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An antifungal mechanism of semi purified fraction of *Hypnea musciformis* lies in membrane-targeted action within *Candida Albicans*

***R. Mary Santhi and S. Thampi Raj**

*Department of Botany and Research Centre, Scott Christian College (Autonomous), Nagercoil - 629 003, Tamil Nadu, India.
Email-rms.santhi@gmail.com
Assistant Professor, Department of Botany, Scott Christian College (Autonomous), Nagercoil – 629 003, Tamil Nadu, India Email: minuthampi17@gmail.com

ABSTRACT

This study analyzes the antifungal properties of semi purified fraction of *Hypnea musciformis* and elucidates its mode of action against pathogenic fungi. Although antifungal effect of *Hypnea musciformism* has been reported, this is the first study for its mode of action underlying disruption of plasma membrane in *Candida albicans*. Semi purified fraction of *Hypnea musciformis* exerts activity against *C. albicans* by directly killing cells and causing others to undergo programmed cell death (apoptosis). Apoptosis is initiated at subinhibitory concentrations, suggesting that strategies to target this process may augment the benefits of antifungal agents.

KEY WORDS: semi purified fraction, *Hypnea musciformis*, *Candida albicans*, apoptosis, antifungal agents

***Corresponding author:**

R. Mary Santhi

R. Mary Santhi, Department of Botany and Research Centre,
Scott Christian College (Autonomous)
Nagercoil - 629 003,
Tamil Nadu, India.
Email-rms.santhi@gmail.com

INTRODUCTION

Candida albicans, the most prevalent human fungal opportunistic pathogen, can cause systemic and mucosal infectious diseases (Berman, 2006). Through a breakdown of host defenses, the organism can cause diseases ranging from superficial skin or mucous membrane infections like thrush and vaginal candidiasis, to the systemic involvement of multiple organs, primarily in patients with compromised immunity (Bergsson *et al.*, 2001). A noteworthy feature of the biology of *C. albicans* is its ability to grow as yeast, pseudohyphal and hyphal forms depending on the conditions (Wightman *et al.*, 2004). The hyphal form plays an important role in the cause of disease by invading epithelial cells and causing tissue damage and the pseudohyphae help *C. albicans* invade deeper tissues after it colonizes the epithelium (Pawaiya *et al.*, 2011).

Infections have become the leading cause of death worldwide which has led to an increase in antibacterial resistance, making it a global growing problem. Thus, there is an urgent need to discover new antimicrobial compounds from plants with diverse chemical structures and novel mechanisms of action for new and reemerging infectious diseases. The new therapeutic agents should be effective and have a novel mode of action that renders them impervious to existing resistance mechanisms (Westh *et al.*, 2004). The revolutionized therapy of infectious diseases by the use of antimicrobial drugs has certain limitations due to changing patterns of resistance in pathogens and side effects they produced. These limitations demand for improved pharmacokinetic properties which necessitate the continued research of new antifungal compounds for the development of drugs.

MATERIALS AND METHODS

Cleaning, drying and extraction

Samples of the selected seaweed were collected from coastal areas of Rameswaram. *Hypnea musciformis* was rinsed with fresh water to eliminate foreign materials such as sand and shells. The selected seaweed was air dried at room temperature 27±2^{o}C below 30^{o}C to avoid decomposition of thermolabile compounds. The dried algal material was stored in sealed containers in dry and cool place. The aim of grinding is to improve the efficiency of extraction by increasing the surface area of algal material, facilitating the penetration of solvent into the cells. Soxhlet apparatus is used in bulk extraction of algal metabolites. Finally reduced to thick oily natural crude extract in a rotary evaporator (Buchi) at 40^{o}C, collected in air tight plastic vials and stored in refrigerator for further studies.

Purification of bioactive compound(s) using silica gel column chromatography

The crude methanolic extract of *H. musciformis* (8 g) was subjected to bioassay guided silica gel column chromatography to separate the extract into its component fractions. The fractions (50 mL) were collected and subjected to TLC. The eluted fractions with distinct spots and same Rf value in TLC analysis were pooled for each solvent system and condensed using rotary evaporator (Buchi). A total of six fractions were obtained based on the TLC profile. In this assay the active fifth fraction was subjected to detailed studies.

Test pathogen

The test microbe used in the present study is medically important fungi: *Candida albicans* MTCC 277. The test microorganism were purchased from Microbial Type Culture Collection Centre (MTCC), IMTECH, Chandigarh, India. The test fungi were maintained on Potato Dextrose Agar (PDA) slants.

Time killing assay

Killing kinetics of the semi purified fraction against *C. albicans* was determined by diluting the SPF with RPMI medium to a final concentration of 0.5 of MIC, 1x and 2x MIC. *C. albicans* suspension was adjusted to 0.5 McFarland turbidity standard with cell density of 1x 10^6 to 5x 10^6 cells/mL. Hundred µL of adjusted yeast suspension was exposed to 900 µL of RPMI medium containing SPF, yielding a starting inoculum of approximately 10^5 cells/mL. The solutions were incubated at 37ºC with agitation. At the predetermined time interval of 0, 2, 4, 6, 12 and 24 h, 10 µL of the samples were removed, serially diluted with phosphate-buffered saline (PBS) plated on PDA plates, incubated for 24 h and the colony counts were determined after the incubation period. Controls for yeast growth and antifungal agents were also performed. The lower limit of accurate and reproducible quantitation was 100 CFU/mL. Results were obtained from three independent experiments (Lum *et al.*, 2015).

Biofilm reduction assay

Biofilms of *C. albicans* were formed on commercially available pre-sterilized, polystyrene, flat bottomed 96 well microtitre plates as described by Jin *et al.* (2004) with modification. Firstly, a standardized yeast suspension (10^7 cells/mL) of *C. albicans* was prepared by suspending colonies from 24 h old culture in RPMI 1640 medium and adjusted to an optical density of 0.38-0.39 at 520 nm. Hundred µL of yeast suspension was dispensed into each well of a microtitre plate using a multichannel pipette and the plates were incubated in a shaking incubator at 37ºC with 75 rpm for 90 min to allow adherence of yeast on the surface of each

well. After the adhesion phase, the non-adherent cells were removed and each well was washed twice with 150 µL PBS. Hundred µL of RPMI 1640 medium was transferred to each washed well and the plates were incubated at 37ºC in a shaking incubator at 75 rpm for 24 h to allow biofilm formation. Following biofilm phase, the medium was aspirated and each well was washed twice gently with 200 µL PBS to remove non-adherent cells. Residual PBS was removed by inverting the plates over an absorbent paper before addition of SPF. SPF (200 µL) with concentrations ranging from 0.5 of MIC, 1x and 2x times of the MIC determined previously was added to respective wells and the plates were incubated at 37ºC in a shaking incubator at 75 rpm for 24 h. Antifungal agent-free wells and biofilm-free wells were included as positive and negative controls. After treatment with SPF, the medium was removed and each well was washed twice with 200 µL PBS. The biofilm formation was quantified by using XTT reduction assay as described below. Each experiment was repeated three times in duplicate.

XTT reduction assay was performed according to the method adapted from Jin *et al.* (2004). Briefly, XTT (Sigma) was dissolved to a concentration of 1 mg/mL using sterile PBS. XTT solution was then filter-sterilized using a 0.22 mm-pore-size filter and kept at -70ºC prior to use. Menadione solution (0.4 mM) was prepared by dissolving menadione (Sigma) with acetone. The menadione solution was filter-sterilized and kept at -70ºC before use. Prior to each assay, XTT solution was thawed and mixed with menadione solution at a volume ratio of 20:1. After washing, a total volume of 200 µL of XTT-menadione mixture (158 µL of PBS, 40 µL of XTT and 2 µL of menadione) was added to each prewashed biofilm and control wells. The plates were incubated in the dark for 2 hours at 37ºC. Hundred µL of the solution was transferred to new wells and the colorimetric change in the solution was measured using a microtiter plate reader at 490 nm. Antibiofilm activity of the SPF and conventional antifungals was expressed as the biofilm-eradicating concentration 2 (BEC-2), which is defined as the minimum concentration of the SPF resulting in 50 % reduction of the biofilm viability compared to the growth control.

Annexin V and PI staining

C. albicans cells exposed to SPF were washed in phosphate-buffered saline (PBS) and incubated at 30°C for 10 min in 0.02 mg/mL Zymolyase 20T in 0.1 M potassium phosphate buffer (PPB: 0.5 mL of 50 mM K_2HPO_4, 5 mM EDTA, 50 mM dithiothreitol [DTT], 50 mM KH_2PO_4, 40 mM 2-mercaptoethanol) with sorbitol at a final concentration of 2.4 M and at pH 7.2 (Phillips *et al.*, 2003; Rieger *et al.*, 2010). Thereafter, 100 µL of permeabilization solution (0.1 M sodium citrate [pH 6.0] with 0.1% Triton X-100) was added to the washed protoplasts, which were placed on ice for 2 min and washed again. Protoplasts were fixed with 70 % ethanol at 30°C for

20 min and subsequently washed with Annexin-V (Sigma-Aldrich) incubation buffer. Annexin V/propidium iodide (PI) binding assays were performed according to the staining kit protocol, using 10 % annexin reagent, 10 % PI reagent, and 1 mg/mL of RNase A at 37°C for 30 min. Cell analysis for these and other assays in the study was performed using a confocal scanning microscope. All assays were performed at least in triplicate and repeated at least three times.

RESULTS

Killing assay of SPF

Antifungal activity of the SPF of *H. musciformis* was further assessed by determining their killing kinetics on *C. albicans* strain at 0.5 of MIC, 1× and 2× MIC concentrations. The best killing kinetics, by eradicating the fungal cells rapidly within 4 to 6 h **(Figure 1)**. The results clearly indicated that the time kill assay was totally dependent on the concentration of SPF used for testing. From the results, it was clear that regrowth of *C. albicans* strain was noticed at 0.5 of MIC concentration (Figure 1). Whereas regrowth of *C. albicans* was not recorded in any other concentration.

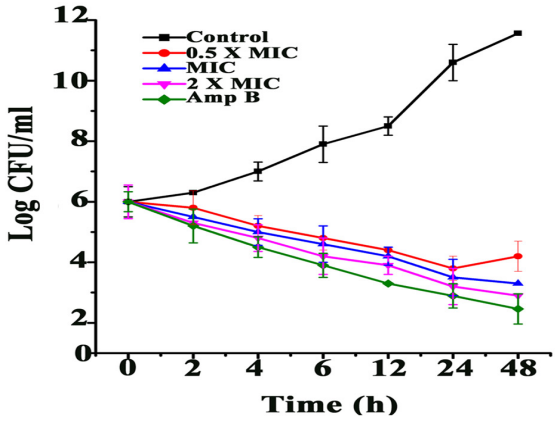

Figure 1 Time kill curve of SPF against *C. albicans* strain

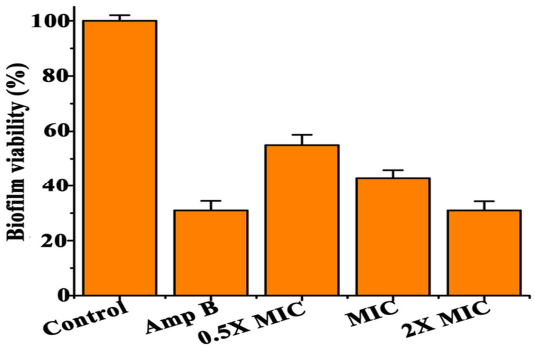

Figure 2 Biofilm viability after treatment with SPF at concentration ranging from 0.5 × to 2 × MIC concentration against *C. albicans* strain. Each data point represents mean result 6 standard deviation (error bars) from two experiments in triplicates

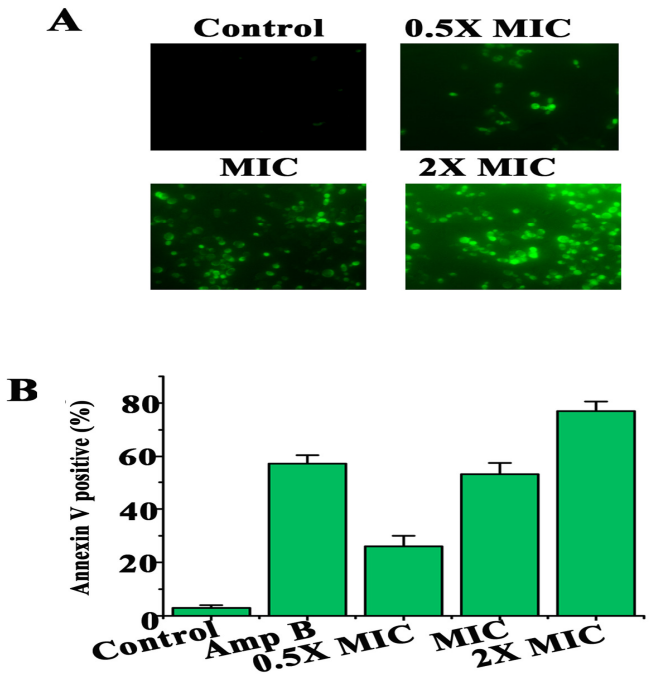

Figure 3 Phosphatidylserine externalization showed by Annexin-V staining in SPF-treated *C. albicans.* [A] Confocal image of *C. albicans* [B] Percentage of phosphatidylserine externalization positive cells.

Biofilm reduction assay of SPF

Biofilm formation is often associated with antifungal resistance as compared to planktonic cells and requires drug concentrations of 30-2000 times the corresponding MIC values to reduce 50 % of the biofilms metabolic activity. The susceptibility of the fungal biofilms to the SPF was assessed by using XTT reduction assay, which enables quantitation of the number of living cells in 24 h old biofilms after treatment. Based on the results shown in Figure 2, the biofilm metabolic activity of *C. albicans* decreased as the concentration of SPF increased. From the results it was clear that SPF of *H. musciformis* exhibited good antibiofilm activity. In contrast, the conventional antifungal agent, amphotericin B displayed the most potent antibiofilm activity.

Phosphatidylserine externalization (induction of apoptosis) by SPF in C. albicans

To differentiate between apoptotic and necrotic *Candida* cell death induced by SPF, Annexin V double staining assay using the FITC - Annexin V and PI was selected. As revealed in Figure 3[A] and [B] *C. albicans* exposed to SPF were considerably stained green fluorescence (positive FITC - Annexin V and negative PI) at the edge of the cell after digestion of the cell wall, representing a very clear externalization of phosphatidylserine. FITC - Annexin V stained *C. albicans* cells were not recorded in experiments conducted without SPF (control). The exposure of phosphatidylserine from

the inner to the outer leaflet of plasma membrane in cells is an early morphological marker of apoptosis. Therefore, these results suggest that SPF of *H. musciformis* induced early apoptosis in *C. albicans*, as shown by a significant staining in Annexin V-positive apoptotic cells. From the results obtained it was established that SPF induces the production and accumulation of intracellular ROS and which finally induces apoptotic features in *C. albicans.*

DISCUSSION

C. albicans readily forms biofilms, which are characterized by resistance to standard antifungal therapy and host immune responses (Ramage *et al.,* 2005), enabling the colonization of mucosal surfaces, with the potential for subsequent invasion and dissemination. *C. albicans* also forms biofilms on catheters and medical devices, which are difficult to eradicate unless the device is removed (Richards *et al.,* 1999; Mermel *et al.,* 2001; Ramage *et al.,* 2005; Miceli *et al.,* 2009; Silva *et al.,* 2010; Falagas *et al.,* 2010). A variety of antifungal agents have been evaluated for their *in vitro* activities against *C. albicans* biofilm, including supra therapeutic concentrations of antifungals used for systemic infections, such as echinocandins, polyenes and azoles, as well as a wide range of other agents (Miceli *et al.,* 2009; Ku *et al.,* 2010; Miceli *et al.,* 2012). However, resistance to azoles and echinocandins has been well documented in *Candida* species and amphotericin B is limited by substantial toxicity. These shortcomings have spurred an investigation for new antifungal agents (Wiederhold *et al.,* 2011; Pierce *et al.,* 2013). In this study the effect of SPF for its ability to inhibit the biofilm formation by *C. albicans* and found that the SPF of *H. musciformis* exhibited excellent antibiofilm formation property when treated *in vitro.*

Apoptosis is a highly regulated cellular suicide program crucial for development and homeostasis in metazoan organisms, resulting in the removal of unwanted, mutated, damaged or simply dispensable cells without occurring an inflammatory reaction (Rockenfeller and Madeo, 2008; Oh *et al.,* 2008). Apoptosis has been accepted as a process that is not exclusive to multicellular organisms, but rather is a universal mechanism of cell elimination operating according to a basic program, including in simpler and more ancient forms of single-celled eukaryotes. The full apoptotic program comprises two phases, one of which has necrotic features (Silva, 2010). Therefore, the effect of *H. musciformis* SPF on the activation of apoptosis by Annexin V staining was analyzed. From the results it was clear that the SPF exert significant enhancement in the activation of apoptosis as evidenced by Annexin V staining.

CONCLUSION

The present observations identify a beneficial role of SPF of *H. musciformis* induced apoptosis in *C. albicans* cells via the exposure of phosphatidylserine from the inner to the outer leaflet of plasma membrane in cells is an early morphological marker of apoptosis. In summary, the experimental evidence herein strongly supports that digestion of the cell wall, representing a very clear externalization of phosphatidylserine induced apoptosis pathway in *C. albicans*.

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