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In vitro assessment of anti - dermatophytic effect of active fraction of methanolic extracts of *Acorus calamus*

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Dermatophytes, active plant extracts, *Acorus calamus*

1 SUMMARY

Dermatophytes are a group of closely related fungi that have the capacity to invade keratinized tissue (skin, hair, and nails) of humans and animals causing dermatophytosis. Treatment of these fungal infections requires prolonged therapy. Hence, new drug formulations especially of bio-based compounds that have relatively less or no side effects are currently being investigated to meet the new demands. The present study aimed to evaluate the anti- dermatophytic efficacy of the methanolic extracts *of Acorus calamus* under in vitro condition and to demonstrate the mode of action of the extract using clinical strains (obtained from patients suffering from prolonged severe dermatophytic infections). The results showed that the active fraction of the methanolic extract of *A. calamus* exhibits MIC at the lower concentration of 32ug/mL. To establish infections in the host, virulence factors are pivotal. Hence determining the effect of the active fraction on the virulence factors of dermatophytes such as secreted aspartyl proteases, neutral protease, alkaline protease, acid protease and keratinase, was attempted. The results were promising and proved that the active fraction of *Acorus calamus* reduced the virulence factors in terms of enzyme units.

2 INTRODUCTION

Dermatophytes are a group of fungi that cause infections of skin, hair and nails. They include three genera namely *Epidermophyton*, *Microsporum* and *Trichophyton*. Dermatophytes infections are generally referred to as 'Tinea'. The treatment of the infections is normally by azoles, e.g. Fluconazole, Itraconazole, Ketoconazole; polyenes, e.g. Amphothericin B, Griseofulvin, terbinafine echinocandins. Antibiotics may be necessary to treat secondary bacterial infections that occur in addition to the fungal infections (Weizmann & Summerbell, 1995).

Virulence factors are pivotal in pathogenesis of most organisms, which includes extra and intra cellular enzymes for their morphological switching and biofilm formation. In majority of organisms, phospholipases and proteases are two enzymes always required for virulence. These two enzymes are able to degrade the proteins and damage the host cell membranes thus facilitating entry and invasion by organisms. Secreted aspartyl proteases are capable of degrading epithelial and mucosal barrier proteins such as collagen, keratin and mucin as well as antibodies and cytokines.

Cloning and disruption of genes that code for these enzymes has shown their involvement in the virulence of *Candida* spp.

(Hube *et al*., 1997, Sangalard *et al*., 1997; Leidich *et al*., 1998; Watts *et al*., 1998; Bernandis *et al*., 1999). Controlling of dermatophytic infections requires prolonged therapy, and the existing drugs, on long run seem to exhibit side effects, which necessitates the need for new drug formulations for long term use that are biocompatible. In addition, the drugs should selectively kill the fungal cells without affecting the other cells.

Since ancient times, Indians have used numerous medicinal plants to combat infections. However, either due to non–availability of recorded information, or lack of scientific evaluation, these ancient treasures are not widely used nowadays. The plant *Acorus calamus*, apart from its other potential values, has been used in newborn babies, to prevent fungal and dermatophytic infections. However, no scientific validation is available for its antifungal and antidermatophytic activities. Hence, this study attempted to evaluate the efficacy of this plant as an antifungal agent.

3 MATERIALS AND METHODS

3.1 Organisms: The strains isolated from patients suffering from dermatophytic infections (General Hospital, Chennai, India). The isolates were characterized using Kaminski scheme and identified as *Trichophyton ajelloi, Trichophyton rubrum, Trichophyton mentogrophytes, Trichophyton tonsurans*. All the cultures were maintained in Sabouraud's dextrose agar slants at -20ºC until use.

3.2 Active fraction of *Acorus calamus*: Rhizomes of *Acorus calamus* were obtained from medicinal farms of South India and voucher specimen deposited in Plant anatomy research centre, Chennai. The rhizomes were shade dried and coarsely powdered, the crude methanolic extract fractionated using column chromatography, into two active fractions. However, for this study we used one fraction to assess the antidermatophytic effect.

3.3 Antidermatophytic efficacy of active fraction: The antidermatophytic effect of active fraction of *Acorus calamus* was determined using broth dilution method. Briefly, conidia were harvested from colonies by covering plates with sterile saline containing 1% Tween 80, and the spore suspension was filtered through glass wool to remove residual hyphae or long chains of arthrospores. The number of conidia was estimated microscopically using heamocytometer and the conidial suspensions were diluted in RPMI 1640 (Sigma) buffered with MOPS. The tests were performed in 96 well polystyrene plates. Each microdilution well containing 100 µL of diluted (twice) drug concentrations was inoculated with 100 µL of diluted (twice) conidial inoculum suspensions (final volume in each well was 200 µL) to bring dilutions of inoculum to 10 - 10^4 cfu/mL. Growth and sterility controls were included for each isolate tested. Plates were incubated at 28ºC and MICs was recorded after 5 days of incubation. The susceptibility endpoint was recorded for each strain and for each drug. MIC was defined as the lowest drug concentration resulting in total inhibition of visible growth. Tests were performed in duplicate and all experiments were repeated three times (Pfaller *et al*., 1995; Martinez-Rossi, 2008; Aranjo *et al*., 2009).

3.3.1 Effect of methanolic extract of *A. calamus* on virulence factors of dermatophytes: Conidia were extracted from fungal colonies as described above. One ml of conidial suspension was added to 100 ml of Sabouraud's Dextrose (SD) Broth and incubated at 28ºC. Active fraction of *A. calamus* was added at MIC concentration and none in the control. Every week an aliquot was withdrawn, the enzyme assay for virulence factor was carried out. The extracellular enzymes (crude) were tested for the following virulence factors.

3.4 Effect of *A. calamus* (active fraction) on the virulence factors of dermatophytes

3.4.1 Secreted aspartyl protease (SAP): SAP was assayed as per the protocol of Mac Donald and Odds (1980). The enzyme unit was measured spectrophotometically (Cary 100 UV spectrophotometer) following the digestion of BSA (Bovine Serum Albumin fraction V, Himedia , India) as substrate. A typical reaction mix contained 250 µl of enzyme, 500 µl of 2.0% (w/v) BSA in 50mM Sodium citrate buffer (pH 3.2), 100 µL 50mM sodium citrate buffer (pH 3.2) and 200 µL 2M Perchloric acid and incubated on ice for 15 min. Precipitated protein was removed by centrifugation at 14000g for 5min. and the enzymes activity was measured by determining the increase in A_{280} of supernatant.

3.4.2 Alkaline Protease: Alkaline protease was determined according to Gupta *et al.* (2002), in brief, the reaction mixture consists of Casein (500 µL, dissolved in carbonate-bicarbonate buffer, 0.1M, pH 9.5), crude Enzyme (500 µL) and incubated at 60ºC for 20 minutes. The reaction stopped by the addition of TCA (500 µL) and free amino acids released by protease from casein hydrolysis were estimated by standard Lowry method. The protease activity was defined as mol of tyrosine released per minute per ml of enzyme.

3.4.3 Acidic Protease Assay: This was done as per Larson & Whitaker (1970). The reaction mixture contained Haemoglobin 1ml in citrate buffer (pH 3) to which added crude enzyme (400µL) and incubated at 37ºC for 30 min. TCA (1mL) was added to stop the reaction and centrifuged at 5000 rpm for 5 min and the supernatant read at 280 nm.

3.4.4 Keratinase: The most important virulence factor of dermatophytes is keratinase. This was estimated by the protocol of Brandelli (2005). The reaction mixture consists of enzyme (100µL) Tris (800µL) and Azokeratin (200µL); followed by incubation at 40ºC for 15 min; added TCA (1ml) to arrest the reaction and centrifuged at 5000 rpm for 15 min. To the supernatant added Sodium carbonate (5 mL) and incubated for 10 min and added 1 ml of Folins reagent (1 mL) and incubated for 30- 60 min and read at 660 nm.

4 RESULTS AND DISCUSSION

In recent years, the incidence of skin infections has increased throughout the world. The existing drugs are able to control, but are unable to eradicate them completely. Most of the infections worsen due to poor eating habits and climatic conditions. Dermatophytes are slow growing organism that require at least four weeks for complete growth. The MICs of antifungal agents can be determined after four days for *T. mentagrophytes* and five days for *T. rubrum, T .tonsurans* and *T. ajelloi* when incubated at 28 ºC. The MIC is regarded as the lowest concentration of the extract that does not show any viable growth after incubation (compared to the control). The MIC value of active fraction of *Acorus calamus* was found to be in range of 30-35 µg/ml, the individual values are given in Table 1. From the literature surveyed, the antidermatophytic effect of the *Acorus calamus* is potentially high. In this study we observed, the active fraction of *A. calamus* reduced by upto 90% the virulence factors compared to the control.

Table 1: Minimum Inhibitory Concentration (MIC) of Active fraction of *Acorus calamus* against dermatophytes.

Sl no.	Isolate	MIC (µg/ml)
1	T. rubrum	32
2	T. mentagrophytes	32
3	T. tonsurans	30
4	T. ajelloi	35

The secreted aspartyl proteases (SAPs) are the potent virulent factor of dermatophytic infections and genes SAP 1 – 9, code for these virulence factors (Mc. Donalds & Odds, 1980). These proteases are produced extracellulary as well as intracellularly. However, in the samples treated with active fraction of *Acorus calamus* there was a drastic reduction in the SAPs. This reduction was found to be evident in all the species. Of the four dermatophytic species, *T. mentagrophytes* was found to contain maximum content of the acidic proteases.

The content of alkaline proteases was found to vary with all species. The enzyme content was high during the fourth week, in case of *T. ajelloi* it was gradually decreasing from first week to the next weeks, while in case *T. rubrum* initially it was less and gradually it increased to more than for the other species. The proteolytic activity has also been confirmed in some saprophytic fungi, yeasts and even bacteria (Descamps *et al.*, 2002).

Filamentous fungi can synthesize a diverse range of hydrolytic enzymes such as as proteases, carbohydrases and lipases. Keratinases are the key enzymes in fungal invasion of skin and have been mostly studied in dermatophyte species belonging to the *Trichophyton* and *Microsporum* genera, some pathogenic yeasts as *Candida albicans* and also some other fungi and bacteria. Keratinases are the enzymes that are required to colonize the hair, which is the most important factor that is responsible for manifestation of infection. Ghahfarokhi *et al.* (1996) showed that the extracts of garlic and onion have an effect on the virulence factors such as keratinase activities in *T. mentagrophytes*. These extracts disrupt the fungal ultra structure causing massive necrosis and disorganise some cellular compartments. From the results, it is

clear that the active fractions of *Acorus calamus* also reduces or inhibits the activity of virulence factors to a greater extent.

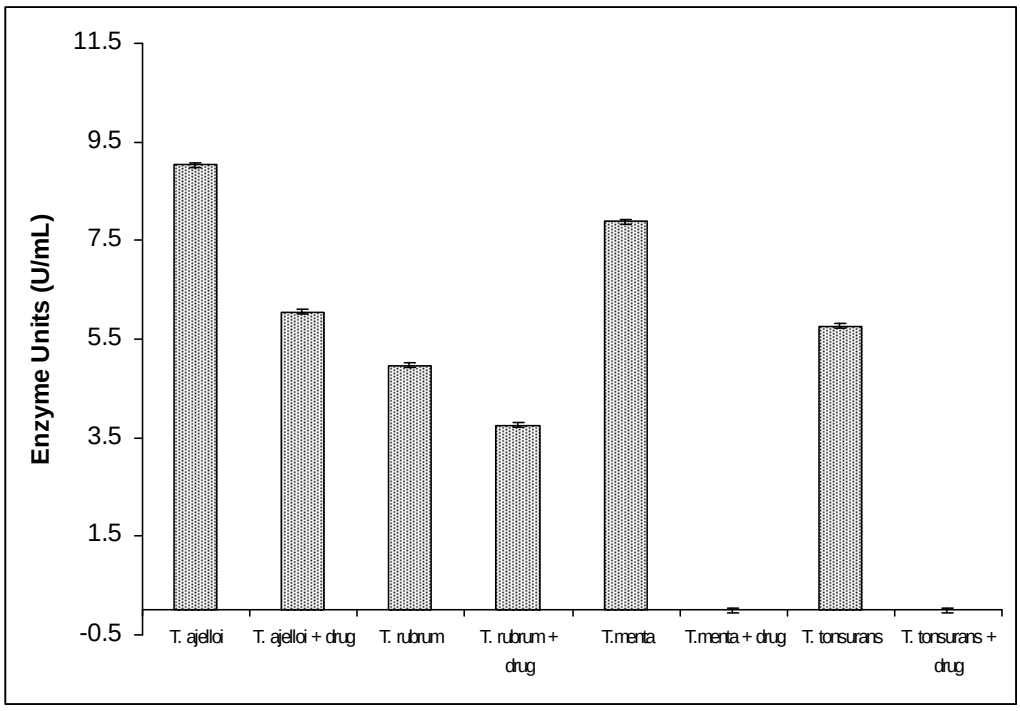

Figure 1: Effect of Active fraction of *Acorus calamus* on Secreted Aspartyl Protease.

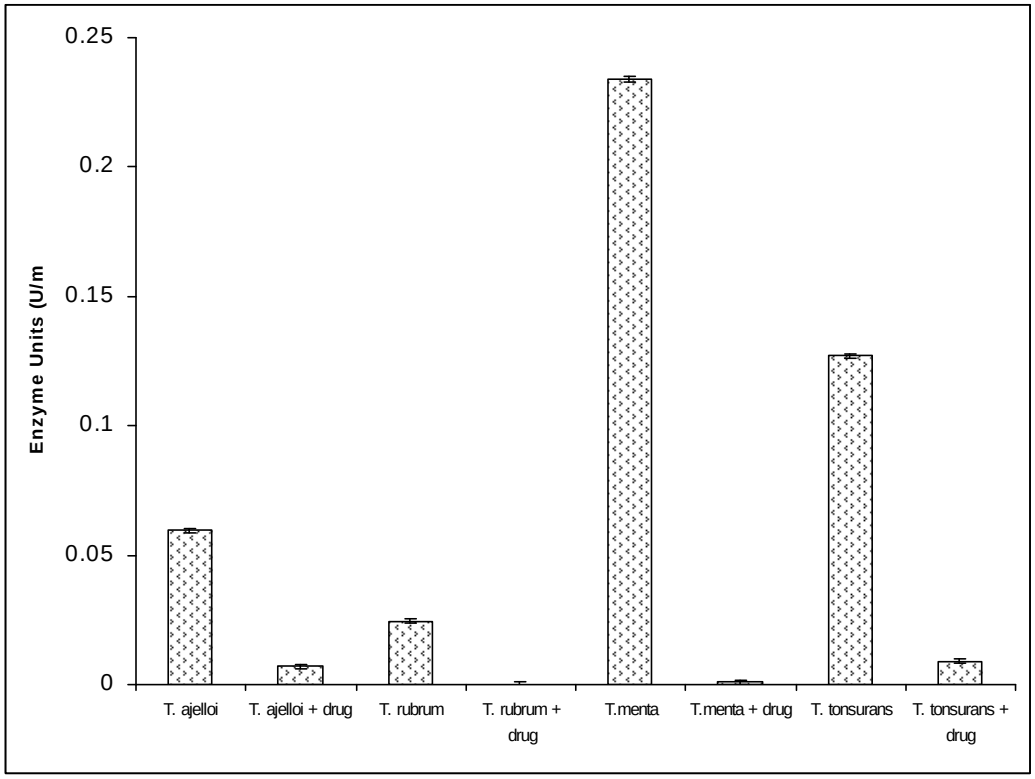

Figure 2: Effect of Active fraction of *Acorus calamus* on Acid Protease of dermatophytes.

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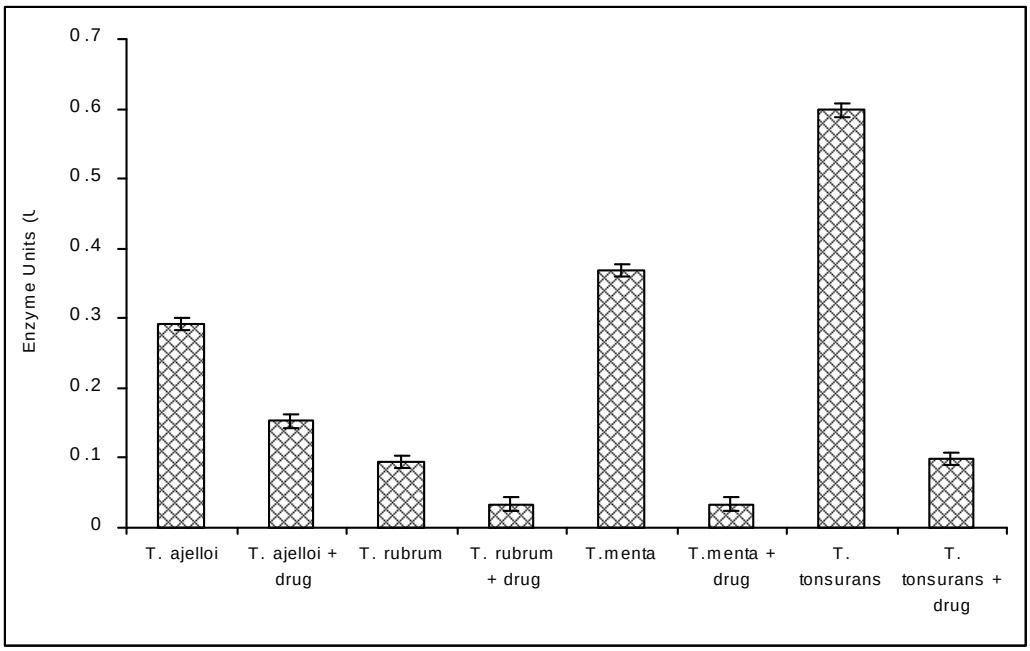

Figure 3: Effect of Active fraction of *Acorus calamus* on Alkaline Protease of dermatophytes.

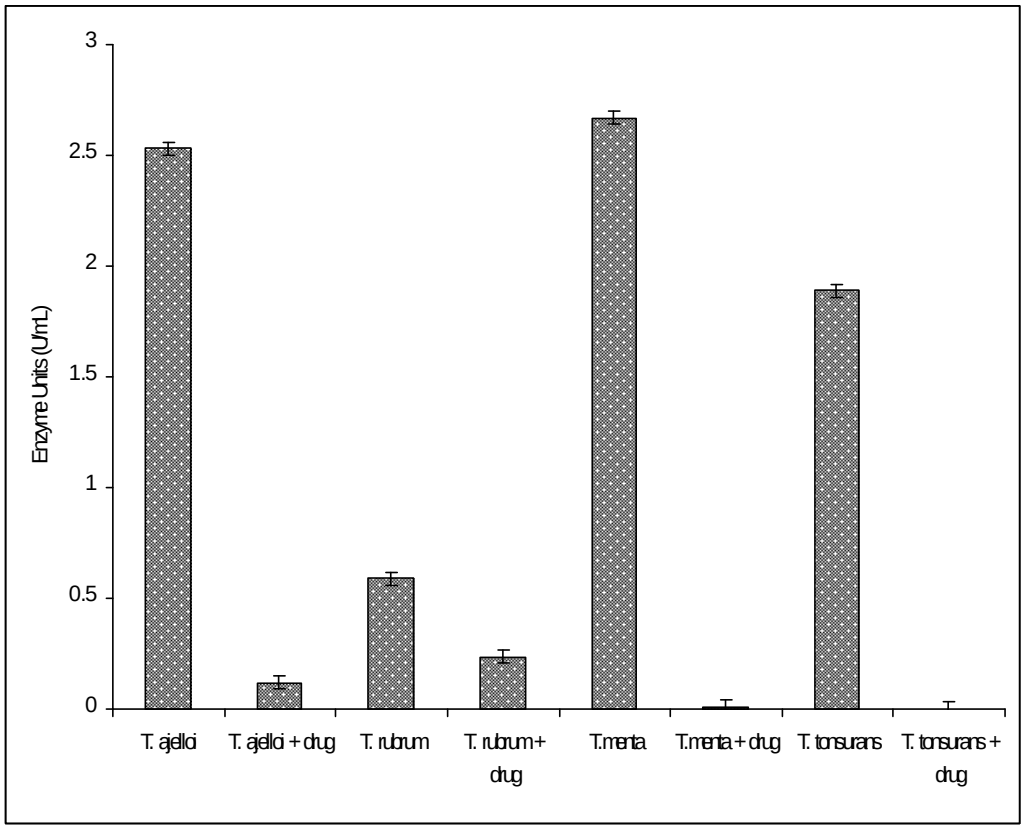

Figure 4: Effect of Active fraction of *Acorus calamus* on Keratinase of dermatophytes.

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