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Antifungal and antibiofilm activity of Persian shallot (*Allium stipitatum* Regel.) against clinically significant *Candida* spp.

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Abstract. *Candida* species are the most common cause of fungal infections that range from non-life-threatening mucocutaneous illness to life-threatening invasive processes that may involve virtually any organ. Such a broad range of infections requires an equally broad range of therapeutic approach. Persian shallot (*Allium stipitatum* Regel.) is a medicinal plant that has been widely used in tradition Persian medicine for various ailments. *Allium stipitatum* is also used in modern medicine and has been reported to have a range of health benefits including antibiotic (antifungal) properties. The present study assessed the in *vitro* anticandidal and antibiofilm potential of hexane (ASHE) and dichloromethane (ASDE) extracts of *Allium stipitatum* (Persian shallot) against planktonic and biofilm forms of 5 medically important *Candida* spp. Antifungal activity was assessed by disk diffusion, minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and time-kill assay. The antibiofilm activity of ASHE and ASDE against reference strain *C. albicans* ATCC 14053 was determined by XTT [2,3-*bis*(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay. The zone of inhibition ranged from 22 to 40 mm, while the MICs ranged from 8 to 32 μg mL^{-1}. The MFCs of ASHE and ASDE were in the range of 16 to 32 μg mL^{-1} each respectively. Time-kill kinetics showed that both extracts were strongly fungicidal against planktonic cultures of *C. albicans* with ~ 1.45 log reduction in CFU at 4 h post-treatment (hpt). In addition, both ASHE and ASDE were shown to inhibit preformed *C. albicans* biofilms in a concentration-dependent manner. The results demonstrated that ASHE and ASDE were broad-spectrum in action, and could be developed as a promising alternative to synthetic antifungals in controlling infections due to *Candida* spp. of clinical significance.

List of Abbreviations

ASHE - *Allium stipitatum* hexane extract; ASHE - *Allium stipitatum* dichloromethane extract; MIC - minimum inhibitory concentration; MFC - minimum fungicidal concentration; XTT - 2,3-*bis*-(2-methoxy-4-nitro-5-sulfophenyl)-*2H*-tetrazolium-5-carboxanilide; ATCC - American Type Culture Collection; CFU - colony forming units; BSIs - bloodstream infections; MRSA - methicillin resistant *Staphylococcus aureus*; DMSO - dimethyl sulfoxide; SDA - Sabouraud's dextrose agar; SDB - Sabouraud's dextrose broth; RPMI - Rosewell Park Memorial Institute; HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); MOPS - 3-(N-morpholino) propanesulfonic acid); PBS - phosphate buffered saline; CLSI - Clinical and Laboratory Standards Institute; NaCl - Sodium chloride; BHI - brain heart infusion; SD - standard deviation; GC-MS - gas chromatography mass spectrometer; HWP - hyphal cell wall.

INTRODUCTION

Candida yeast is a clinically significant human pathogen that endures in the normal mucosal flora of the gastrointestinal tract in about 80% of healthy adults. The organism can be pathogenic at times by causing candidemia and systemic candidiasis in hosts with compromised immunity (Calderone, 2002). Candidal infections are caused by a group of clinically important pathogenic fungi which are difficult to treat due to its high drug resistance and increase in prevalence (Akeme Yamamoto *et al.*, 2012). Although *C. albicans* remains the predominant species causing bloodstream infections, non-*albicans Candida* such as *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* account for more than 90% of invasive and bloodstream infections (Richardson & Lass-Florl, 2008; Chi *et al.*, 2011). For the past two decades, the wide use of fluconazole for the treatment of bloodstream infections (BSIs) in immuno-compromised patients have decreased the incidences of *Candida*-associated BSIs. However, an increased incidence (10%) of *C. albicans* along with non-*albicans* candidal infections in Asia have been reported and 20% of the BSIs are caused by *C. glabrata* alone (Pfaller *et al.*, 2004).

The second main challenge is the increasing incidence of biofilm-related infections by *Candida* spp. Biofilms of *Candida* species are notoriously resistant and/or refractory to conventional antifungals with high recurrence rates compared to their planktonic counterparts (Casarato & Lara, 2010). As a result, it is imperative to search for alternative antifungals with new targets to overcome the increased mortality rate associated with candidal infections which thereby will reduce the associated toxicity and resistance (Escalante *et al.*, 2008; Zhang *et al.*, 2006). Several plant extracts including *Allium* have been reported to have antifungal activity against *C. albicans* (Elsom *et al.*, 2003; Iwalokun *et al.*, 2004; Khodavandi *et al.*, 2010; Zarei Mahmoudabadi & Gharib Nasery, 2009).

Anticandidal activity of aqueous extract of garlic (garlic tablet formulation of *A. sativum*) added with 2.56 μg mL^{-1} of total thiosulphinate completely inhibited the viability of *C. albicans* in 4 h (Elsom *et al.*, 2003). The fresh crude juice of *Allium ascalonicum* was reported to have moderate antifungal activity against clinical isolates of *C. albicans* (Zarei Mahmoudabadi & Gharib Nasery, 2009). Alcoholic and aqueous extracts of *A. stipitatum* are strongly fungicidal against several dermatophytes with MICs ranging from 0.058 - 0.8 mg mL^{-1} for alcoholic extract and 0.26 - 3.84 mg mL^{-1} for water extract, respectively (Fateh *et al.*, 2010). In our recent study, we have shown that *A. stipitatum* exerts strong antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and wound healing activity in a burn wound mouse model (Karunanidhi *et al.*, 2017; 2018). The excellent anti-MRSA and wound healing properties prompted the investigation on the anticandidal and antibiofilm activity of *C. albicans* and non-*albicans Candida* spp.

MATERIALS AND METHODS

Plant material and preparation of extracts

Fresh bulbs of Persian shallot (*Allium stipitatum*) were collected at 1750 m above sea level in Arak, Marzaki Province of Iran (33°05'N 49°42'E). *A. stipitatum* was authenticated by taxonomist Dr. Mitra Noori and the voucher specimen (CMN 10, 02 May 2007) was deposited at the Department of Biology, Faculty of Science, Arak University, Iran. Plant materials were washed with water, sliced to pieces and dried completely under shade for 2-3 weeks. Five kilograms of dried Persian shallot bulbs were ground into fine powder and sequentially extracted with hexane and dichloromethane for 72 h using maceration. The extract was filtered through Whatman No.1 paper to remove solid plant materials and the filtrates were dried (BÜCHI Rotovapor R-200, Flawil, Switzerland) at 40°C under vacuum. Upon filtration and

solvent volatilization, the hexane and dichloromethane extracts (ASHE and ASDE) of *A. stipitatum* yielded 221 g (4.42%) and 164 g (3.28%) of residue, respectively. The concentrates were transferred to glass scintillation vials (Wheaton Brand, USA) and used for further assays.

Chemicals

Merck supplied hexane, dichloromethane, dimethyl sulfoxide (DMSO), fungal growth media Sabouraud's dextrose agar (SDA) and Sabouraud's dextrose broth (SDB). Rosewell Park Memorial Institute (RPMI-1640) medium with HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)] (25 mM) & L-Glutamine and MOPS [3-(N-morpholino) propanesulfonic acid] buffer was obtained from Sigma Chemicals Co. St Louis, MO, USA). Resazurin and phosphate buffered saline (PBS) were purchased from Fisher Scientific, Malaysia. Antibiotic control, amphotericin B (Ampho) was obtained from Sigma Chemicals and filter paper discs (6 mm diameter) (GE Healthcare, Malaysia), sterile swabs, 96-well polystyrene microtitre plates (©TPP, Trasadingen, Switzerland). Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide, monosodium salt) reagent was purchased as resazurin sodium salt powder (Acros Organic NV). Resazurin was prepared as a stock solution of 100 μg mL^{-1} and was used at a final concentration of 0.01% (w/v) in PBS (pH 7.2). The stock solution was filtered sterilized in a 0.20 μm-pore filter and stored in dark at 4°C. XTT was obtained as XTT sodium salt (Sigma Aldrich, MO USA). The stock solution of XTT (1 mg mL^{-1} in PBS) was prepared and was used at a final concentration of 0.01% (w/v) in distilled water. The stock solution was filter sterilized in a 0.20 μm-pore filter and stored at -20°C in dark.

Fungal strains and culture conditions

Isolates of *C. albicans* ATCC 14053, *C. glabrata* ATCC 2001, *C. krusei* clinical isolate, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 750 were obtained from Mycology Laboratory, Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Stock cultures were prepared in glycerol (20%) and were stored at -80°C. Yeast cultures were propagated on SDA plates and passaged twice to ensure viability and purity before antifungal susceptibility testing. The inoculum was prepared according to the document M27-S4 CLSI guidelines (CLSI, 2012). From the 24 h cultures grown on SDA plates at 37°C, five colonies (> 1 mm diameter) were selected and suspended in 1 mL of sterile saline (0.85% NaCl). The suspension was vortexed for 15 s and the cell densities were adjusted to 0.5 McFarland standard at 530 nm (0.08 - 0.13) using a Biophotometer (Eppendorf, Hamburg, Germany). From the above procedure, a yeast stock suspension of 1×10^6 to 5×10^6 cells mL^{-1} was obtained.

Antifungal assays

Disk diffusion assay

The antifungal activity of ASHE and ASDE were determined by disk diffusion method following the guidelines recommended by CLSI (CLSI, 2012). Standardized overnight cultures of the test *Candida* isolates were spread evenly on SDA plates by lawn culture. The culture plates were allowed to stand for 15 min at room temperature in a laminar flow cabinet to allow for any excess moisture surface to be absorbed into the agar before the drug-impregnated discs were placed. Sterile antibiotic assay filter paper discs of 6 mm diameter were placed on SDA plates and 20 μL (corresponding to 200 μg extract/10 mg mL^{-1}) of ASHE and ASDE from the freshly prepared stocks were carefully loaded onto the filter paper discs. Filter disc impregnated with 20 μL of amphotericin B (1.6 mg mL^{-1}) was included as the positive control, while filter disc containing 20 μL of DMSO (10%) served as negative control. The plates were incubated at 35°C and the inhibition zones were measured after 48 h of growth.

Determination of MIC and MFC by modified broth microdilution

The modified version of broth microdilution was adapted from Schwalbe *et al.* (2007)

using RPMI-1640 medium (pH 7.0) enriched with MOPS buffer (0.165 M). ASHE and ASDE were prepared in DMSO (10%) and subsequent 2-fold serial dilutions were performed yielding to a final concentration ranging from 16-8192- μg mL^{-1} of ASHE and ASDE respectively. The microtitre wells plates were incubated at 35°C for 24 h, and 30 μL of resazurin (0.01%) was loaded to all wells and the plates were incubated for an additional 6-8 h. A colour change from blue to pink indicated the fungal growth, and the MIC was determined as the lowest concentration that did not cause visible growth (or) the lowest concentration of an antibiotic that prevented this colour change. MFC was considered to be the concentration of the drug that inhibited 90% of fungal growth. It was determined by spread plating 100 μL of the broth from clear wells (blue colour) onto SDA plates followed by incubation at 37°C for 48 h. The experiment was performed in triplicates.

Time-kill assay for detecting the fungicidal effect of ASHE and ASDE

The killing kinetics of ASHE and ASDE on *C. albicans* was carried out by following the method described earlier (Argemi *et al*., 2013) with slight modifications. Yeast suspensions were diluted to 1 × 10^6 CFU mL^{-1} and ASHE/ASDE concentrations were adjusted to 0.5x, 1x, 2x and 4x MICs. Cultures were incubated at 35°C for 0, 2, 4, 6, 8, 12, and 24 h. Aliquots of 100 μL were pipetted out from each tube, serially diluted 10 fold in sterile distilled water (100 μL mixture + 900 μL sterile distilled water), and 100 μL was plated on SDA plates. Tubes without ASHE/ASDE served as growth controls (0x) and tube with DMSO (10%) served as diluent control. The plates were incubated at 37°C for 48 h followed by the enumeration of viable colony counts. Killing curves were constructed by plotting the $\log_{10}$CFU mL^{-1} against time over a 48 h time period. A positive fungicidal activity was defined by a $\geq$ 3-$\log_{10}$ reduction in colony counts (Klepser *et al*., 1998) and the experiment was performed in triplicates.

Antibiofilm assays

Biofilm formation

Biofilms were produced by following the previously described method (Pierce *et al*., 2008) with minor modifications. *C. albicans* cells (5 × 10^6 cells mL^{-1}) were seeded in a 96-well microtitre plate and allowed to grow for 48 h at 37°C to ensure proper adhesion. Upon 48 h of adhesion and biofilm formation, the supernatant of each well was pipetted out using a multichannel pipette without disrupting the biofilm formed on the polystyrene surface. The wells were rinsed with 200 μL of sterile PBS followed by the addition of 100 μL of ASHE/ASDE at increasing concentration (0x, 1x, 2x and 4x MIC) to the biofilm wells. Wells without ASHE/ASDE (0x) served as biofilm control. The plates were incubated at 37°C for an additional 24 h to determine the antibiofilm potential of ASHE and ASDE. The experiment was performed in triplicates.

XTT-reduction assay

Following 48 h of adhesion/biofilm formation and 24 h of ASHE/ASDE treatment, the contents of the wells were pipetted out and biofilm quantification was determined by XTT reduction assay similar to the method as described previously (Pierce *et al*., 2008). Before each assay, fresh XTT solutions were prepared by dissolving 4 mg XTT in 10 mL pre-warmed (37°C) PBS. This solution was supplemented with 100 μL menadione stock solution, containing 55 mg menadione (Sigma) in 100 mL acetone. The effect of ASHE and ASDE was tested at concentrations of 0x (negative control), 1x, 2x, and 4x MICs to 24 h old biofilms formed earlier. Post-treated biofilm plates were washed three times with 200 μL of sterile PBS and completely dried. One hundred microliters of XTT/menadione solution was added to each well containing the pre-washed biofilms as well as to negative controls. The plate was covered with aluminium foil and incubated in the dark for 3 h at 37 °C. The contents of the wells (~ 80 μL) were transferred to a new microtitre plate and the absorbance of the adherent

biofilm was read at 490 nm in a microtitre plate reader (BioTek EL808, USA). Wells containing BHI served as blank and no positive control was included since a commercial antibiofilm agent is not available.

Statistical analysis

Values are expressed as mean ± SD. All experiments were performed in triplicates and the differences between the treated and untreated (control) groups were analysed using Graph pad prism 6.0. Statistically significant differences ($p<0.05$) between groups (control vs treated) were assessed using one-way (ANOVA).

RESULTS

Antifungal activities of ASHE and ASDE (Zone of inhibition, MICs, and MFCs)

Based on the results obtained in disk diffusion assay, ASHE and ASDE displayed strong antifungal activity against all the yeast strains tested. The antifungal activity of ASDE was slightly higher than ASHE with larger inhibition zones at 10 mg mL^{-1} concentration. The inhibition zones for ASHE and ASDE ranged from 23-30 mm and 22-40 mm, respectively (Fig. 1 & Table 2). The zone of inhibition formed by ASDE was comparatively larger than amphotericin B (32 μg $disc^{-1}$), especially against non-*albicans Candida* spp., which were in the range of 35-40 mm diameter. The maximum zone of inhibition was observed in ASDE treated *C. krusei* plate (40 mm) followed by *C. glabrata* (39 mm), *C. parapsilosis* (37 mm) and *C. tropicalis* (35 mm). The MICs of ASHE and ASDE ranged from 10 - 100 μg mL^{-1} and the MFCs ranged from 8-32 μg mL^{-1} (Table 2). All four non-*albicans Candida* were highly susceptible to low concentrations of ASDE (MIC/MFC 8/16 μg mL^{-1}). However, *C. albicans* was inhibited at a slightly higher concentration of ASHE (16 μg mL^{-1}) and ASDE (32 μg mL^{-1}), respectively.

Analysis of fungal killing kinetics

C. albicans treated with 1x MIC of ASHE (16 μg mL^{-1}) and ASDE (32 μg mL^{-1}), 2x

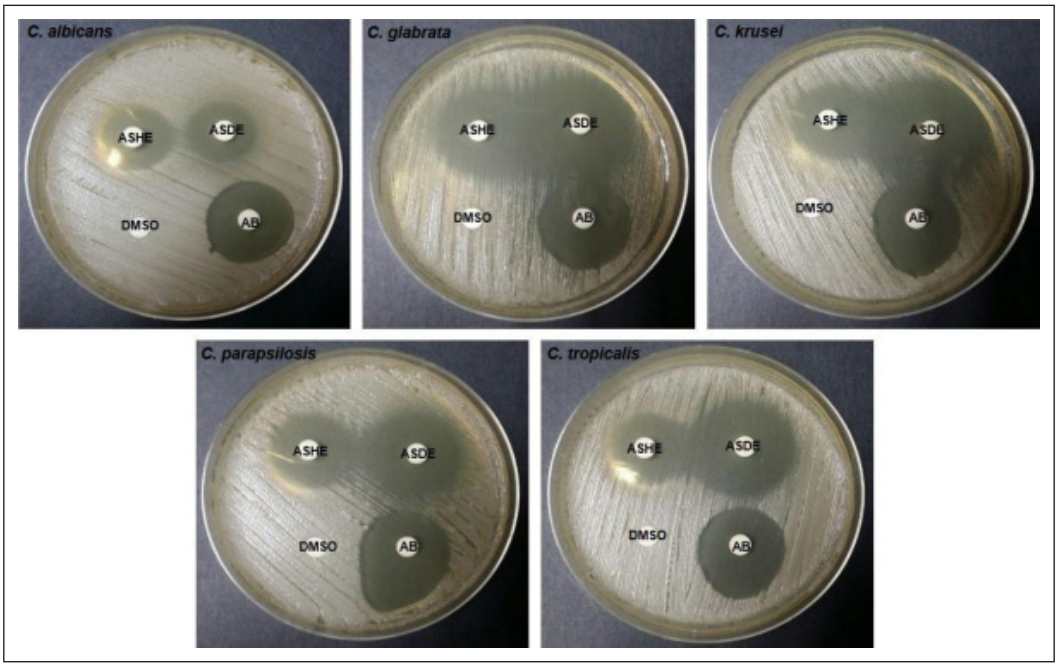

Figure 1. Effect of ASHE (200 μg $disc^{1}$) and ASDE (200 μg $disc^{1}$) applied to a blank filter paper disk on SDA plate inoculated with *C. albicans* ATCC 14053,*C. glabrata* ATCC 2001, *C. krusei* clinical isolate, *C. parapsilosis* ATCC 22019, and *C. tropicalis* ATCC 750. ASHE - *A. stipitatum* hexane extract; ASDE - *A. stipitatum* dichloromethane extract; AB- Amphotericin B; DMSO - dimethyl sulfoxide (10%).

Table 1. Zone of inhibition, MIC and MFC of ASHE and ASDE against test microorganisms (20 μL corresponding to 200 μg/disc)

Strain	Zone of inhibition in diameter ± SD (mm)[a]			DMSO (10%)	MIC (μg mL^{1})[b]		MFC (μg mL^{1})[c]	
	ASHE	ASDE	AmpB		ASHE	ASDE	ASHE	ASDE
C. albicans ATCC 14053	25 ± 0.5774	22 ± 0.2887	29 ± 0.5774	–	16	32	32	32
C. glabrata ATCC 2001	30 ± 0.2887	39 ± 0.5774	30 ± 0.5000	–	8	8	16	16
C. krusei ATCC CI	23 ± 0.0	40 ± 0.2887	28 ± 0.5774	–	16	8	32	16
C. parapsilosis ATCC 22019	28 ± 0.7638	37 ± 1.000	32 ± 2.03	–	16	8	32	16
C. tropicalis ATCC 750	25 ± 0.7638	35 ± 0.8660	29 ± 0.5000	–	16	8	32	16

Values are given as mean ± S.D. of triplicates.
[a]Determined by disk diffusion assay.
[b]Determined by broth microdilution method.
[c]Determined by plate colony count technique.
AmpB - amphotericin B (1.6 mg mL^{-1}).
– No zone of inhibition.

MIC of ASHE (32 μg mL^{-1}) and ASDE (64 μg mL^{-1}) and 4x MIC of ASHE (64 μg mL^{-1}) and ASDE (128 μg mL^{-1}) demonstrated significant reduction in yeast viability. It is apparent from the time-kill studies, that ASHE and ASDE were strongly fungicidal in killing >90% of *C. albicans* cells. Fungicidal endpoints were achieved at 4 h for ASHE (Fig. 2a) and 8 h for ASDE (Fig. 2b), respectively. ASHE and ASDE at 1x MIC showed an average 1.16 $\log_{10}$ reduction in CFUs at 8 h post-treatment. While, at higher concentrations (2x and 4x MICs), an average of 1.45 $\log_{10}$ reduction in CFU was observed (99.9%) at 4 h post-treatment (p<0.05) (Fig. 2). Increasing concentrations of the ASHE/ASDE exhibited higher killing rate indicating a concentration-dependent antifungal activity. No recurrence of yeast colonies was observed after 4 h post-treatment in 2x and 4x treated plates and after 8 h in 1x MIC groups.

Inhibition of biofilm formation

Challenging preformed biofilms of *C. albicans* with ASHE and ASDE showed significant reductions in biofilm viability. Compared to the control biofilms, *C. albicans* biofilms treated with 1x, 2x and 4x MICs of ASHE/ASDE were disrupted/removed in a concentration-dependent manner. ASHE/ASDE treated groups showed lesser absorbance at 490 nm which implied that both extracts effectively removed the adhering biofilms on polystyrene surfaces. Control biofilms had more absorbance values which denoted more living cells. ASHE and ASDE at 1x MIC showed a slight reduction in biofilm viability. However, at 4x MICs, biofilms of *C. albicans* were highly susceptible to ASHE and ASDE (p<0.05) (Fig. 3).

DISCUSSION

Fresh crude juice of *A. ascalonicum* was reported to exhibit antifungal activity against clinical isolates of *C. albicans* with inhibition zones of 13-20 mm at 0.25% concentration (equivalent to 2500 μg mL^{-1}) (Zarei Mahmoudabadi & Gharib Nasery, 2009). However, ASHE/ASDE used in the present study showed much stronger anticandidal activity at a comparatively lesser concentration of 8-32 μg mL^{-1}, which is ~80 fold less than the previous report on aqueous extract. Recently, the anticandidal effect of ethyl

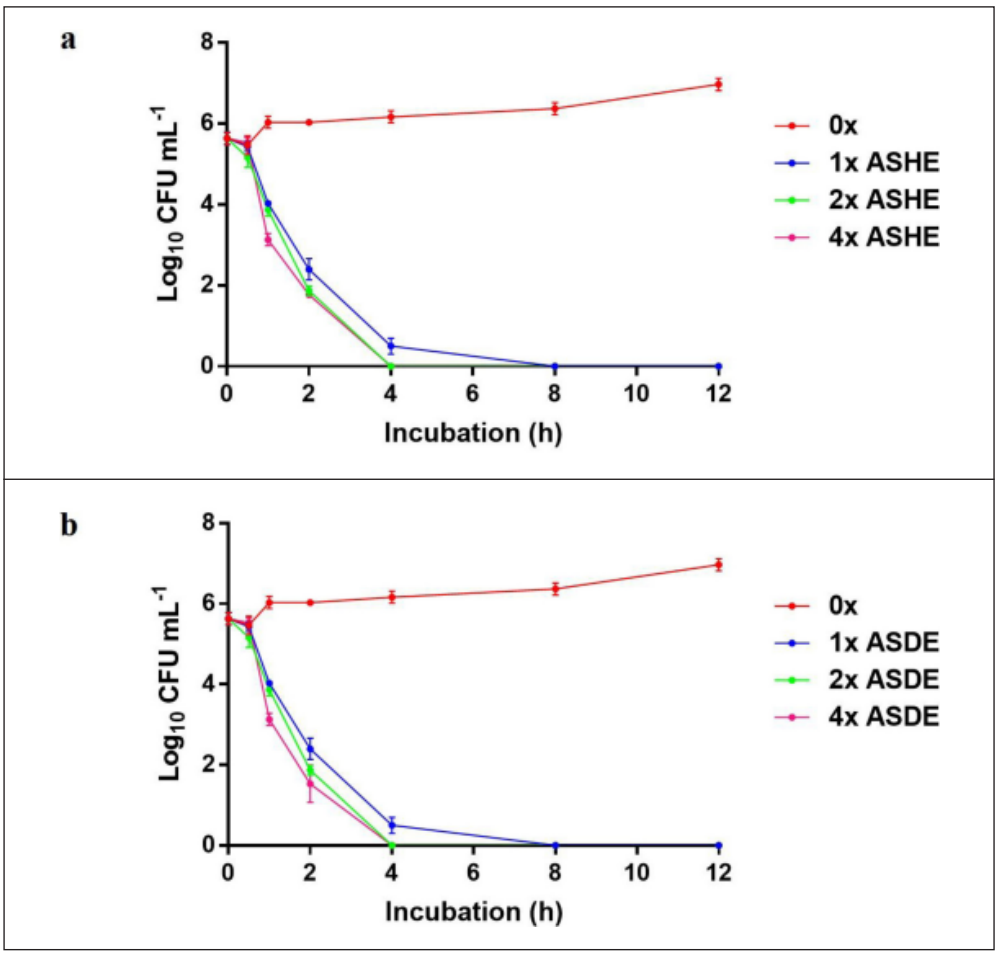

Figure 2. Effect of (A) ASHE and (B) ASDE on the viability of *C. albicans* ATCC 14053 in liquid medium (time-kill curve) treated with ASHE and ASDE at concentrations of 1x, 2x and 4x MICs with control (0x MIC). MIC - minimum inhibitory concentration; CFU - colony forming units.

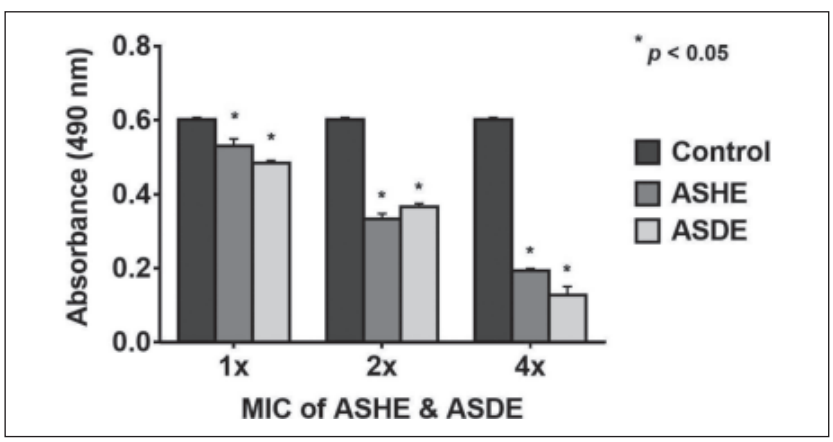

Figure 3. Effect of ASHE and ASDE on *C. albicans* ATCC 14053 biofilms at concentrations of 1x, 2x and 4x MIC with control (0x MIC). Comparison of absorbance between control and treated samples at 490 nm by XTT assay. $^{*}p<0.05$; MIC: minimal inhibitory concentration. Values are expressed as mean ± SD.

acetate, hexane, methanol and water extracts of Persian shallot against different clinically important *Candida* spp. was reported (Khodavandi *et al*., 2014). The inhibition zones of ASHE (23-30 mm) and ASDE (22-40 mm) observed in the present study was much similar to the inhibition zones reported for hexane extract (22-37 mm at 25 mg mL^{-1} per disc). Another significance of the present study is that ASDE exhibited a much stronger antifungal activity with a maximum zone size of 40 mm against *C. krusei*. The inhibition zones exerted by ASHE/ASDE were similar and both the extracts were equally potent in killing *C. albicans* and non-*albicans Candida* spp. Aqueous extract of *A. hirtifolium* has also been reported to have excellent anti-dermatophyte activity against *Trichophyton* and *Microsporum* spp. (Mahboubi & Kazempour, 2015). In our earlier investigation on the *in vivo* antibacterial and burn wound healing activities of *A. stipitatum*, both ASHE and ASDE were subjected to gas chromatography-mass spectrometric (GC-MS) analysis (Karunanidhi *et al*., 2017). Based on the GC-MS analysis, several sulphur containing compounds like S-methyl methanethiosulphonate, 2,4,5-trithiahexane, 2,4-dithiapentane, 2-pyridinethione and methane (chloromethylthio) (methylthio)- were reported in our recently published work (Karunanidhi *et al*., 2017). The aforementioned compounds are known to exhibit strong antibacterial activities against gram-positive and gram-negative bacteria. These broad-spectrum antibacterial compounds could be responsible for the growth inhibition of *Candida* biofilms and their planktonic counterparts. The presence of the above compounds in *A. stipitatum* has also been reported by other researchers (Ismail *et al.*, 2012). However, GC-MS analysis of ASHE and ASDE in our earlier investigation by Karunanidhi *et al.* (2017), showed a higher concentration of 2-pyridinethione (3.38-3.87%), thiosulfuric acid (0.15-1.52%), and methanesulfonamide (5.23%) which has not been reported by other authors. This could be one of the reasons for the higher activity compared to previous reports.

In comparison, a recent antifungal study by (Bagiu *et al*., 2012) using *A. ursinum* (a closely related *Allium* member to *A. ascalonicum* and *A. stipitatum*) reported a MIC of 0.5-4.0 mg mL^{-1} against *Candida* spp. The MICs and MFCs of ASHE and ASDE of the present study were in agreement with a previous report on the antifungal activity of *A. ascalonicum* hexane extract (Khodavandi *et al*., 2014).

Time-kill assay remains crucial in determining the mechanism of action of an antifungal drug. In our study, cell counts were found to be zero after 4 h post-treatment with ASHE/ASDE at 4x and 2x MICs, while at 1x MIC the colony counts were too few and a further 4 h incubation of SDA plates showed a complete fungicidal activity of ASHE and ASDE. No evidence of recurrence or regrowth of *C. albicans* was observed even after 8 h post-treatment, which indicates that ASHE and ASDE were strongly fungicidal by completely killing *C. albicans* in 4 to 8 h post-inoculation with ~1.16-1.45 $\log_{10}$ reduction in yeast inoculum. *A. sativum* extract and allyl alcohol, a metabolic product present in garlic cloves induced oxidative stress in *C. albicans*. In a previous study by Lemar *et al.* (2005), allyl alcohol of *A. sativum* was reported to be fungistatic at 58 μg mL^{-1}, however, increasing the concentration to 2-fold (116 μg mL^{-1}) resulted in the fungicidal effect of allyl alcohol at 10 h of incubation. This pattern of fungicidal activity was observed in the present study, and ASHE/ASDE were not completely fungicidal at 1x MIC. However, increasing concentrations of ASHE/ASDE (2x and 4x) showed a significant difference ($p<0.05$) in fungicidal effects which further implies the concentration-dependent killing effects of ASHE/ASDE on *C. albicans*. Compared to a previous report by Khodavandi *et al.* (2014), on the time-kill kinetics of hexane and ethyl acetate extracts of *A. ascalonicum*, the killing rate of ASHE and ASDE for *C. albicans* was slightly higher (Khodavandi *et al.*, 2014). This could be either due to the minor difference in the MICs (16-32 μg mL^{-1}) against different *Candida* spp., or the sequential extraction method followed in the present

study. ASHE/ASDE was strongly fungicidal in killing *C. albicans* reference strain in a time period of 8 h. However, a slow fungicidal effect (24 h post-treatment) of hexane, ethyl acetate, methanol and aqueous extracts was also reported (Khodavandi *et al.*, 2014) which was slightly contradictory to the present time-kill results.

C. albicans biofilms in hospital settings remains a crucial stage in its pathogenesis, and biofilm forms of *Candida* are less sensitive to antifungals thereby increasing the intricacy of antifungal therapy (Inigo *et al.*, 2012). Biofilm-related casualties due to *C. albicans* are increasingly prevalent in hospitalized patients with indwelling catheters (Ramage *et al.*, 2009). Therefore, development of new antifungals which inhibits early biofilms of *C. albicans* would be advantageous. Earlier investigation on the effect of fresh garlic extract on planktonic, adherent and sessile phases of *C. albicans* provided promising anticandidal and antibiofilm activity of *Allium* extracts (Shuford *et al.*, 2005). Allicin, a major bioactive component of several *Allium* members exhibited synergistic effect along with azoles against different *C. albicans*, *C. glabrata* and *C. tropicalis* (Khodavandi *et al.*, 2010). Allicin and alcoholic extract of Persian shallot inhibit mature biofilms of *C. albicans* effectively in a dose-dependent manner. The activity of allicin and the shallot-alcohol extract was superior to fluconazole by significantly down-regulating the hyphal cell wall protein (*HWP1*) gene involved in the biofilm formation of *C. albicans* (Khodavandi *et al.*, 2014; Khodavandi *et al.*, 2011). The anticandidal and antibiofilm activity of ASHE and ASDE appear promising and merit further investigation for determination of the biofilm inhibitory mechanism of ASHE/ASDE.

CONCLUSION

The results demonstrated the broad-spectrum anticandidal property of *A. stipitatum* against *C. albicans* and non-*albicans* *Candida* species. Considering the broad-spectrum antifungal property, *A. stipitatum* could benefit as a potential antifungal agent. The inhibition zones ranged from 22-40 mm, and the MICs from 8-32 μg mL^{-1}. Both ASHE and ASDE were strongly fungicidal against planktonic cells of *Candida* spp. and complete fungicidal activity was achieved at 8 h post-treatment. ASHE/ASDE inhibited preformed biofilms of *C. albicans* biofilms at 2x and 4x MICs. Further investigations on the effect of ASHE/ASDE on *C. albicans* biofilm-associated genes could afford some useful insights into the molecular targets of these antifungal extracts. The results further extended the antibiofilm potential of ASHE/ASDE and foreshadow the benefits of this medicinal plant in controlling infections associated with *Candida* spp.

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Conflict of Interest:
None.

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