medical sciences forum

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Proceeding Paper

Signature Garlic Phytochemical as a Potential Anti-Candidal Candidate Targeting Virulence Factors in *Candida albicans* †

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† Presented at the 2nd International Electronic Conference on Biomedicines, 1–31 March 2023; Available online: https://ecb2023.sciforum.net/.

Abstract: Resistance to presently available antifungals, and their toxicity is a severe concern throughout the world. It is necessary to investigate innovative, more effective molecules especially derived from medicinally active plants with lesser side effects. Allyl methyl sulfide (AMS) an organosulfur derived from garlic oil, is explored for its activity against *Candida albicans*. The minimum Inhibitory Concentration (MIC) of AMS was found to be 200 µg/mL and 250 µg/mL and the Minimum Fungicidal Concentration (MFC) values of AMS are 400 µg/mL and 500 µg/mL for the selected strains respectively. Fungal growth in *C. albicans* was 90% inhibited at their respective MIC values, as demonstrated by micro broth dilution experiments. After treatment with AMS, *C. albicans'* release of extracellular proteinases, phospholipases, and biofilm formation was significantly inhibited. In *C. albicans*, AMS treatment also reduces attachment to buccal epithelial tissues as measured microscopically. In addition, AMS exhibited significant control over yeast to hypha transition in *C. albicans* cells, which constitute one of the major virulent morphological features of *Candida* species. All the findings of this study indicate that AMS might be a potential alternative to commonly used antifungals.

Keywords: *Candida albicans*; antifungal; allyl methyl sulfide; virulence factors

Citation: Hasan, Z.; Islam, A.; Khan, L.A. Signature Garlic Phytochemical as a Potential Anti-*Candida*l *Candida*te Targeting Virulence Factors in *Candida albicans*. *Med. Sci. Forum* **2023**, *3*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s):

Published: 1 March 2023

1. Introduction

Candida is a prominent contributor to nosocomial and mucocutaneous infections in immunocompromised persons. *Candida albicans* is the frequently isolated and studied species of the genus *Candida* and is responsible for a wide range of ailments, including thrush, diaper rash, and systemic candidiasis [1]. Despite the availability of antifungal drugs, growing levels of resistance to conventional antifungal treatments have been recorded, necessitating the development of novel therapeutic approaches to combat *Candida* infections [2]. Phytochemicals, or substances produced from plants, have been extensively investigated for their potential use as antifungal agents. Garlic is a good *Candida*te for the treatment of *Candida* infections due to its broad-spectrum antifungal action [3].

This work aims to examine the antifungal activity of garlic phytochemical against *C. albicans* and the effects of these compounds on *C. albicans* virulence factors. Using in vitro assays, the antifungal activity of garlic phytochemical against *C. albicans* was investigated along with its effect on virulence factors.

Allyl Methyl Sulfide (AMS) is present in garlic, onions, and other Allium species. Recent research has demonstrated that AMS has antimicrobial characteristics, implying it can suppress the growth of fungi [4]. This feature makes AMS a possible natural alternative for managing fungal infections in a wide range of settings including health and med-

icine, agriculture and food preservation. The efficacy of AMS against several fungi, including *Candida albicans, Aspergillus flavus, and Rhizopus stolonifer*, has already been investigated by different researchers at the initial level [5–7]. To fully understand the mechanism of its antifungal activity and its potential effects of virulence-causing mechanism, additional research is required.

In this work, the effectiveness of the allyl methyl sulfide (AMS) against *C. albicans* is investigated in vitro. The purpose of the study was to examine the effect of AMS on virulence factors and its potential as an anti-Candidal.

2. Materials and Methods

2.1. Growth Parameters, Strain, Media and Chemicals Used

The standard laboratory strain of *C. albicans* ATCC 5314 and 90028, were employed in the current study and is obtained from the National Centre for Cell Sciences (NCCS), Pune, India. On YPD plates (1% yeast extract, 2% peptone, 2% dextrose, 2.5% agar) all the strains were maintained for experiments keeping at 4 °C. All chemicals used in the study are of analytical grade and were purchased from Merck (India). MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide), and other media components, Trypsin, EDTA, N-acetyl glucosamine and Dulbecco's modified Eagle's medium (DMEM) were purchased from Himedia (India). Allyl methyl sulfide (AMS) and fluconazole were obtained from Sigma Aldrich (USA). Gibco (Grand Island, NY, USA) provided the fetal bovine serum (FBS).

2.2. Determination of Antifungal Susceptibility

Minimum Inhibitory Concentration and Minimum Fungicidal Concentration Evaluation of AMS

The micro broth dilution method was used in accordance with the recommendations found in CLSI reference document M27-A3 [8] to determine the minimum inhibitory concentration (MIC) values of AMS for the *Candida* strains. MIC was defined as the lowest concentration that causes a 90% decrease in absorbance as compared to that of the control. 20 μL aliquots were obtained from each well containing different concentrations of AMS and control. In order to determine the minimal fungicidal concentration (MFC), these were subcultured on YPD agar plates for 3–4 days at 35 °C until growth was visible in control samples. The MFC for AMS was defined as the lowest AMS concentration that resulted in no fungal growth on agar plates [9].

2.3. Determination of AMS Impact on Virulence

2.3.1. Proteinase and Phospholipase Assay

The *Candida* culture was cultivated overnight and its concentration was standardized using saline. Then, a desired concentration of AMS equivalent to MIC, MIC/2 and MIC/8 was applied to the standardized culture. To determine proteinase secretion, 2 μL aliquots were placed at equal intervals on agar plates containing 2% agar, 0.2 g of BSA fraction V, yeast nitrogen base, 20 g of glucose, and enough distilled water to reach a final volume of 1000 mL. Similarly, to determine phospholipase secretion, aliquots were placed on agar peptone media comprising 2% agar, 10 g of peptone, 30 g of glucose, 57.3 g of NaCl, 0.55 g of CaCl2, and distilled water in a total volume of 900 mL, which was subsequently supplemented with 10% egg yolk emulsion. Plates were incubated at 37 °C for 2-4 days. Measuring the degradation or precipitation zones (Pz) allowed for the estimation of proteinase and phospholipase secretion [10].

2.3.2. Buccal Epithelial Cells Adhesion Assay

Candida cells that had been grown overnight were washed in sterile PBS (pH 6.8) and resuspended in spider media (pH 7.2). The first author voluntarily donated epithelial cells

by gently scraping the cheek mucous membrane with sterile cotton swabs and gently stirring and washing them in PBS. Adherence assays were performed by mixing 1 mL of each suspension in a test tube, then incubating for 2 h at 37 °C with gentle stirring in the presence of AMS (200 μg/mL). Two drops of trypan blue solution (0.4%) were added to each tube after incubation. Under confocal microscopy, 10 μL of the stained suspension was transferred to a glass slide and examined at 40× magnification [11].

2.3.3. Biofilm Formation Assay

Candida biofilms were examined on the polystyrene surface of 96-well plates. A cell suspension of 1 × 107 cells/mL was produced in PBS and 100 μL was injected into each well for overnight culture. The plates were incubated for 90 min at 37 °C and 50 rpm to adhere the cells to the surface. To eliminate non-adhered cells, the wells were gently washed 2–3 times with PBS. The plates were incubated at 37 °C for 24 h after being filled with 200 μL of YPD medium and 20 μg/mL of AMS. Developed biofilms were treated with AMS (20 μg/mL) for 24 h. Biofilms were measured using the MTT test. 100-μL aliquot of MTT was added to each well and mixed gently for 1 min. The plates were then incubated in the dark for 5 h at 37 °C in a CO2 incubator. The sample was recorded at 450 nm using a microtiter plate reader (BIO-RAD, iMark, Hercules, CA, USA) and the results are expressed as percentage viability [12].

2.3.4. Yeast to Hypha Virulence Trait

The cells that were developed overnight were collected, and then they were washed twice. After that, the cells were starved for six hours at 37 degrees Celsius while suspended in PBS. After the cells had been incubated, they were moved to the media for hyphal growth in the presence of AMS, which included 10% (*v*/*v*) horse serum, and glucosamine. Hyphae were observed under a confocal microscope at magnifications of 40× [13].

2.4. Statistical Analysis

All experiments were carried out at least three times independently. To investigate the statistical difference between control and treated samples, the statistical significance of data was determined using the Student's *t*-test. The significance level was set at $p \leq 0.05$.

3. Results

3.1. Antifungal Activity Determination

Allyl methyl sulfide (AMS) had minimum inhibitory and fungicidal concentrations of 200 μg/mL and 400 μg/mL against *C. albicans* ATCC 5314 and 250 μg/mL MIC and 500 μg/mL MFC against *C. albicans* ATCC 90028, respectively. (Table 1)

Table 1. MIC90 and MFC of isolates as determined by broth microdilution assay.

Strains	Allyl Methyl Sulfide MIC90/MFC (μg/mL)	Fluconazole MIC90/MFC (μg/mL)
C. albicans ATCC 5314	200/400	8/16
C. albicans ATCC 90028	250/500	10/18

3.2. Effect of AMS against Virulence Traits of C. albicans

3.2.1. Effect on Proteinase and Phospholipase Secretion

Extracellular hydrolytic enzymes, primarily proteinases and phospholipases, are crucial in the development of fungal infection because they promote adhesion, invasion, and tissue damage in the host. Therefore, proteinase and phospholipase secretory activity was examined at MIC and sub-MIC concentrations (MIC/2 and MIC/4) of AMS along with untreated cells. As shown in Figure 1A *Candida* cells grown in agar plates show different

precipitation zones (Pz) in presence of AMS. However, the reduction at MIC/4 is negligible with inhibition of only 6% in proteinase secretion. But at MIC/2 and MIC concentrations it demonstrated a 13% and 55% reduction in proteinase secretion (Figure 2A). On the other hand, the inhibition of phospholipase secretion is less prominent at MIC and sub-MIC concentrations of AMS. It accounts for only 3%, 6% and 10% of MIC/4, MIC/2 and MIC of AMS respectively (Figures 1B and 2B). The inhibition was more pronounced in the case of proteinase rather than phospholipase secretion.

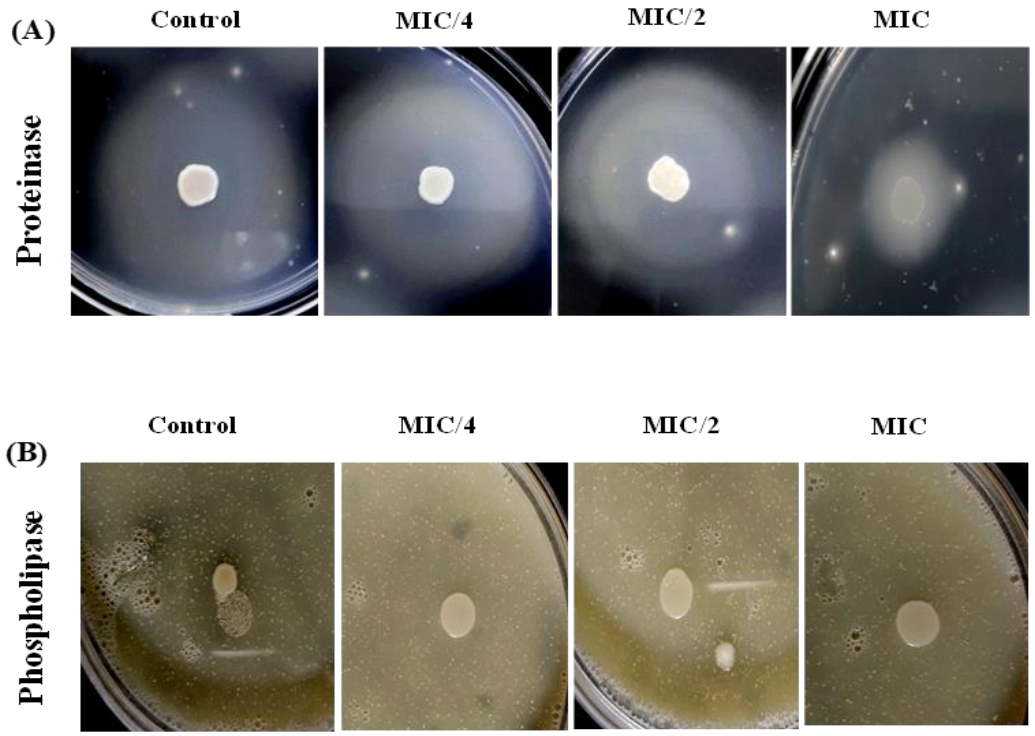

Figure 1. Petri plate pictures showing (**A**) Proteinase secretion and (**B**) phospholipase secretion in *C. albicans* in the presence of MIC and sub-MIC concentrations of AMS.

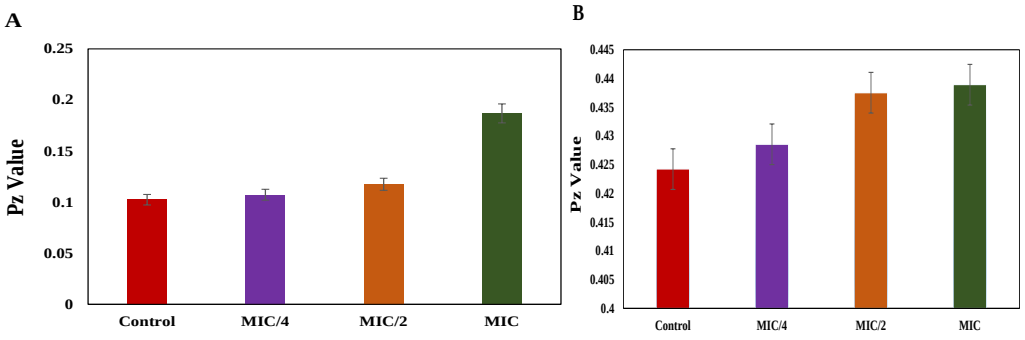

Figure 2. (**A**) Proteinase secretion and (**B**) phospholipase secretion in *C. albicans* in the presence of MIC and sub-MIC concentrations of AMS. Pz value is the mean of three different recordings and is the ratio of the diameter of the colony to the diameter of the colony plus zone of clearance and zone of precipitation (Data are presented as means ± SD. Student *t*-test, $p \leq 0.05$).

3.2.2. Effect of AMS on Adherence to Host Epithelial Tissue

To infect the host, adherence to the mucosal and outer epithelial tissues is the prerequisite for the *C. albicans* to exert its pathogenic potential. Thus, buccal epithelial cells (BECs) adhesion assay plays a crucial role in testing the efficacy of antifungals targeting virulent factors. At MIC concentration, AMS can detach the *C. albicans* cells from the BEC

surface and also cause a significant reduction in cell population as compared to the regular pattern of attached cells in the case of the control sample (Figure 3).

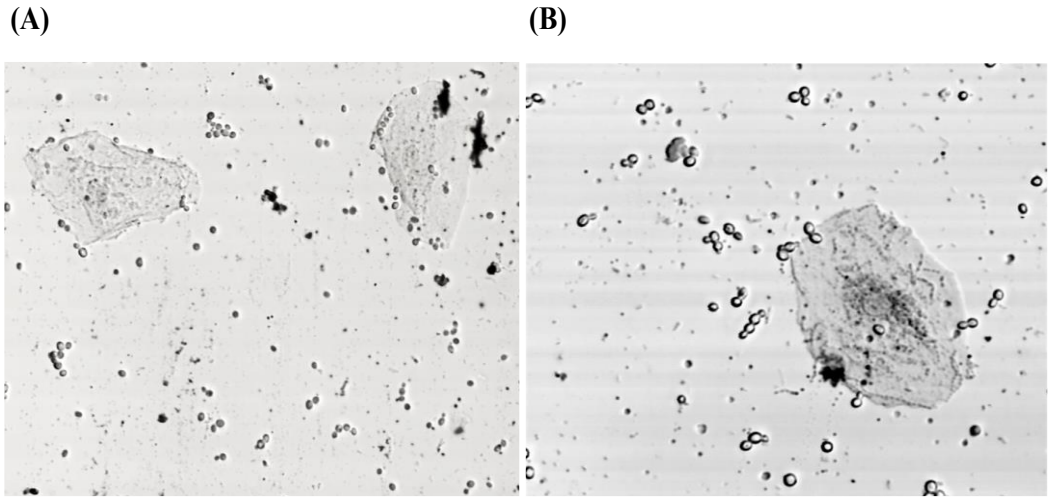

Figure 3. Adhesion to buccal epithelial tissues microscopically monitored in *C. albicans*. (**A**) represents control cells while (**B**) shows AMS treated at its MIC (200 μg/mL) concentration.

3.2.3. Effect of AMS on Biofilm Formation

In comparison to untreated controls, AMS at various inhibitory and sub-inhibitory concentrations was found to reduce biofilm formation in a concentration-dependent manner. The biofilm formation was estimated in terms of cell viability percentage which corresponds to the value of absorbance at 490 nm. As Figure 4 suggests untreated control cells show 100% cell viability in terms of biofilm formation, the rest of the concentrations are taken in reference to it. The results show that biofilm formation was inhibited by 53%, 68% and 81% at MIC/4, MIC/2 and MIC concentrations of AMS respectively.

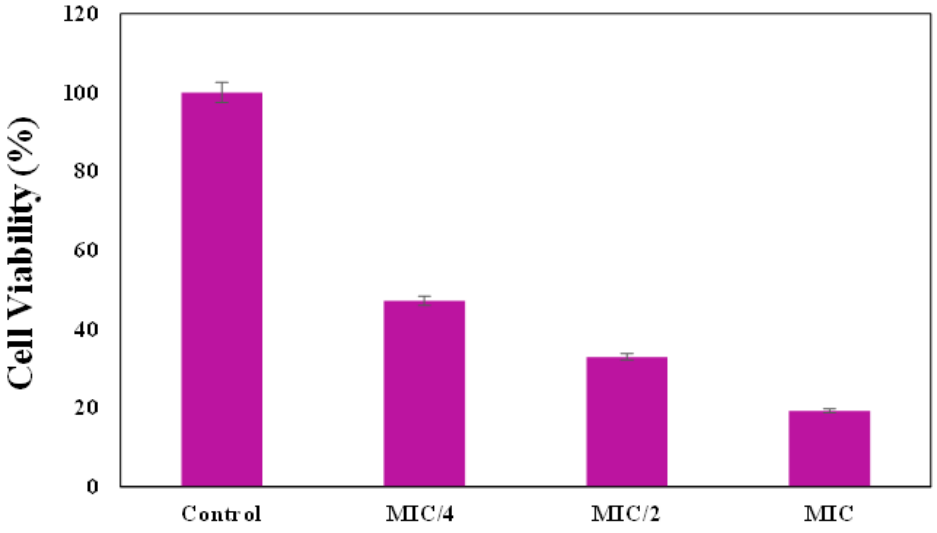

Figure 4. Effects of varying concentrations of AMS (MIC/4, MIC/2, and MIC) on biofilm formation in *C. albicans* determined through MTT assay. (Data are presented as means ± SD. Student *t*-test, $p \leq 0.05$).

3.2.4. Effect of AMS on Morphological Switching

To avoid a hostile host environment and stress situation *C. albicans* cells undergo yeast to hypha morphological switching. In the presence of 10% serum as well as glucosamine-containing hypha-inducing media at 37 °C, the induction of germ tube or hyphae was significantly reduced at the MIC value of AMS. Furthermore, a significant reduction in the number of cells along with the disappearance of hypha was observed under confocal microscopy. Therefore, it is clear that at MIC, AMS blocked *C. albicans* filamentation, a mechanism essential to fungal pathogenesis.

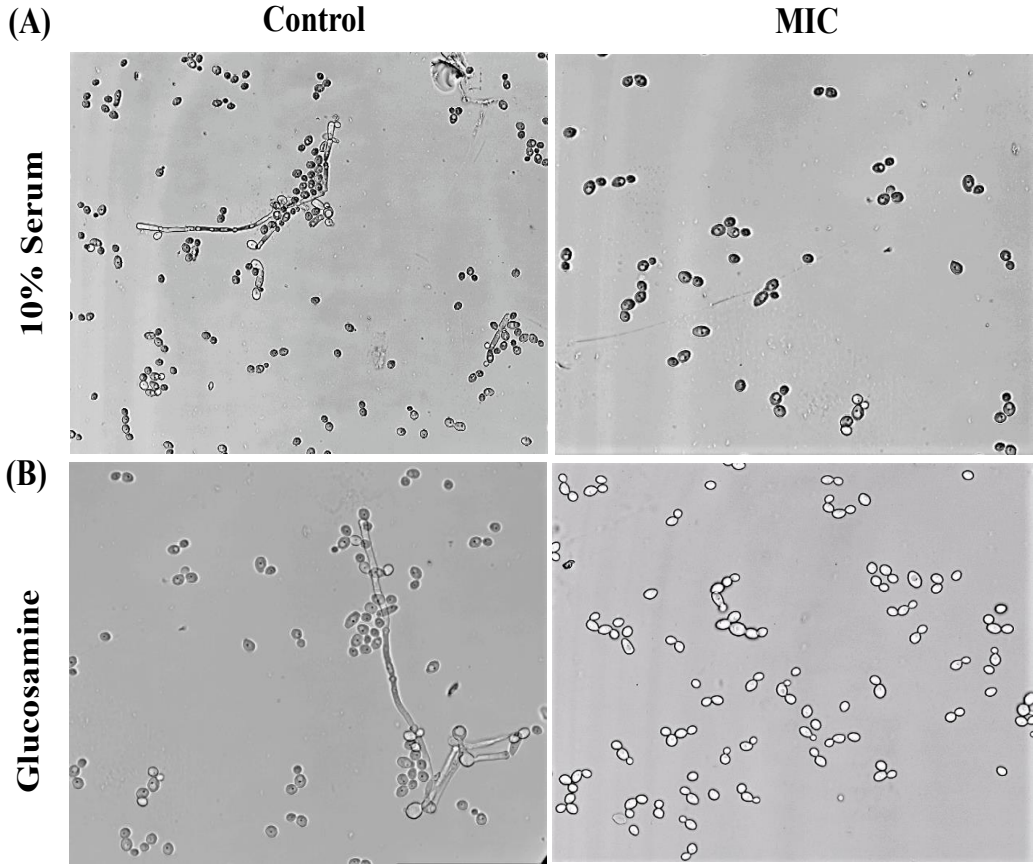

Figure 5. Effect of AMS on morphogenesis in hyphae inducing liquid media 10% serum in YPD (**A**), glucosamine (**B**) in *C. albicans* after an incubation of 2 h. Cells without AMS showed a significant number of cells undergoing hyphal induction.

4. Discussion

The most prevalent human fungal pathogen, *Candida* albicans, can cause systemic infections as well as mucosal infections. *C. albicans's* pathogenicity is linked to a number of virulence factors, such as adhesion, phenotypic and morphological alterations, the production of numerous hydrolases (secreted proteinase and phospholipases), the presence of efflux pumps [14]. Additionally, cells are covered in adherent biofilms that make it more difficult for conventional antifungals to work and build host defences that are resistant, which raises the risk of multidrug resistance [15]. Therefore, a different approach to treating *Candida* infections that are linked to virulence attributes and biofilms is required.

Garlic is said to have medicinal, insecticidal, antibacterial, and antifungal effects. Additionally, there has recently been a surge in interest in garlic as an anti-fungal agent. It has been shown that garlic extract is fungicidal to pathogenic yeasts, particularly *C. albicans*. Allicin was one of the components of garlic that was shown to prevent the proliferation of fungi by blocking succinate dehydrogenase [5]. According to reports, the main active metabolite component of allicin is AMS [5,16]. It has been demonstrated that AMS,

a key constituent of volatile garlic metabolites, possesses antibacterial, antioxidant, and anticancer effects [3,6,17]. However, no concrete study is available highlighting its effect on virulence factors in *C. albicans*.

We show that AMS had a MIC of 200 μg/mL against *C. albicans* ATCC 5314 and 250 μg/mL against *C. albicans* ATCC 90028. In both the strains the value of MFC was found to be double of MIC which is 400 μg/mL and 500 μg/mL respectively. We also put the negative control of standard antifungal fluconazole and it shows a similar ratio to AMS between MIC and MFC. Although the concentration of MIC and MFC for AMS is quite high compared to fluconazole but its natural origin and negligible toxicity in that range shouldn't limit its efficiency.

C. albicans secretes virulence agents including proteases and phospholipases that increase its pathogenicity. These hydrolases effectively break down host membrane and surface membrane proteins, which promotes *C. albicans* invasion of host tissue [15]. AMS was successful in preventing *C. albicans* from producing these virulence enzymes. This finding inspires us to hypothesise that AMS might be able to prevent candidemia from developing. The adherence of *C. albicans* to the host epithelial surface is considered to be an important factor in the development of infection [18]. Also, the ability of *C. albicans* to produce biofilms is linked to the majority of infections caused by this organism [13]. Our study reports that AMS is able to reduce the adherence of *Candida* cells to host epithelial tissues and inhibit biofilm formation at the abiotic surface at its MIC concentration. In addition to the above-mentioned factors, the yeast-to-hyphal transition is thought to be critical for *C. albicans* pathogenicity. The hyphal cells colonise endothelium and epithelial tissues and promote systemic infection [19]. Our results show that AMS-treated cells had less hyphal development while control cells had a higher filamentous structure. Overall, the results of this study show that AMS has the potential to be employed as an antifungal agent, particularly against *C. albicans*.

5. Conclusions

The study suggests that Allyl Methyl Sulfide (AMS) has the potential as an antifungal Candidate against *Candida albicans*. The effectiveness of AMS against *C. albicans* originates from the inhibition of proteinase and phospholipase secretion. In addition, it also inhibits biofilm formation, yeast-to-hyphal transition and adhesion of *Candida* cells to the epithelium.

Author Contributions: Conceptualization, Z.H. and L.A.K.; methodology, Z.H.; validation, L.A.K., A.I. and Z.H.; formal analysis, Z.H.; investigation, Z.H.; resources, A.I. and L.A.K.; data curation, Z.H.; writing—original draft preparation, Z.H.; writing—review and editing, Z.H., A.I. and L.A.K.; visualization, Z.H.; supervision, L.A.K. and A.I. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available because it is part of the study which will be published in a full-length paper.

Acknowledgments: Z.H. is thankful to Indian Council for Medical Research for awarding the Senior Research fellowship [Sanction No. Myco/Fell./2/2020-ECD-II].

Conflicts of Interest: The authors declare no conflict of interest.

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