Research Article

Induction of Apoptosis in *Candida albicans* by Semi-purified Fraction of *Hypnea musciformis*

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ABSTRACT

The leading cause of death worldwide is microbial infections. Amidst the rise of new species of *Candida*, *Candida albicans* leads the list with high morbidity and mortality rates. One of the most opportunistic pathogens responsible for infectious diseases in human beings is *Candida albicans*. To overcome this problem, there is a need to explore new antifungals that could replace the current treatment options. Marine red alga *Hypnea musciformis* was collected from the coastal areas of Rameswaram. The collected alga was cleaned, shade-dried, and powdered and extraction was done using the Soxhlet apparatus. The crude methanolic extract was subjected to bioassay-guided silica gel chromatography. A total of six fractions was obtained, among the six fractions, the fifth fraction was subjected to time killing assay, biofilm reduction assay and phosphatidylserine externalization against *Candida albicans*. The aim of the current study was to evaluate the potential of a semi-purified fraction of *Hypnea musciformis* to induce apoptosis in *Candida albicans.* In time killing assay, 1× and 2× MIC concentrations within 4 to 6 hr, the fungal cells were eradicated rapidly. The corresponding MIC values are required to reduce 50% of the biofilm's metabolic activity 30-2000 times. From the results, it was evident that the SPF of *H. musciformis* exhibited good antibiofilm activity. SPF induced the production and accumulation of intracellular ROS, which finally induces apoptotic features in *C. albicans,* was confirmed using Annexin V double staining assay.

Keywords: *Candida albicans,* FITC – Annexin V, ROS, Phosphatidylserine.

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INTRODUCTION

Systemic and mucosal infectious diseases are caused by the most prevalent human fungal opportunistic pathogen, *Candida albicans*.[1] Through a breakdown of host defences, *Candida albicans* can cause diseases varying from superficial skin or mucous membrane infections like thrush and vaginal candidiasis, to the systemic involvement of multiple organs, mostly in patients with compromised immunity.[2] The ability of *Candida albicans* to grow as yeast, pseudohyphal and hyphal forms depending on the conditions is one of the noteworthy feature on the biology of *C. albicans* is.[3] The hyphal form of *Candida albicans* plays an important role in the cause of disease by invading epithelial cells and causing tissue damage and the pseudohyphae forms help them invade deeper tissues after colonizing the epithelium.[4]

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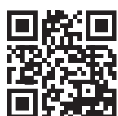

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Increase in antimicrobial resistance is the leading cause of death worldwide makes it a globally growing problem. Thus antimicrobial compounds from plants with diverse chemical structures and novel mechanisms of action developed from plants for new and reemerging infectious diseases is the urgent need of discovery. The new therapeutic agents should be impervious to existing resistance mechanisms and have a novel mode of action.[5] The revolutionized therapy of infectious diseases by the continuous use of antimicrobial drugs has certain limitations due to change in patterns of resistance among pathogens and the side effects produced by them. These current limitations demand the improved pharmacokinetic properties which necessitate continued research of new antifungal compounds in the development of novel drugs.

MATERIALS AND METHODS

Cleaning and drying

The seaweed *Hypnea musciformis,* was collected from the coastal areas of Rameswaram. The seaweed was cleaned and air dried at room temperature 27±2°C below 30°C to avoid the decomposition of thermolabile compounds.

Bulk extraction

Bulk extraction of algal metabolites was done using Soxhlet apparatus. The algal sample placed in the thimble holder and filled with condensed fresh solvent from a distillation flask. When the liquid reaches the overflow level, a siphon aspirates the solution of the thimble holder and unloads it back into the distillation flask carrying extracted solutes into the bulk liquid. Finally, the liquid is reduced to thick oily natural crude extract using a rotary evaporator (Buchi) at 40°C, collected in air-tight vials and refrigerated for further studies.

Purification of bioactive compound(s)

The crude methanolic extract of *H. musciformis* (8 g) was separated into its component fractions using bioassay-guided silica gel column chromatography. A total of six fractions were obtained based on the TLC profile. The 5th fraction was subjected to detailed antifungal studies.

Test pathogen

Medically important fungi: *Candida albicans* MTCC 277 is the test microbe used in the present study. The test microbe was purchased from the Microbial Type Culture Collection Centre (MTCC), IMTECH, Chandigarh, India and was 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. The suspension of *C. albicans* was adjusted to 0.5 McFarland turbidity standard with a cell density of 1x 10^6 to 5x 10^6 cells/mL. A hundred µL of adjusted cell yeast suspension was exposed to 900 µL of RPMI medium containing SPF, yielding a starting inoculum of approximately 10^5 cells/mL. The suspenions were incubated at 37°C with agitation. The samples were removed at the predetermined time interval of 0, 2, 4, 6, 12 and 24 hr, 10 µL and serially diluted with Phosphate-Buffered Saline (PBS) plated on PDA plates were incubated for 24 hr and the colonies were determined after the period of incubation. 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 by performing three independent experiments.[6]

Biofilm reduction assay

Jin *et al.,*[7] method of biofilm reduction assay was performed with modification. Commercially available pre-sterilized, polystyrene, flat-bottomed 96-well flat-bottomed plates were used for the formation of biofilms of *C. albicans*. Standardized yeast suspension (10^7 cells/mL) of *C. albicans* was prepared by suspending colonies from 24 hr old culture in RPMI 1640 medium, and the optical density was adjusted to 0.38-0.39 at 520 nm. Yeast suspension of 100 µL of was dispensed into each well of a microtiter plate using a multichannel pipette and the plates were incubated in a shaking incubator at 37°C 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. To allow the formation of biofilm 100 µL of RPMI 1640 medium was transferred to each washed well and the plates were incubated at 37°C in a shaking incubator at 75 rpm for 24 hr. Following the biofilm phase, the medium was aspirated and each well was washed twice gently with 200 µL PBS to remove non-adherent cells. Before the addition of SPF by inverting the plates over an absorbent paper, residual PBS was removed. 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 37°C in a shaking incubator at 75 rpm for 24 hr. 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 an XTT reduction assay. Each experiment was repeated three times in duplicate. XTT reduction assay was performed according to the method adapted from Jin *et al*.[7]

Annexin V and PI staining

SPF-exposed *C. albicans* cells 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 at pH 7.2.[8,9] Confocal scanning microscope was used to perform cell analysis and other assays in the study. All assays were performed in triplicates.

RESULTS

Killing kinetics of the SPF of *H. musciformis* was assessed by determining the antifungal activity against *C. albicans* strain at 0.5 MIC, 1× and 2× MIC concentrations by eradicating the fungal cells rapidly within 4 to 6 hr (Figure 1). Evidences revealed the regrowth of *C. albicans* strain was noticed at 0.5 MIC concentration. Whereas an increase in the concentration of SPF has prevented the regrowth of *C. albicans*. The results clearly indicated that the time-killing assay was completely dependent on the concentration of SPF used for testing.

Biofilm formation is often associated with antifungal resistance and requires drug concentrations of 30-2000 times the corresponding MIC values to reduce 50% metabolic activity of the biofilms. XTT reduction assay is used to assess the susceptibility of the fungal biofilms to the SPF. XTT reduction assay quantifies the number of living cells in 24 hr old treated biofilms. Evidences indicate that an increase in the concentration of SPF decreased the biofilm metabolic activity of *C. albicans* (Figure 2). The results confirmed the excellent antibiofilm activity of SPF of *H. musciformis* when treated *in vitro*. The conventional antifungal agent, amphotericin B displayed the most potent antibiofilm activity.

Apoptotic and necrotic cell death in *Candida albicans* induced by SPF is distinguished by Annexin V double staining assay using the FITC – Annexin V and PI. SPF exposed *C. albicans* stained green fluorescence (positive FITC – Annexin V and negative PI) at the edges of the cell after digestion of the cell wall, indicating a very clear phosphatidylserine externalization (Figure 3[A] and [B]). FITC – Annexin V stained *C. albicans* cells were not recorded in experiments conducted without SPF (control). Exposure of phosphatidylserine from the inner to the outer leaflet of plasma membrane in cells of *C. albicans* is an early morphological marker of apoptosis. Results evidenced that SPF of *H. musciformis* induced early apoptosis in *C. albicans*, confirmed by significant staining in Annexin V-positive apoptotic cells. In addition, SPF of *H. musciformis* induces the production and accumulation of intracellular ROS, which induces apoptotic features in *C. albicans*.

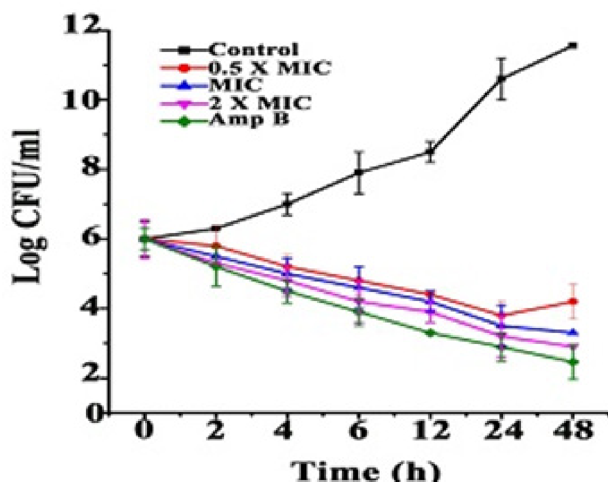

Figure 1: Time kill curve of SPF against *C. albicans* strain.

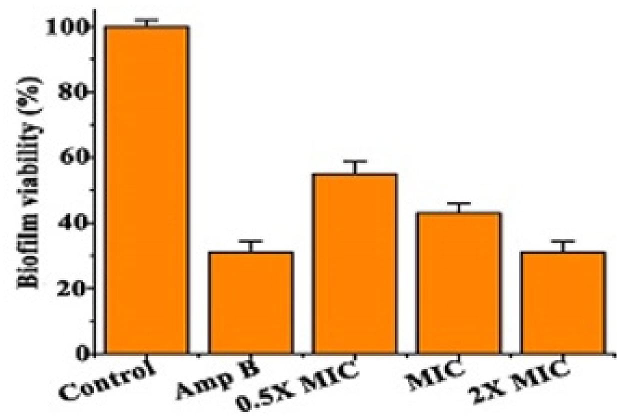

Figure 2: Biofilm viability after treatment with SPF at concentration ranging from 0.5 x to 2 x MIC concentration against *C. albicans* strain.

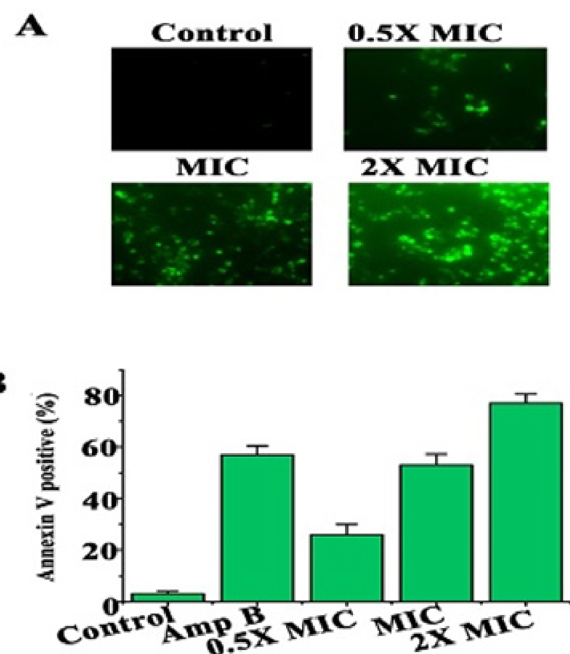

Figure 3: Phosphatidylserine externalization showed by Annexin-V staining in SPF-treated *Candida albicans* [A] Confocal image of *Candida albicans* [B] Percentage of phosphatidylserine externalization positive cells.

DISCUSSION

Host immune responses and resistance to standard antifungal therapy are characterized by biofilm formation in *C. albicans*[10], enabling the colonization of mucosal surfaces with the potential for subsequent invasion and dissemination. Biofilms formed by *C. albicans* on catheters and medical devices are difficult to eradicate unless the device is removed.[10,11]

Supra-therapeutic concentrations of antifungals used for systemic infections and a wide variety of other antifungal agents have been evaluated for their *in vitro* activities against *C. albicans*.[12] Resistance to azoles and echinocandins has been well documented in *Candida* species and amphotericin B is limited by substantial toxicity. These shortcomings paved way for investigation into new antifungal agents.[13]

Highly regulated cellular suicide program, apoptosis is crucial for the development and homeostasis of metazoan organisms, results in the removal of unwanted, mutated, damaged or simply dispensable cells without any inflammatory reaction.[14] Apoptosis is a universal mechanism of eliminating cells, operating according to a basic program, from simpler and more ancient forms of single-celled eukaryotes. The apoptotic phases show necrotic features.[15] Actuate apoptosis and necrosis in *Candida* cells were induced by Eugenol Tosylate Congeners (ETC-5, ETC-6 and ETC-7) via a metacaspase-dependent pathway in a dose-dependent manner. Moreover, ETCs exhibited potential antifungal activity against *C. albicans* by inducing apoptotic and necrotic pathways.[16] Studies by Jia *et al*.[17] revealed that *C. albicans* with coumarin increased the intracellular Reactive Oxygen Species (ROS) levels followed by alterations in mitochondrial functions. Results of Chen and Lan,[18] provided new insights onto the potentials of Hep 25 and its derivatives as novel antifungal agents against *C. albicans*. The results, evidenced the significant enhancement in the activation of apoptosis by SPF. Terrestrial plants has been widely used in the control of *C. albicans,* current study envisages the isolation of individual compound from *H. musciformis*, which is an clear indication that marine algae also has the potential to control.

Vera *et al*.[19] evaluated the potential of microalgal extracts to induce apoptosis in an *in vitro* human liver cancer model. The apoptotic potential was highest in the extracts of *Alexandrium minutum*, *Alexandrium tamarense*, *Gambierdiscus australes*, *Prorocentrum hoffmannianum* and *Prorocentrum reticulatum* at the concentration of 100 μg/mL. In addition, several other studies have reported that some *Alexandrium* species caused detrimental or lethal effects on various marine living organisms by inducing apoptotic responses.[20,21] Lacuna exists in the use of seaweeds as a potential for inducing apoptosis in *C. albicans.* The outcome of this study provides additional scientific data for the selection of several macroalgal species for further investigation and also offers a venue for continued research in the development of new potential drugs for human therapeutics from marine macroalgae.

CONCLUSION

Infections are the leading cause for death in today's world. In the present study potentiality of a semi-purified fraction of *Hypnea musciformis* to induce apoptosis in *Candida albicans* by time-killing assay and biofilm reduction assay. Results confirmed that the SPF of *H. musciformis* exhibited remarkable antibiofilm activity. SPF induces the production and accumulation of intracellular ROS and which finally induces apoptotic features in *C. albicans* ultimately leading to the death of the pathogen.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

ABBREVIATIONS

SPF: Semi-Purified Fraction; **MIC:** Minimum Inhibitory Concentration; **ROS:** Reactive Oxygen Species; **MTCC:** Microbial Type Culture Collection Centre; **TLC:** Thin Layer Chromatography; **PDA:** Potato Dextrose Agar; **PBS:** Phosphate-Buffered Saline; **DTT:** Dithiothreitol; **CFU/mL:** Colony-forming unit per millilitre; **ETC:** Eugenol Tosylate Congeners; **hr:** Hour; **g:** Gram; **M:** Molar; **mM:** Milli molar.

SUMMARY

H. musciformis collected from the coastal areas of Rameswaram, were cleaned, shade dried and powdered. The extraction was carried out using Soxhlet apparatus. The collected extract was subjected to column chromatography a total of six fractions was obtained among which the fifth fraction was evaluated for its potential antifungal activity. The current study revealed the beneficial role of SPF in *H. musciformis.* The experimental evidence herein strongly supports

the digestion of the cell wall, representing a very clear externalization of the phosphatidylserine from the inner to the outer leaflet of the plasma membrane, which is an early morphological marker of apoptosis thereby inhibiting the growth of *C. albicans*.

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