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Effect of active fraction of methanolic extract of *Acorus calamus* on sterol metabolism of *Candida albicans*

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ABSTRACT
Objective: Due to resistance exhibited by *Candida albicans* towards the existing antifungal drugs, new drugs are needed. This study examined the potential of antifungal agents extracted from the plant *Acorus calamus.*
Methodology and Results: The methanolic extract of *Acorus calamus* was found to be most effective, hence by fractionation the active component (fraction 3) was separated and the efficiency studied by bioautography, germ tube assay and sterol profile. Germ tubes were absent in cultures treated with the active fraction. UV-visible spectrophotometric analysis revealed that the configuration of sterols varied with ergosterol biosynthesis being completely inhibited at the squalene level.
Conclusion and application of findings: The active fraction of methanolic extract of *Acorus calamus* effectively reduces ergosterol biosynthesis and thus prevents germ tube formation, finally causing cell death. Antibiotic resistance exhibited by *Candida albicans* could be effectively treated with the active fraction of *Acorus calamus*.

Key words: *Acorus calamus*, *Candida albicans*, sterol, ergosterol, germtube

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INTRODUCTION

Candida albicans is a dimorphic and opportunistic fungal pathogen that causes superficial or systemic infections in immuno-compromised individuals. The antifungal agents that are available for treating *C. albicans* infections can be categorized into several chemical classes with different cellular targets. Among these, polyenes and azoles target the ergosterol biosynthesis of fungal species (Navarro–Martinez *et al.,* 2006; Sanglard *et al.,* 2003).

Ergosterol is an essential component of yeast plasma membrane and plays an important role in fluidity, permeability and the activity of membrane- bound enzymes. Ergosterol is also a major component of secretory vesicles and has an important role in mitochondrial respiration. Genes in the ergosterol pathway exhibit transcriptional regulation in response to mutations in other ERG genes and resulting sterol limitation. In yeast cells, depletion of ergosterol with concomitant accumulation of sterol

intermediates can result in alterations in membrane functions, synthesis, activity of membrane-bound enzymes, mitochondrial activities, and other functions (Shimokawa & Nakayama, 1992).

Different classes of antifungal drugs target the ergosterol biosynthesis pathway. Among these, polyenes such as Amphotericin B act by binding tightly to the ergosterol molecule and damage cell plasma membrane, thus resulting in leakage of intracellular ions. Azoles such as fluconazole, itraconazole, or voriconazole inhibit a cytochrome P450 (Erg11p) that is responsible for 14α demethylation of lanosterol and thus block ergosterol biosynthesis (Sanglard *et al.,* 2003; Gray *et al.,* 1998). Other antifungals with less relevance for treatment of *C. albicans* infections include allylamines (terbinafine) and morpholines (amorolfine). These inhibit ergosterol biosynthesis by blocking activity of squalene epoxidase and sterol α 14-reductase or 7-8-isomerase, respectively (Sanglard *et al.,* 1996; Kelly *et al.,* 1995, 1996, 1997).

Drug resistance exhibited by *Candida* species is a common challenge to clinicians. The azole resistance in *Candida albicans* is mainly due to: (i) active efflux of the antifungals, (ii) alterations of the target enzyme or (iii) the absence of the target enzyme (for Amphotericin B resistance) (Sanglard *et al.,* 1996 & 1997). Drug resistance in *Candida* is possible either by point mutation or over-expression of the genes that encode for drug efflux pumps belonging to the ATP- binding cassette (ABC) super family namely, CDR, CDR2 and transporters belonging to the major facilitator super family (MFS), namely MDRI (Lee *et al.,* 2004).

In addition, increased incidence of invasive mycoses and the emerging problem of antifungal drug resistance create the need for new antifungal drugs, which could circumvent drug resistance mechanisms. This study aimed to assess the efficacy of the active fraction of extracts from indigenous medicinal plants in combating *Candida albicans* infections, and additionally to evaluate the mechanisms of action of the active fraction.

MATERIALS AND METHODS

Plant extracts: *Acorus calamus* was obtained from the medicinal farms of Salem District, Tamil Nadu, India. The plant was identified by P. Jayaraman, Director, Plant Anatomy Research Centre and voucher specimen was deposited in Plant Anatomy Research Centre, Chennai, India. The rhizomes were dried under shade before grinding to coarse particles. The rhizome powders were sequentially extracted with solvents from non-polar to polar, i.e. hexane, ethyl acetate, methanol and water. The solvent free extracts obtained after distillation was subjected to column chromatography using Hexane: Ethyl acetate (9:1). About five fractions were obtained and tested for anticandidal activity, using clinical as well as type strains.

Organisms: Clinical isolates used in the study were *Candida albicans* (CLCA 0591, from a denture stomatitis patient, *SRMC*, Chennai, India) and type strain MTCC 7315 (IMTECH, Chandigarh). The cultures were maintained in Sabouraud's Dextrose Agar slants at 37°C. A single colony taken from the slant was inoculated on Sabouraud's Dextrose Broth and incubated for 24 h before using as inoculum for subsequent experiments.

Bioautography: The anti-candidal activity of fractions (I to V) was evaluated by bioautography technique. In brief, the five fractions were chromatographed using Thin Layer Chromatography. The solvent free TLC chromatogram sheets were overlaid with soft agar with a suspension of *Candida*. The plate was incubated for 48h at 37ºC and then it was

sprayed with MTT (3-(4,5-dimethyl;-2-thio azolyl) –2,5,- diphenyl- 2H – tetrazolium bromide) (2mg/ml) ,and incubated for another 1 h. A zone of inhibition appearing halo yellow was observed where there was activity.

Germ tube assays: To assess the effect of the active fraction on cell wall of *Candida albicans*, germ tube formation studies were carried out. Briefly, blastoconidia were incubated at 37°C in 10% New Born Calf Serum (NBCS) in phosphate-buffered saline for 45 min. to induce germ tube formation and then incubated at 37° C with 0.1µg/mL of active fraction for 15 min. Subsequently, the drug was removed using water and the blastospores were re-incubated at 37°C in 10% NBCS for an additional 60 min, and viewed under light microscope (Brayman & Wilks 2003).

To quantify germ tube formation the cells of *Candida* sp. were washed once with 70% ethanol and then with 200µl of 0.25% Sodium Dodecyl Sulfate (SDS). The plates were then washed twice with distilled water. Germ tubes attached to plates were stained with 100µl of 0.02% filter sterilized crystal violet dissolved in phosphate-buffered saline for 15 min. The dye solution was removed carefully and plates were washed three times with water, once with 0.25% SDS, and twice more with water. After the plates were dried, 200µl of isopropanol containing 0.04 N HCl and 50µl of 0.25% SDS were added to plates and mixed briefly at 160 rpm, and read at 590 nm in spectrophotometer (Cary 100 UV – Visible spectrophotometer) (Brayman & Wilks 2003).

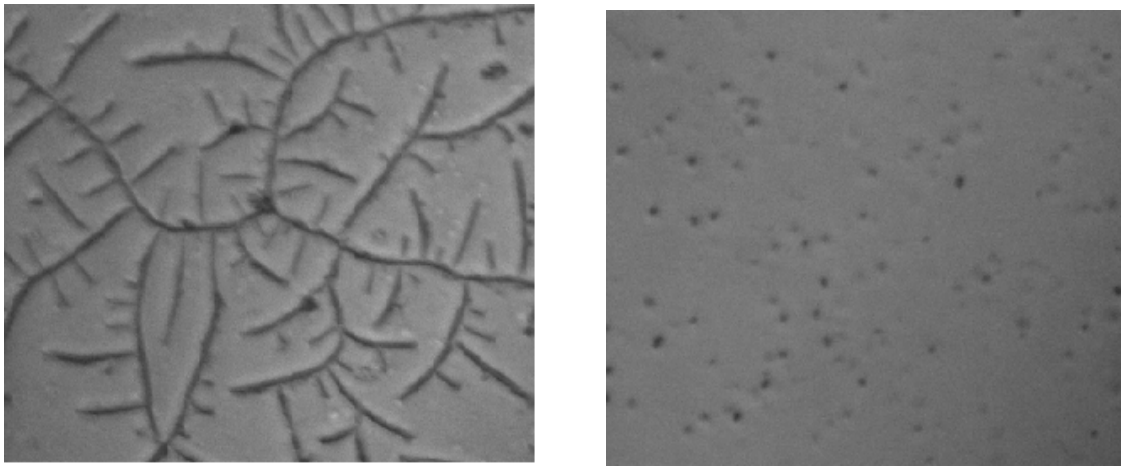

Figure 1: Effect of active fraction of *Acorus calamus* on germ tube formation of *Candida albicans*. On the left is control (untreated) and on the right is the treated fungus.

Sterol extraction: Briefly, 3ml of 25% alcoholic KOH was added to 0.2g (wet weight) of yeast cells. The mixture was refluxed for 3 hr under nitrogen. The refluxed mixture was filtered, diluted with an equal amount of water and extracted four times with heptane. The extract was dried with anhydrous Na_2SO_4. The dried extract was dissolved in chloroform (10mg/ml) and stored under nitrogen at -20°C until analysis (Navarro-Martinez *et al.,* 2006).

Sterol content assay: Ergosterol content was calculated as a percentage of the wet weight of the cells as follows: % ergosterol + % 24(28)DHE = [(A281.5/290) X F]/pellet weight, % 24(28)DHE = [(A230/518) X F]/pellet weight, and % ergosterol = [%ergosterol + % 24(28)DHE]–% 24(28)DHE, where F is the factor for dilution in ethanol and 290 and 518 are the E values (in percentages per cm)

determined for crystalline ergosterol and 24(28)DHE, respectively.

Extraction of non saponifiable lipid: Extraction of non-saponifiable lipid was carried out at room temperature. Cells grown in SDA medium (both control as well as treated) were harvested by centrifugation at 1000g for 10min and washed twice with deionized water. Equal volume (1.5 ml) of water and Ethanolic KOH (15 % (W/V) KOH in 90% (V/V) ethanol) were added to each sample for saponification at 80°C for 1 h in the dark, and then samples were cooled on ice. The non-saponifiable lipids were extracted using petroleum ether (boiling point 40 – 60°C) and were dried under a stream of nitrogen. The sterol fractions were separated in a solvent system of n- heptane: di isopropylether: acetic acid (60:40:4) using Thin Layer Chromatogram procedure. The developed TLC plate was sprayed with a freshly prepared solution of 50 mg Ferric chloride ($FeCl_3$) in a mixture of 90 ml H_2O, 5ml Acetic acid and 5 ml sulfuric acid. After heating at 100°C for 3-5min, the sterol spots were identified by the formation of red – violet color.

RESULTS AND DISCUSSION

Sterol plays a major role in the building and maintenance of eukaryotic cell membranes, regulating both membrane fluidity and permeability, and the cell cycle aerobic metabolism. Ergosterol has a concentration dependent role; at lower concentration it initiates growth while in higher concentration it fulfills another role in maintenance of the membrane (Arthington-Skaggs *et al* ., 2000).

Membrane sterols have diverse functions and their regulation is directly related to cell survival; if etherification of sterol is blocked, intermediate sterol may accumulate in the cells, which would possibly be toxic to cells. Ergosterol is an important constituent of the cell wall, and is a major factor in stability of the cell wall. Damage to the cell wall could certainly affect the ergosterol content and promote leakage which would finally lead to cell lysis (Sanglard *et al* 1996, 1997, 2003).

In our study, the anticandidal efficacy of the active fraction of *Acorus calamus* was assessed and the mechanism of cell lysis evaluated. The results show that the selected plant and its separated fractions affect ergosterol biosynthesis considerably. Initial bioautography studies revealed that only fraction 3 has anticandidal activity.

Results also revealed that the active fraction is able to completely inhibit germ tube formation (Figure 1). Under experimental conditions the control (untreated) exhibited luxuriant growth of germ tube, which was prevented by treatments with the active fraction. Based on these observations, we speculate that the active fraction inhibits glucan synthetase, which is essential for germ tube formation (Sobel *et al.,* 1984; Brayman & Wilks 2003). The results were quantified and further confirmed with crystal violet staining (Table 1).

Table 1: Germ tube assay of *Candida albicans* treated with active fraction of *Acorus calamus* with reference to Crystal Violet staining.

Sl No.	Strains	Control (OD at 590nm)	Treated (OD at 590nm)***
1	*Candida albicans* (Type strain)	2.0325	0.0135
2	*Candida albicans* (Clinical strain)	2.0156	0.0123

*** (P<0.001)

The role of the active fraction in combating candidal activity was confined to the cell membrane, with the major target site being ergosterol. The sterol content of the treated cells was significantly ($P \leq 0.001$) reduced to 81% when compared to the control.

Spectroscopic analysis of the obtained sterol showed 4 absorption maxima at 262, 271, 281 and 293. Similar observations were made by Lu *et al.,* (1999). The results were authenticated by an analysis of pure ergosterol procured from Sigma, India. However, the sterols of treated cells exhibited absorption maxima at 253 and 302 (Figure 2), which clearly reveals changes in structural conformity of ergosterol. Further circular dichroism (CD) analysis of sterol in the control experiments at open circuit showed three characteristic peaks at Δε (-0.75) 261nm, (-0.25) 293 nm and (-0.25) 300 nm, which correspond to UV absorption peaks at 271, 281 and 293 nm (Figure 3). These values coincided with the values of the authentic ergosterol samples (Lu *et al*., 1999).

Analysis of non-saponifiable lipids in the untreated candidal cells exhibited a wine red spot in the control, which was absent in the treated cells. The results clearly indicate that the active fraction affects sterol metabolism and changes its structural conformation. Figure 4 illustrates the action of different drugs in the biosynthesis of ergosterol. The exact stage at which the active fraction of *Acorus calamus* affect ergosterol biosynthesis could be predicted from the results obtained form GLC analysis (data not shown). The active fraction strongly prevented the formation of squalene epoxide and thus completely affected ergosterol synthesis, leading to fungal cell lyses.

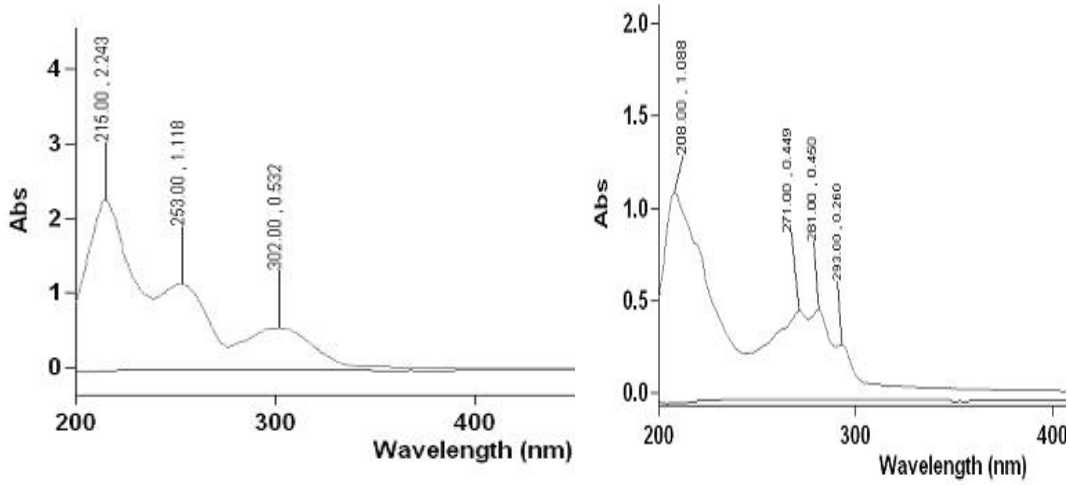

Figure 2: UV- visible spectrum of sterols obtained from the cells treated with the active fraction of *Acorus calamus* (left); right is the untreated control.

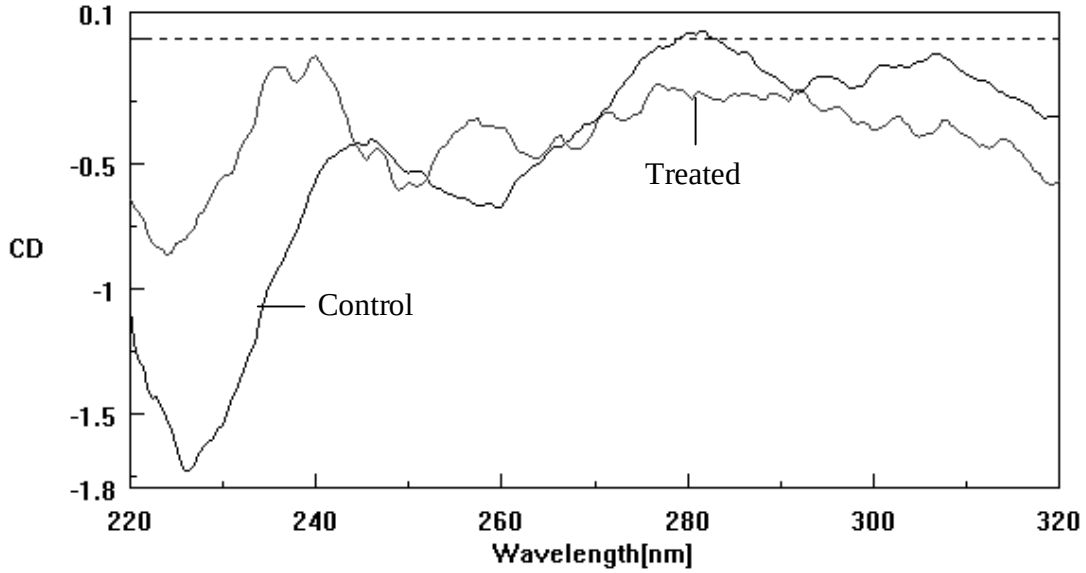

Figure 3: Circular Dichoroism analysis of sterols obtained from the cells treated with the active fraction of *Acorus calamus*.

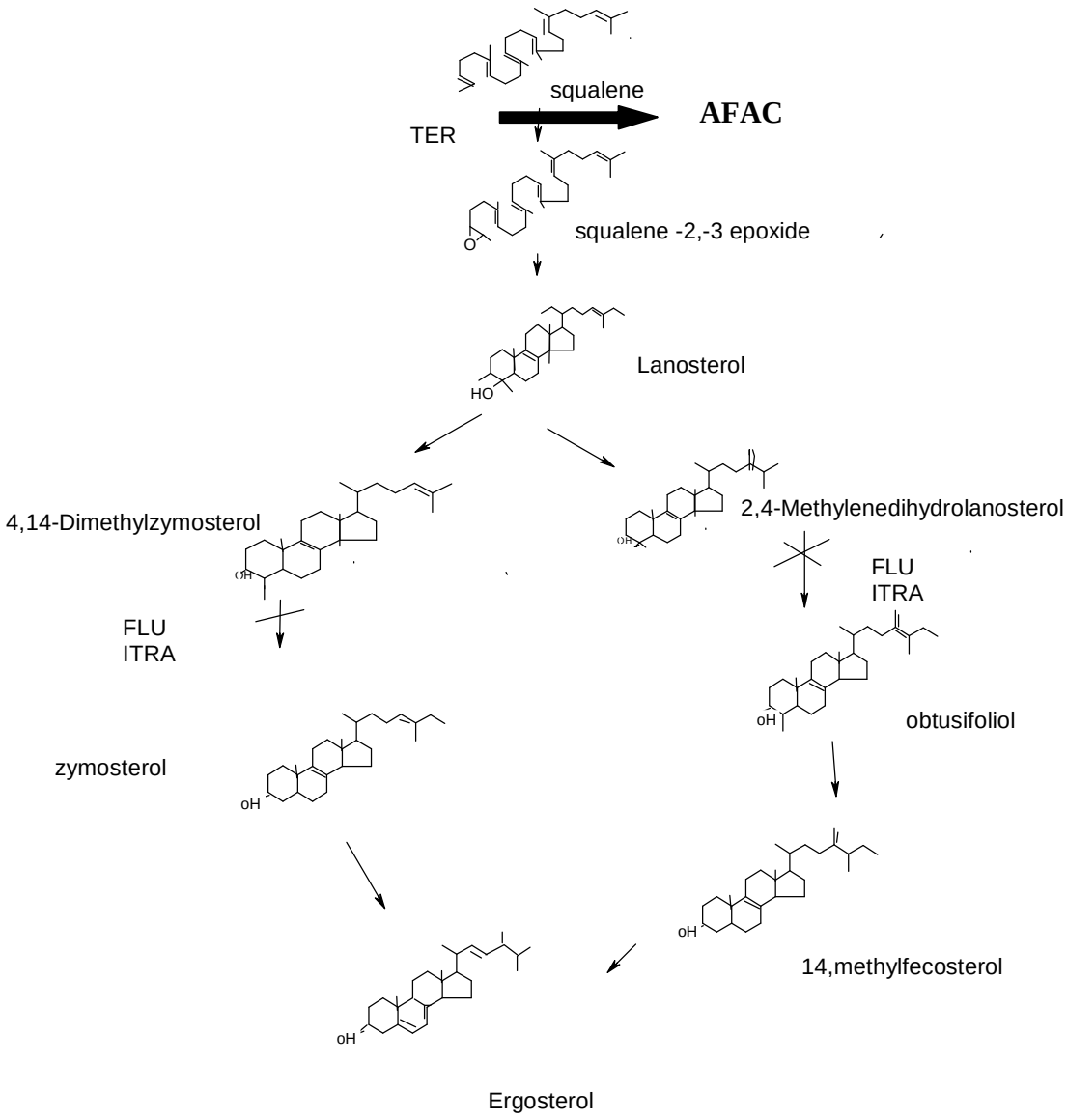

Figure 4: Predicted Ergosterol biosynthesis pathway exhibiting the involvement of active fraction of *Acorus calamus*. The steps of inhibition by various other standard drugs at various levels of ergosterol synthesis are indicated: FLU = Flucanazole ; ITRA = Itraconazole ; TER = Terbinafine AFAC = Active fraction of *Acorus calamus*.

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