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Role of TMPMP and TMAP on virulence factors of *C. albicans*

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Abstract

During last decade, human fungal diseases gained importance as few pathogens cause life-threatening infections and *Candida albicans* is one among them. The recent antifungal resistance exhibited by C.albicans and other related fungi demand new drugs. Since natural products are potent source of antimicrobial compounds, an attempt was made using two terpenoids, viz., 1-2, 4, 5- Tri Methoxy Phenyl -1'- Methoxy Propionaldehyde (TMPMP) and 1-2, 4, 5- Tri Methoxy Acetophenone (TMAP), extracted from traditional Indian medicinal plant, Acorus calamus for its antifungal efficacy. The present study demonstrates the efficacy of these two terpenoids by exploring their effect on the virulence factors of the chosen organism. Results reveal the major virulence factors, namely, acidic, alkaline and neutral proteases, secreted aspartyl protease, lipase, phospholipase, elastase and keratinase activities have been completely reduced in the presence of TMPMP and TMAP compared to control. The reduction in virulence factors was on par with standard antifungal drug Fluconazole.

Keywords: *Candida albicans*; Virulence factors; Acorus calamus; Terpenoids; Antifungaldrugs

1. Introduction

Candida albicans, an opportunistic fungal pathogen can cause either systemic or mucosal infections in humans. In immunocompromised patients, *Candida* infections may lead to life-threatening circumstances [1, 2]. Recent reports claim that *C. albicans* accounts for 72% of nosocomial fungal infections and have surpassed Gram negative bacteria and as third most frequently isolated organism from bloodstream infections [3].

Increased incidence of invasive mycoses and emerging problems of antifungal drug resistance have encouraged the search for new antifungal drugs. Present day therapeutic options are limited to three major classes of compounds namely, polyenes, azoles, and candins [4, 5]. Utility of polyenes are limited by their nephrotoxicity [6]. Though azoles are relatively safer, the cross-resistance exhibited by fungal species restricts their usage. Later, during 2001, echinocandins - a lipopeptide with high fungicidal activity and negligible toxicities was introduced. Within four years, *in vitro* echinocandin resistance, along with cross-resistance to other echinocandins, is reported by Moudgal *et al.,* [7]. Ghannoum *et al.,* [8] reported during the past decade considerable increase in the emergence of antifungal resistance had implication on morbidity, mortality, and health care, and thus demands new, safe, and more efficacious antifungal drugs.

Despite the existing synthetic drugs, natural products have always been a choice for potent antimicrobial source. Antifungal efficacy of plant derived antimicrobial compounds is reported by number of researchers [9-14] and the antifungal efficacy of terpenoids is reported by [15]. In our preliminary studies we found, methanolic extracts and unidentified active fractions exhibited antidermatophytic as well as anticandidal properties [16, 17]. In the present study, an attempt was made on the evaluation of anticandidal efficacy of novel terpenoids 1-2, 4, 5- Trimethoxy phenyl -1'- methoxy propionaldehyde (TMPMP) and 1-2, 4, 5- Trimethoxy acetophenone (TMAP) isolated from rhizomes of *A.calamus L* (The extraction procedures and the structural elucidation based on physico-chemical characterization of these two compounds were submitted for filing as CSIR - Indian Patent). In brief, the present study emphasizes, anticandidal efficacy of TMPMP and TMAP, and their impact on virulence factors of *Candida spp* including, acidic protease, neutral protease, alkaline protease, secreted aspartyl protease, lipase, phospholipase, keratinase, elastase and the antioxidant enzyme catalase.

2. Experimental

2.1. Chemicals

Microbial growth media and supplements were purchased from Himedia, India. Chemicals for various buffers, reagents were purchased from SRL, India. All Solvents and acids were of AR grade purchased from Merck, India.

2.2. Test organisms

Antifungal efficacy of TMPMP and TMAP was evaluated using of 17 strains of *Candida* spp, including Type and clinical strains. Clinical isolates of *Candida* species were obtained from hospitals and characterized by growth in selective media, germ tube, and gram staining techniques [16, 17]. For clarity of presentation results of *C.albicans* SRM0510 (clinical isolate) was alone discussed in detail.

2.3. Determination of Minimum Inhibitory Concentration of TMPMP and TMAP

Minimum Inhibitory Concentration (MIC) of TMPMP and TMAP for *Candida spp.* was determined by broth microdilution technique M27-A as recommended by the NCCLS.

2.4. Effect of drugs on virulence factors and enzymes

Effect of TMPMP and TMAP on virulence factors of C.*albicans* were evaluated at their sub-lethal concentration.

2.5. Acidic Protease Assay (ACP)

Acidic Protease activity of *C.albicans* of control and drug treated experiments was carried out using the protocols of Nelson and Young [18]. The assay mixture consists of 500 µl of 0.1M citrate buffer, 400 µl hemoglobin, and 100 µl of sample. Mixture was incubated at 37ºC for 30 minutes subsequently; 1ml of TCA (Trichloroaceticacid) was added and centrifuged at 5000 RPM for 5 minutes. The supernatant was then read at 280 nm for determining acidic protease.

2.6. Neutral Protease Assay (NuP)

Neutral Protease activity of *C.albicans* of control and drug treated experiments was carried out using the protocols of Kray and Wildi, [19]. Assay mixture consists of 300 µl of phosphate buffer, 500 µl of casein, 200 µl of sample; mixture was incubated at 60ºC for 10 minutes. After incubation, 1 ml of TCA was added and centrifuged at 5000 RPM for 15 minutes. Supernatant was then estimated for protein [20].

2.7. Alkaline Protease Assay (Alp)

Alkaline Protease activity of *C.albicans* of control and drug treated experiments was carried out using the protocols of Gupta et al [21]. Assay mixture consists of 500 µl of casein and 500 µl of sample; mixture was then heated at 60ºC for 20 minutes, 1 ml of TCA was added and centrifuged at 5000 RPM for 15 minutes. The supernatant was then estimated for protein content [20].

2.8. Secreted Aspartyl Protease (SAP)

Secreted Aspartyl Protease activity of *C.albicans* of control and drug treated experiments was carried out using the protocols of Mac Donalds and Odds [22]. The assay mixture consists of BSA (2%) in 50 mM sodium citrate buffer, 200 µl of sample; mixture was then incubated at 37ºC for 30 minutes. After incubation, 200 µl of 2 M perchloric acid was added and incubated in ice for 15 minutes, centrifuged at 14000 RPM for 15 minutes. The culture supernatant was then read at 280 nm.

$$\text{Enzyme activity} = \frac{\text{(Test OD} - \text{Control OD} \times 1000)}{\text{(Volume of enzyme} \times \text{incubation time} \times \text{Mol.wt of Tyrosine)}}$$

(1 unit of enzyme activity was defined as an amount of enzyme that liberates 1 µm tyrosine from substrate BSA/ml of enzyme under assay condition).

2.9. Lipase assay

Lipase activity of *C.albicans* of control and drug treated experiments was carried out using the protocols of Xu et al [23]. Assay mixture consists of 2 ml of 3% polyvinyl alcohol (PVA)/olive oil (3:1) and 0.025 M phosphate buffer (pH 7.5) in 200 µl of sample, incubated for 15 minutes at 40ºC. The reaction was stopped by the addition of 7.5 ml 95% ethanol. Fatty acid liberated was titrated with standard 0.05 M NaOH in the presence of phenolphthalein as indicator. One unit of lipase activity was defined as the amount of enzyme that produced 1 µmole of fatty acid per minute under the assay conditions described.

Lipase activity
= (Volume of NaOH consumed × Normality of NaOH) / Time of incubation

Volume of NaOH consumed = Difference (Test-Control)

2.10. Phospholipase assay

Phospholipase activity of *C.albicans* of control and drug treated experiments was carried

out using the protocols of Leidich et al [24]. Briefly, assay mixture consists of 1ml of sample, 1 ml of 60% perchloroacetic acid and heated in a sand bath at 200ºC until the solution turns colourless. Subsequently, one ml of 60% perchloroacetic acid was added and the final volume was made to 9.1 ml using distilled water, to this 0.5 ml of molybdate II reagent and 0.4 ml ANSA reagent mixture of was added. The tubes were shaken well and heated in a boiling water bath for 8 minutes exactly. The blue colour developed was read spectrophotometerically at 680 nm.

2.11. Keratinase Assay

Keratinase activity of *C.albicans* of control and drug treated experiments was carried out using the protocols of Brandelli [25]. The assay mixture consists of 100 µl of sample, 800 µl of Tris and 200 µl of azokeratin. The reaction mixture was incubated at 40ºC for 15 minutes in water bath and subsequently 1 ml of TCA was added. In case of control, TCA (1 ml) was added prior to incubation. The assay mixtures were then centrifuged at 5000 RPM for 15 minutes. To the supernatant, 5 ml of sodium carbonate was added, incubated for 10 minutes. Subsequently, 1 ml of Folin's reagent was added, incubated for 30 - 60 minutes, and measured the optical density at 660 nm.

2.12. Elastase activity

Elastase activity of *C.albicans* of control and drug treated experiments was carried out using the protocols of Mc Iver et al [26]. Briefly, 200 µl of substrate (SANA- N-succ-(Ala)3-p-nitroanilide) in 1.2 ml of 1 mM Tris buffer (pH 8) was added to 100 µl of sample. The optical density was measured at 410 nm. In control experiments distilled water was added instead of sample.

2.13. Catalase activity

Catalase activity of *C.albicans* of control and drug treated experiments was carried out using the protocols of Aebi et al [27]. Briefly, 0.56 µl of hydrogen peroxide (AR grade, SRL, India) was added to 10 ml of 0.1 M Potassium phosphate buffer. To 1ml of the above-mentioned hydrogen peroxide-buffer solution, 2ml of distilled water, and 100 µl of sample were added.

$$\text{Enzyme unit/ml} = \frac{\text{Absorbance at 240 nm} \times 100}{43.6 \times \text{ml of enzyme} \times \text{ml of the reaction mixture}}$$

2.14. Statistical Analysis

All experiments were carried out in triplicates. Results were carefully analyzed and statistically defined using ANOVA.

3. Results and Discussion

In our preliminary studies we reported, the crude methanolic extracts of *A.calamus* rhizome and the unidentified active fractions exhibited antidermatophytic as well as anticandidal properties [16,17]. From our continuous studies, we received two novel terpenoids of *A.calamus*. These two terpenoids are not reported elsewhere and the patent protection was made accordingly. The present study emphasizes the antifungal efficacy of those two novel terpenoids and further demonstrates the mode of action of these two drugs by assessing their effect on virulence factors of the chosen pathogen. Both clinical and type strains of *C.albicans* (9 strains), *C. tropicalis* (3 strains), *C. dublinensis* (1 strains), C. *sake* (2 strains), C. *boidnii* (2 strains) were chosen for the present study. However for better understanding, results of *C.albicans* SRM 0510, alone was presented and discussed. The antifungal efficacy of acidic terpenoids extracted from natural sources, reported by Onishi *et al* [28] and Inouye *et al* [15] reported the antidermatophytic efficacy.

3.1. Antifungal efficacy of TMPMP and TMAP

Minimum Inhibitory Concentration (MIC) of the chosen drugs for *C.albicans* SRM 5210, was the lowest drug concentration required to cause a prominent reduction in growth. MIC of TMPMP and TMAP was identified as 1.0 and 1.5 µg/ml respectively. Similar to our findings, Onishi et al [28] reported new MIC in the range of 0.25 to 1 µg/ml for acidic terpenoids extracted from natural sources. The MIC of standard drug was estimated as 0.7 µg/ml.

3.2. Virulence factors

Pathogenicity greatly depends on complex array of microorganism-related putative virulence factors [29, 30]. *C.albicans* expresses several virulence factors contributing to their pathogenicity, which includes, yeast – to – mycelium transition, antigenic variability, phenotypic switching, adhesion to host cells surface hydrophobicity, molecular mimicry and production of extracellular enzymes. Most of the biological functions related to pathogenesity and virulence resides in fungal cell wall [29, 30]. Expression of virulence factors may be associated with specific characteristics of *Candida* isolates. The role of virulence factors in establishing the infections was vital in disease manifestations. Figure 1 illustrates the overall

scenario of the role of virulence factors during different stages of infection caused by *Candida* spp.

The effect of TMPMP and TMAP on the virulence factors of *C.albicans* SRM 0510 was evaluated at their respective sub-lethal levels. It has been evidenced from the experimental results that *C.albicans* produce enzymes responsible for their virulence within a limited time of 48 hours. Secreted aspartyl proteases, in general, are capable of degrading epithelial and mucosal barrier proteins such as collagen, keratin and mucin, cloning and disruption of genes for these enzymes have shown their involvement in virulence of *Candida* species [31-33, 24, 34]. About 35 and 45% reduction in SAP activity were observed upon treatment with TMPMP and TMAP respectively (in comparison with control as 100%) (Figure 2a). Similarly, neutral, acidic and alkaline proteases are pivotal for establishing infections, treating *C.albicans* with TMPMP and TMAP exhibited reduced level (50%) of expression of said enzymes. Morschhauser *et al* [35] reported that acid proteases might be of importance in *C.albicans* penetration into deep organs. About 0.2 enzymes units/ml of acid and neutral proteases was observed in control cells and upon treatment 50% reduction in activity in TMPMP and TMAP treated cells respectively was observed (Figure 2a). With regard to alkaline proteases, control cells showed 0.6 units/ml; whereas 15 and 11% reduction in activity were observed with TMPMP and TMAP treated cells respectively (Figure 2a). The values observed were on par with the results obtained with standard drug.

Since skin is composed primarily of lipids, lipases and phospholipase are prime virulence factors of skin colonising pathogens. Phospholipases are the most likely to contribute to pathogenicity of *Candida* species, by damaging host cell membranes, and thereby facilitates invasion into host tissues [32]. In control cells phospholipases content was 0.4 units/ml and upon treatment with TMPMP and TMAP, the content reduced by 75%. Similarly, lipases activity was 0.4 units/ml in control cells and on treating with TMPMP and TMAP, the reduction observed was 75% (Figure 2b). In addition, keratinase and elastase are the two important virulence factors required for the colonization of skin. In *C. albicans* treated with TMAMP and TMAP, keratinase activity was reduced by 35-46% and 28% reduction in elastase activity (Figure 2c). Results on reduction in enzyme activities were on par with the standard drug. An overview of effect of TMPMP and TMAP on the virulence factors suggests, there was a reduction of enzymatic virulence activity.

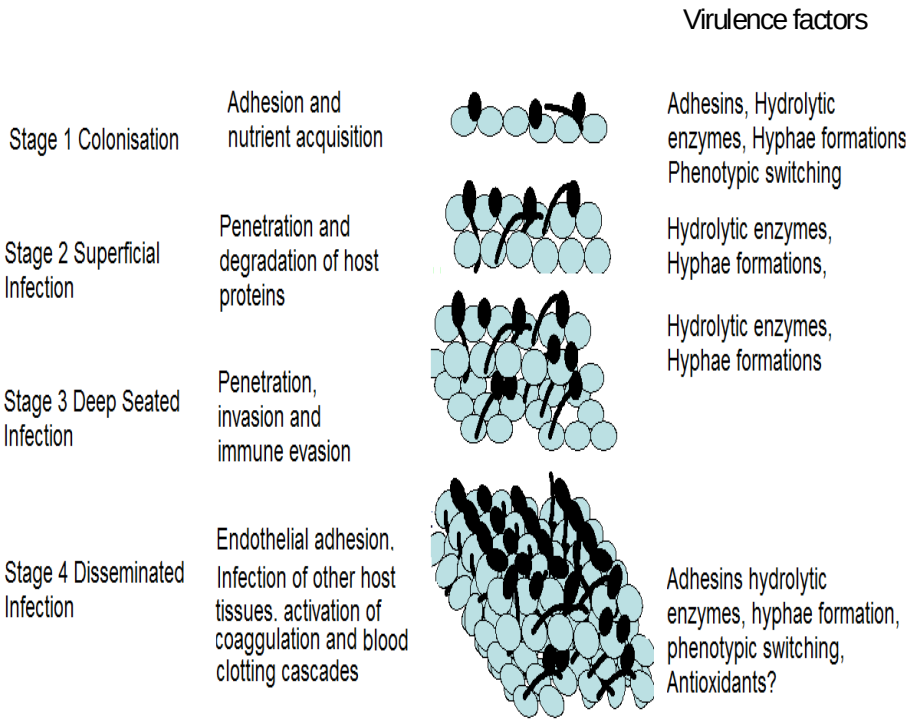

Figure 1. Role of virulence factors in pathogenesis of Candida spp.

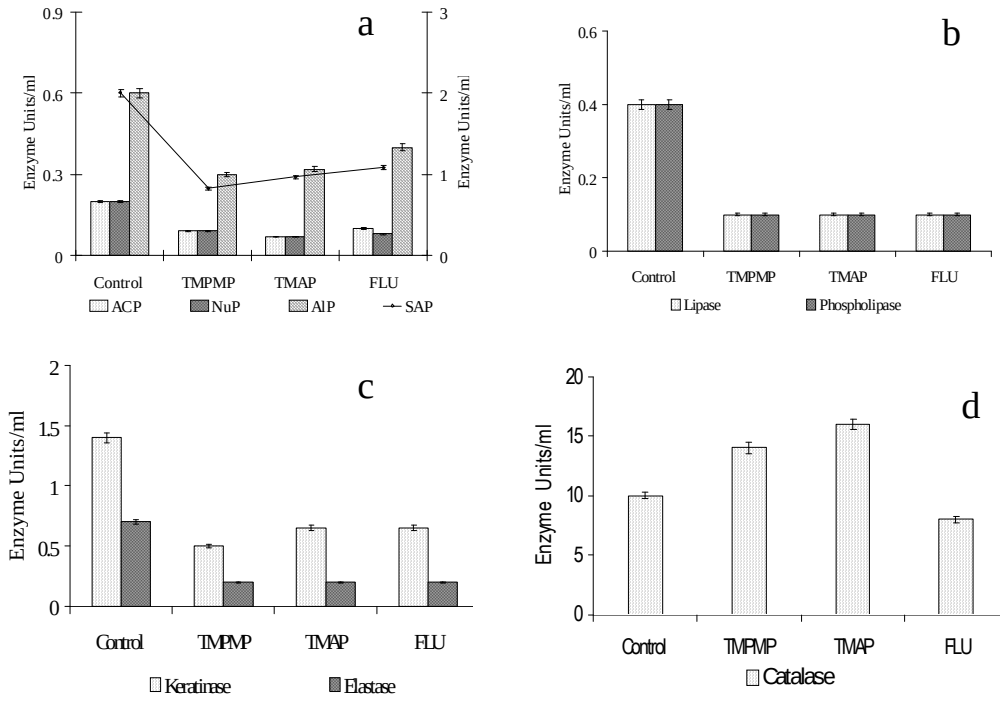

Figure 2. Effect of TMPMP and TMAP on Virulence factors of *C.albicans* SRM0510(**2a**) Effect of TMPMP and TMAP on acid, alkaline, neutral and secreted aspartyl proteases of *C.albicans* SRM0510(**2b**) Effect of TMPMP and TMAP on lipase and phospholipase of *C.albicans* SRM0510(**2c**) Effect of TMPMP and TMAP on Elastase and Keratinase of *C.albicans* SRM0510(**2d**) Effect of TMPMP and TMAP on catalase of *C.albicans* SRM0510.

As far as the antioxidant enzyme catalase was concerned, in contradiction to enzymatic virulence factor content, in the present study, there was an increase in the expression of catalase in *C.albicans* treated with TMPMP and TMAP. Catalase content in control experiments was 10 units/ml, whereas an increase of 140, 160% was observed in TMPMP, TMAP treated cells (Figure 2d). Miyasaka *et al* [36] reported that extracellular catalase activity could be detected in yeast in oxidative stress conditions. Further, various authors also report that the effects of stresses on *Candida* spp are transduced through a variety of membrane receptors and elicit responses through conserved signaling pathways, of which most important being the MAP kinase (MAPK) signal transduction network [37]. Two important components of the MAPK network are high-osmolarity glycerol (HOG; Hog1p) pathway and protein kinase C cell wall integrity (Mkc1p) pathway, which respond to oxidative, osmotic and heavy metal stress [38,39]. The response to oxidative stress transduced via both Mkc1p and Hog1p and involved in the increased expression of antioxidants such as catalase, superoxide dismutase and glutathione [40].

4. Conclusions

Invitro antifungal efficacy of TMPMP and TMAP was evaluated using *C.albicans SRM0510*. The antifungal efficacy of TMPMP and TMAP was comparable with that of standard drugs. Studies on the virulence factors showed a reduced extracellular enzyme production upon treatment with TMPMP and TMAP. While in the case of antioxidant enzymes there was an enhanced production. It is evident from the studies that the active components TMPMP and TMAP can combat fungal infections in all stages of infections, as they were pleomorphic in function.

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