermatophytes after 7 days of incubation on SDA medium containing 100, 200, 300 and 400 μg/ml was studied. The size of the colonies decreased when inoculum size was decreased from 107 to 104 spores/ml with the increase in the compound concentration from 100 to 400 μg/ml, but

decreased colony growth was exhibited by T. tonsurans only with 104 spores/ml at 100, 200 and 300 g/ml, whereas other fungi could not grow even at 100 g/ml of the compound (Fig. 1.3). The surfaces of the colonies of T. tonsurans in the presence of MICs of the compound were granular, irregular in outline, and highly wrinkled (Plate-I).

Figure 5

Plate I: Effect of compound on the colony growth of



A= 100 µg/ml; B= 200 µg/ml; C= 300 µg/ml;

-> Indicates the colony with granular, irregular in outline and highly wrinkled

BROTH DILUTION METHOD

EFFECT OF ON MYCELIAL DRY WEIGHT

The effect of the compound in SD broth on mycelial dry weight is presented in figure 2.1. The mycelial dry weight of all test dermatophytes decreased with the increase in the concentration of the compound, which could inhibit all the fungi, except T. tonsurans at 100, 200, 300 g/ml. The mycelial dry weight differed significantly at all concentrations (P< 0.05).

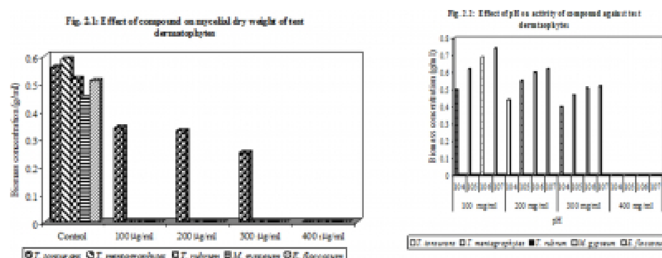
EFFECT OF PH ON ACTIVITY

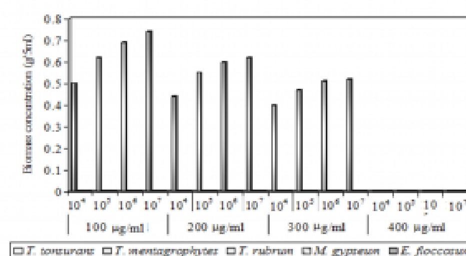
Various pH i.e. 6.0, 6.5, 7.0 and 7.5 of broth medium were tested for the growth of dermatophytes. The compound could inhibit all organisms except T. tonsurans and pH 6.5 was found suitable (Fig. 2.2).

EFFECT OF INOCULUM SIZE ON ACTIVITY

The biomass concentrations of the test dermatophytes were assessed with varying sizes of inoculum i.e., 104, 105, 106 and 107 spores/ml. Only T. tonsurans could grow at 100, 200 and 300 g/ml concentrations (Fig. 2.3). Decline in biomass of all the fungi along with increase in concentrations of the compound were observed which were significantly different (P<0.05).

Figure 6





DISCUSSION

Recently, the use of medicinal plants covering the basic health needs in developing countries is increasing, which offer a new source of antibacterial, antifungal and antiviral agents with significant activity against infective microorganisms.

The antifungal effect of the isolated compound from ethyl acetate leaf extract of Caesalpinia bonducella (L.) Flem. was tested on all the dermatophytes of clinical origin by agar and broth dilution methods. This provided the in vitro assessment of the MICs of the compound. The results show that the compound has an interesting activity even if it differed between the test fungi. In agar dilution method, the conidia germinated less along with the increase in concentrations. At various concentrations of the compound a few were germinated, but had been inhibitory after 7 days. This was probably due to the normal decomposition of the compound, but the small number of spores that germinated at high concentration could not continue to develop into normal hyphae. The high activity of the compound was found at pH 6.0 and as the inoculum size increased the

colony growth increased with the decrease in concentration of the compound. Whereas, in broth dilution, optimum pH was 6.5 with the same inoculum size. The biomass concentration of fungi decreased with increase in concentration i.e. from 100 □g/ml–400 □g/ml in broth medium. The MIC of the compound was considerably affected by inoculum size and medium pH. Also, there were some differences in MIC with to whether solid or liquid medium used, i.e. more concentration of the compound was required to inhibit growth in liquid medium than in solid medium.

The compound, except T. tonsurans, inhibited the germination of all the fungi tested. Concentrations approaching the inhibitory level resulted from the delay in swelling and germination of spores, and the restriction of the radial growth of fungal colony was noticeable inhibition of longitudinal extension of hyphae.

The dermatophytes used in this study are the most common and widespread of this group. They represent the three genera of dermatophytes and their respective ecological types geophilic (Microsporum gypseum), zoophilic (Microsporum canis and Trichophyton mentagrophytes) and anthropophilic (Trichophyton tonsurans, Trichophyton mentagrophytes and Epidermophyton floccosum). The morphological nature of these fungi, which grow as hyphae containing various numbers of micro and macroconidia, accompanied by thick-walled chlamydospores, causes difficulty, when attempting to standardize inocula. Hence, to produce evenly dispersed suspensions, they were subjected to mechanical fragmentation and homogenization. This process resulted in homogenous suspensions containing small septate fragments and gave reproducible results. All the dermatophytes tested, proved to be more susceptible to the compound. The less biomass concentration in broth at all concentrations tested, may probably be due to homogenous dispersion of particles in a liquid medium which enabled closer contact between the fungal elements and the active agent.

At a particular concentration, the potency of a fungitoxicity of the compound can thus depend on the inoculum density of the test fungus. For example, the antifungal activities of citral and menthol were found to vary with inoculum density (Venkataraman and Pattisapu, 1987). This suggests that a high inoculum density of a fungus may either degrade or convert the compound into detoxified forms. The nature of detoxification of the fungitoxic products will also depend on the detoxifying enzymes produced by them. Thus, the detoxification of fungitoxic products at varying inoculum densities could be one of the main factors limiting their efficiency as ideal antifungal agents, leading to the minimum inhibitory concentration of the compound depending on inoculum density. However, an increased inoculum density did not affect the antifungal potency of the compound in the present study.

Although it is desirable to develop the compound having a broad spectrum of activity, it is also important to bear in mind that the treatment of chronic different tineas with the same broad spectrum antifungal agent leads sometimes to a high resistance to the available antifungal agents (Ghannoum and Rice ,1999). Thus, one of the strategies for overcoming this problem is the treatment of fungal infections with the appropriate narrow spectrum agent when the ethiological agent is known (Di Domenico, 1999).

Tinea pedis is one of the most frequent mycoses; it occurs in most classes of patients, especially in immunosuppressed patients (Soares, et al., 1995). T. rubrum is the most common superficial fungus, accounting for at least 60% of all superficial fungal infections in humans. This organism may remain viable in environment for over six months, thus accounting for widespread infection. In this study, this organism has been inhibited by the compound. Therefore, antifungal activity of the compound reported in this study against the dermatophytes open the avenues that it could be helpful for developing the new antifungal agents for treating dermatomycoses caused by T. tonsurans, T. mentagrophytes, T. rubrum, M. gypseum and E. floccosum. Since, there is no scientific evidence to support the medical use of □-(2-hydroxy-2-methylpropyl)-□-[2-hydroxy-3-methylbut-2-en-1-yl] polymethylene} [the compound used in the present study], further studies are needed in order to elucidate the mechanism(s) of action of these compounds and their derivatives, as well as the antimicrobial activity against other microbial strain in particularly antibiotic resistant dermatophytes.

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Author Information

Kavitha Sagar
Central Institute of Medicinal and Aromatic Plants, Allalasandra, GKVK, PO, Bangalore

GM Vidyasagar
Central Institute of Medicinal and Aromatic Plants, Allalasandra, GKVK, PO, Bangalore