

Anti-*Candida* Potential of *Allium ascalonicum* Linn: Antibiofilm Activity and Biomolecular Mechanism of Action

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Persian Shallot (*Allium ascalonicum* Linn.) has been proven to possess some antimicrobial properties against some of bacteria, viruses and fungi. Indeed the aim of this study was to investigate the antifungal properties of several Persian Shallot extracts against important *Candida* species. In the first stage the preliminary screening of antifungal activity was determined by disc diffusion test. The effective extracts (hexane, ethyl acetate, methanol, and water) were proceeded to obtain minimum inhibitory concentration (MIC) using broth microdilution test and time-kill study was also performed at time intervals. Results showed that the Shallot-hexane extract was more effective in terms of *Candida* species growth. Subsequently, the effects of the extracts on *C. albicans* biofilm were determined using XTT assay. Finally the expression level of a crucial gene involved in biofilm formation in *C. albicans* was analysed by semi-quantitative RT-PCR. The MIC was ranged from 5-600 µg/mL for different species of *Candida*. Moreover in time-kill study, no colonies were observed at 1× MIC after 24 h of incubation period. On the other hand, XTT assay showed the significant reduction of biofilm in *Candida*-treated by Shallot at level $p < 0.05$. Eventually the expression level of *HWP1* was down regulated through the semi-quantitative RT-PCR ($p < 0.05$). The present study indicates that *HWP1* could be one of the possible targets of Shallot to inhibit biofilm in *C. albicans*.

Key words: *Candida* species, *Allium ascalonicum* Linn., *HWP1*, Biofilm.

The incidence of candidiasis has increased over recent years, especially in immunocompromised patients^{1,2}. Reports show that there are possible antifungal drug resistance of *Candida*³.

Among *Candida* species, *C. albicans* is more available to form biofilm on the surface of medical devices such as stents, shunts, prostheses, implants, endotracheal tubes,

pacemakers resulting in resistance to antifungal therapy^{3,4}.

Besides, conventional medicine may pose side effects and abusive or incorrect usage of synthetic drugs result in complications. To further worsening the issue, large percentage of world's population do not have access to conventional pharmacological treatment, natural products are the preferred choice to treat candidiasis⁵.

Clearly the alternative therapies should be considered as a way to overcome drug resistance of fungi. One of the alternative therapies is the research and development of natural products. New and effective natural products that

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show antifungal properties without causing significant adverse side effects are necessary for treatments.

There are some researches that focus on exploration of antifungal properties of plants. *Allium ascalonicum* Linn., or commonly known as Persian Shallot, is a herb widely used in preparing food. Many studies have shown that it has the properties of antimicrobial, antiviral, and antifungal⁶⁻⁸. The dried and autoclaved extracts of Persian Shallot showed more antimicrobial properties than garlic and onion. Fungi are more sensitive to Shallot than bacteria. In addition, Persian Shallot compounds are stable at high temperature⁹. Despite the fact that nowadays most of the studies are conducted on the purified materials, in this study for first time, several whole extracts were used rather than isolated active compound, testing the hypothesis that the naturally occurring family of compounds present in the Shallot and used in the diet might have synergistic properties.

Moreover, the anti-*Candida* activity of Shallot extracts was investigated and then *C. albicans* was selected for biofilm study. Finally the gene expression analysis was performed to find the possible target of Shallot extract on *C. albicans* biofilm.

MATERIALS AND METHODS

Extraction of antifungal compounds

The fresh Persian Shallot was washed with distilled water, sliced and dried in the oven for at least two days and then powdered. Subsequently, 100 g of powder was added to 500 mL of organic solvents of hexane, ethyl acetate, methanol, and water respectively and finally the extracts had undergone a sequential extraction using soxhlet. The organic solvents were filtered by Whatman No. 1 filter paper and the filtrate was then collected and concentrated using the rotary evaporator. The concentrated and sticky extracts were left in dessicator for two to three days to remove moisture. Eventually, the different concentrations of extracts were made by dissolving in 5% DMSO.

Candida species isolates

Five clinical isolates from different *Candida* species from microbiological laboratories

of University of Malaya were obtained. *Candida albicans* ATCC 14053, *C. rugosa* ATCC 10571, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 750 were also used as the control strains.

Disc diffusion agar method

Candida species were cultured on Sabouraud Dextrose Agar (SDA) and passaged twice to ensure viability and purity, and then incubated overnight at 37 °C. Five colonies that are more than 1 mm in diameter were selected and inoculated into 5 mL of sterile 0.85% saline. The resulting suspensions were vortexed for 15 s and adjusted the cells density with a spectrophotometer by adding sufficient saline to increase the transmittance to that produced by 0.5 McFarland standards at 530 nm wavelengths. This procedure yielded a yeast stock suspension of 1×10^6 to 5×10^6 cells/mL.

The prepared cell suspension was poured and spread on SDA and then 20 μ L of the different extracts with the concentration of 25 mg/mL were impregnated on separate paper discs. Positive control disc was impregnated with antifungal drug, amphotericin B with the concentration of 1.6 mg/mL. The culture plates were incubated at 37°C for 48 h and then the inhibition zone was measured^{8,10}.

Broth microdilution test for determination of MIC

Hundred μ L of the twofold dilution of the different concentrations of extracts were dissolved in standard RPMI 1640 medium with 0.2% glucose [buffered to pH 7.0 with 0.165 M morpholinophosphonyl sulfate (MOPS)] using 96 microplates according to CLSI document M27-A3 for yeast cells with some slight modifications. Subsequently, 100 μ L of a suspension containing 5×10^2 to 2.5×10^3 yeast cells/mL was added to former mixture and incubated at 35°C for 24 h. The endpoint was defined as the lowest concentration of each antifungal that caused 50% and 90% growth inhibition compared to control-growth.

Time kill study

Four mL of each extracts ($1 \times \text{MIC}$ concentration) was added to the same volume of *Candida* species cell suspensions (10^6 cell/mL). At predetermined time points of 0, 2, 6, 12, 24, 36 and 48 h, 100 μ L of samples was obtained from each tube, serially diluted 10 fold in sterile water (100 μ L mixture + 900 μ L sterile water), and 50 μ L was plated on SDA plates and then incubated at 35°C for 24 h for determination of viable colony counts. The

lower limit of reproducibly quantifiable CFU according to these methods was 50 CFU/mL^{11,12}.

Biofilm formation and quantification using XTT assay

Among *Candida* species, *C. albicans* was selected for biofilm study as more able to form biofilm on the surface of medical devices and would be harmful for health of human^{3,4}. According to a standard protocol with slight modification, 100 µL of *C. albicans* ATCC 14053 cells suspension containing 1×10^6 to 5×10^6 cells/mL [in standard RPMI 1640 medium (with 0.2% glucose and buffered to pH 7.0) with MOPS (0.165 M)] was added to 100 µL of antifungals with different concentrations using 96-well microplate (Brand 781660, Wertheim, Germany) and incubated at 37°C for 90 min with no shaking and finally continued with shaking at 37°C for 24 h to complete the pre-formed biofilm of *C. albicans*¹³⁻¹⁵.

XTT assay was performed for quantification of treated pre-biofilm of *C. albicans*. In summary, the biofilm formed was washed three times with 200 µl of sterile PBS and the plates were dried. A pre-made XTT with concentration of 0.5 g/L was thawed. Fifty µL of menadione was added to 4.95 mL of thawed XTT solution and then 100 µL of XTT/menadione solution was added to the pre-washed biofilm and then incubated in the dark at 37°C for 3 h. Eventually, 80 µL of the supernatant was transferred to a new microplate and the optical density was measured at 490 nm using EMax® microplate reader¹⁶.

Microscopic observation

Four mL of a suspension containing 1×10^6 to 5×10^6 cells/mL of *C. albicans* ATCC 14053 (dissolved in RPMI 1640) were mixed to 4 mL of antifungals with $1 \times$ MIC concentration using coverslips placed in the 6-well cell culture plates, and incubated as described above. Finally, the media were discarded and coverslips were washed twice by PBS. The coverslips were visualized using (Leica, DMRA II, Germany) at $\times 40$ magnification.

Total RNA extraction, cDNA synthesis and semi-quantitative RT-PCR

Approximately 5-10 mL *C. albicans* ATCC 14053 suspensions containing 1×10^6 to 5×10^6 yeast cells/mL [in standard RPMI 1640] was treated by the same volume of antifungals and incubated to induce hyphae as described earlier. Total RNA was extracted using the RNeasy mini kit (Qiagen,

Germany) and treated with 1 U DNase I (Promega). Subsequently, cDNA was synthesized using Moloney-Murine Leukemia Virus (MMLV) reverse transcriptase kit (Qiagen, Germany). Continually, semi-quantitative RT-PCR was performed using the *HWPI* forward, 5'-GGTAGACGGTCAAGGTGAAACA-3' and reverse 5'-AGGTGGATTGTGCGAAGGTT-3' primers (283bp PCR product). Beta actin was also used as the house-keeping gene [forward: 5'-ACCGAAGCTCCAATGAATCCAAAATCC-3', and reverse: 5'-GTTTGGTCAATACCAGCAGCTT CAAA-3', 516 bp PCR product size] with 25 cycles of denaturation and 60°C annealing temperature. The intensity of PCR products were quantified using AlphaImager HP software. The fold change in target gene expression was calculated from the expression ratio of target gene/ratio of reference gene¹⁷.

RESULTS

The result of preliminary screening test (disc diffusion assay) on Persian Shallot (*A. ascalonicum* Linn.) extracts showed that all extracts tested, water, hexane, ethyl acetate and methanol were active against *Candida* species in terms of formation of inhibition zone. Indeed the diameter of inhibition zones was ranged from 7.47 to 37.33 mm (Table 1).

From these studies, shallot extract in the range of 25 to 0.049 mg/mL reveal strong inhibitory properties for *Candida* species growth. Nevertheless further dilutions of Shallot extract (0.04 to 0.0000781 mg/mL) were tested against *Candida* species tested to determine the relative MICs. The MICs were ranged from 5-600 µg/mL and 0.0125-1 µg/mL for different Shallot extracts and amphotericin B, respectively against different species of *Candida* tested (Table 2).

Fig. 1 shows the significant reduction the number of *Candida* species (ATCC strains) treated by antifungals tested at time intervals in compare to control growth ($p \leq 0.05$).

It is indicated that Shallot extract with hexane was more effective against *Candida* species in terms of growth inhibitory potential. Hence we have selected Shallot extract by hexane for biofilm formation.

Table 1. *In vitro* activities of different Shallot extracts against five species of *Candida*

Isolates	Inhibition zone (mm) \pm standard deviation				
	Hexane extract	Ethyl acetate extract	Methanol extract	Aqueous extract	Amphotericine B
<i>C. albicans</i> ATCC14053	37.33 \pm 0.33	34.55 \pm 0.90	30.44 \pm 0.45	17.67 \pm 0.88	38.33 \pm 0.77
<i>C. albicans</i> 3092	22.65 \pm 0.44	33.80 \pm 0.45	28.55 \pm 0.76	15.30 \pm 1.44	29.59 \pm 0.44
<i>C. tropicalis</i> ATCC 750	25.85 \pm 0.50	31.25 \pm 0.65	7.67 \pm 0.88	10.50 \pm 0.89	22.45 \pm 0.55
<i>C. tropicalis</i> 5483	27.10 \pm 0.65	30.35 \pm 0.75	7.47 \pm 0.46	11.55 \pm 0.99	33.23 \pm 0.97
<i>C. parapsilosis</i> ATCC 22019	35.50 \pm 0.45	31.25 \pm 0.85	18.67 \pm 1.33	8.00 \pm 0.58	31.34 \pm 0.44
<i>C. parapsilosis</i> 2707	34.33 \pm 0.33	30.45 \pm 0.34	19.50 \pm 0.66	9.50 \pm 0.59	34.56 \pm 0.89
<i>C. rugosa</i> ATCC 10571	29.42 \pm 0.44	30.65 \pm 0.55	15.33 \pm 0.89	14.25 \pm 0.44	31.33 \pm 0.65
<i>C. rugosa</i> 3114	29.50 \pm 0.55	31.20 \pm 0.88	17.64 \pm 0.77	15.76 \pm 0.78	33.44 \pm 0.97
<i>C. krusei</i> ATCC 6258	33.56 \pm 0.65	27.44 \pm 0.45	24.44 \pm 0.95	14.67 \pm 0.76	32.45 \pm 0.57
<i>C. krusei</i> 3109	31.75 \pm 0.50	29.36 \pm 0.65	27.75 \pm 1.20	16.35 \pm 0.88	31.30 \pm 0.66

*Values are mean \pm standard deviation of three independent experiments

Quantification of preformed *C. albicans* biofilm by XTT has indicated significant reduction ($p \leq 0.05$) in all of concentrations of Shallot hexane extract compared to the control (Table 3).

Fig.2 shows the light microscopy images from *C. albicans* ATCC 14053 treated with Shallot-hexane extract in $1 \times \text{MIC}$ concentration at different time intervals. *C. albicans* cells treated showed a

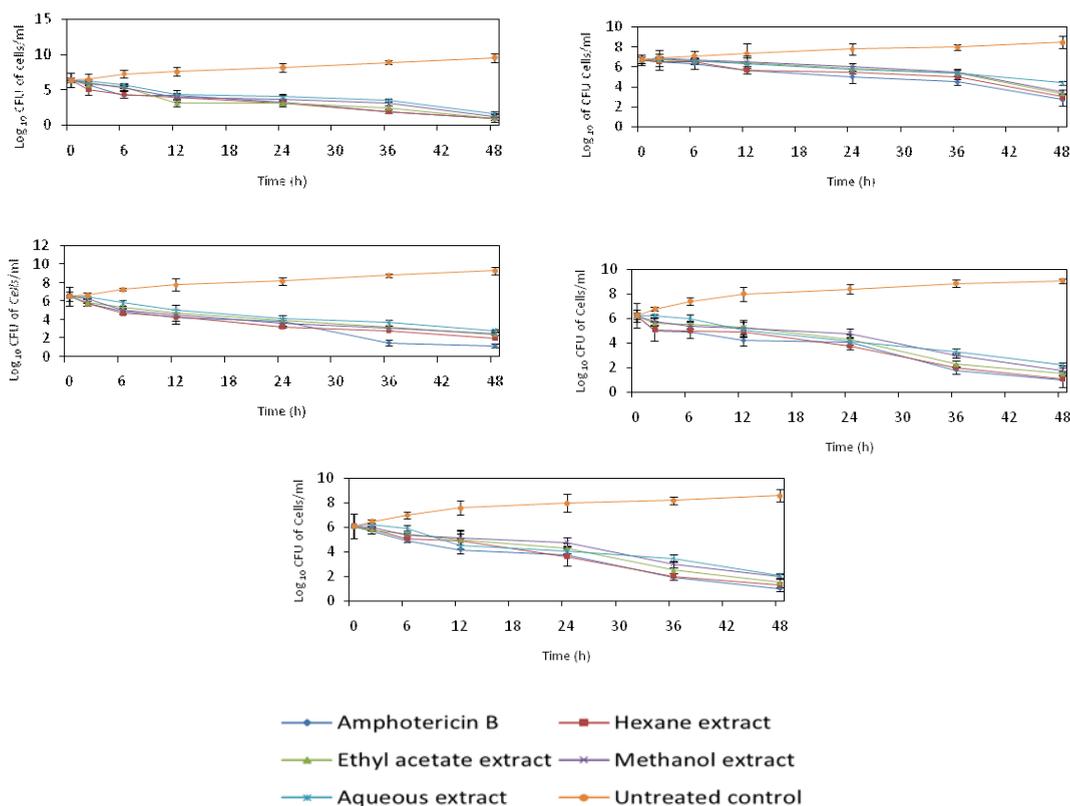


Fig. 1. Time dependent killing of sessile *Candida* species cells treated by different antifungals tested. (a) *C. albicans* ATCC 14053, (b) *C. rugosa* ATCC 10571, (c) *C. krusei* ATCC 6258, (d) *C. parapsilosis* ATCC 22019 and (e) *C. tropicalis* ATCC 750

significant reduction in the number of yeast form and lost their potential to produce filamentous hyphae, while the untreated control was able to form hyphae and structured to show the pre-biofilm after 24 h incubation.

The results of RT-PCR showed that Shallot-hexane extract was able to decrease the expression level of *HWPI* (in *C. albicans* ATCC 14053) in all concentrations tested (based on MICs) significantly ($p < 0.01$) and ranged from 1.8, 3.1, 4.3 and 5.5 fold at $1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, $1 \times \text{MIC}$ and $2 \times \text{MIC}$, respectively (Fig. 3).

DISCUSSION

Due to emergence of resistance to *Candida*, finding the novel anti-*Candida* agents have been one of the main tendencies of medical centers. Nowadays, investigations on medicinal plants such as Shallot are on the rise because it has a curative potential against debilitating conditions and diseases⁹. The antimicrobial activity of Shallot was reported earlier by some of investigator against bacteria and fungi¹⁸⁻²⁰. This study was conducted to examine and explore the

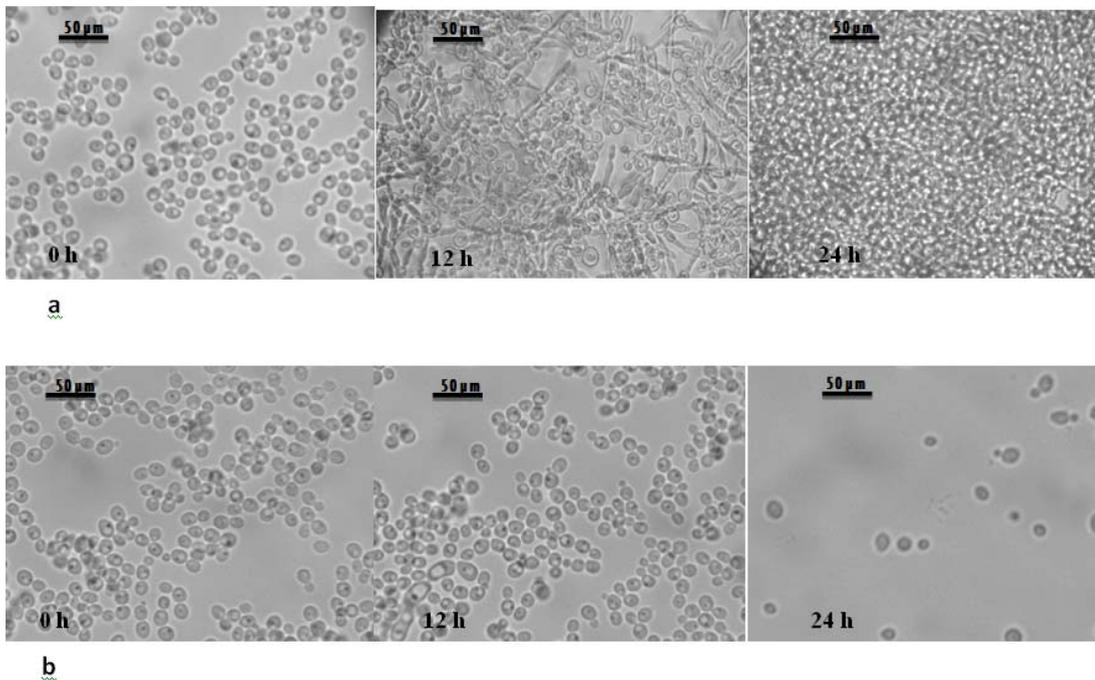


Fig. 2. Microscopic view of *Candida albicans* ATCC 14053 pre-biofilm production at different time intervals incubated in 37°C. (a) Untreated control, (b) Treated by hexane-Shallot extract. Magnification $\times 40$, Bar = 50 μm .

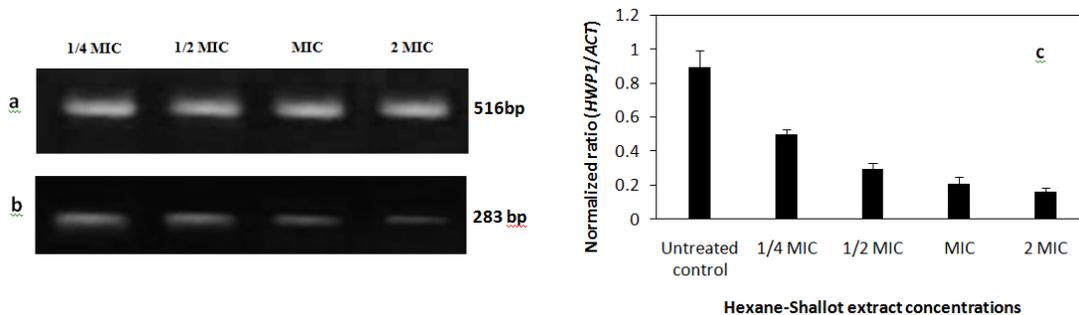


Fig. 3. Gel electrophoresis of semi quantitative RT-PCR products of pre-biofilm related gene from *Candida albicans* ATCC 14053 treated by hexane-Shallot extract in different concentrations based on MICs value, (a) Beta actin gene, (b) *HWPI*. (c) Relative quantitation of *HWPI* expression (normalized to β -actin) in *Candida albicans* ATCC 14053 after 24 h of treatment with different concentrations of hexane-Shallot extract

Table 2. Relative MICs ($\mu\text{g/ml}$) of alkaloid extract of Shallot and amphotericin B on selected *Candida* species

Isolates	Hexane extract		Ethyl acetate extract		Methanol extract		Aqueous extract		Amphotericin B	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
<i>C. albicans</i> ATCC 14053	10-15	18-30	18-30	40-60	20-40	80-120	90-150	250-300	0.025-0.12	0.5-0.75
<i>C. albicans</i> 3092	5-10	18-40	10-20	40-60	20-40	80-90	80-120	200-250	0.0125-0.12	0.25-0.5
<i>C. tropicalis</i> ATCC 750	20-30	40-50	30-40	50-60	40-50	50-80	120-250	300-400	0.05-0.12	0.25-0.75
<i>C. tropicalis</i> 5483	10-20	30-40	20-30	50-60	20-30	50-60	80-120	250-300	0.0125-0.05	0.12-0.25
<i>C. parapsilosis</i> ATCC 22019	10-20	30-50	10-30	30-40	30-40	60-80	80-120	250-300	0.025-0.12	0.25-0.5
<i>C. parapsilosis</i> 2707	10-20	30-50	10-20	40-60	40-50	60-90	120-250	300-400	0.0125-0.12	0.25-0.5
<i>C. rugosa</i> ATCC 10571	30-50	80-100	30-40	80-100	20-30	60-80	250-300	400-500	0.12-0.25	0.5-0.75
<i>C. rugosa</i> 3114	20-30	60-80	30-40	80-90	20-40	80-90	250-300	500-600	0.025-0.12	0.5-0.75
<i>C. krusei</i> ATCC 6258	40-50	80-90	30-40	80-120	30-40	100-120	250-300	400-500	0.12-0.25	0.5-0.75
<i>C. krusei</i> 3109	30-40	80-100	20-40	80-100	40-50	100-120	300-400	500-600	0.12-0.25	0.5-1

*MIC values represent the average of 3 independent experiments.

antifungal properties of Persian Shallot (*A. ascalonicum* Linn.) against several clinically important *Candida* species.

Disc diffusion method was applied for preliminary screening of active antifungal crude extracts as described earlier by some reports²¹⁻²³. Validity of the experiment could be determined without misinterpretation as contamination can be easily recognised. Our findings demonstrated that all isolates tested were susceptible towards different Shallot extracts and amphotericin B in terms of inhibition zone and this anti-*Candida* activity was ranged from 15.30- 37.33 mm, 7.47-31.25 mm, 8-35.5 mm, 11.33-31.20 mm and 11.30-33.56 mm for *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. rugosa* and *C. krusei*, respectively (Table 1). These findings were confirmed by determination of MICs. The MICs value were ranged from 5-100 $\mu\text{g/mL}$, 10-120 $\mu\text{g/mL}$, 20-120 $\mu\text{g/mL}$ and 80-600 $\mu\text{g/mL}$ for Shallot-hexane extract, Shallot-ethyl acetate extract, Shallot-methanol extract and Shallot-aqueous extract, respectively against *Candida* species tested (Table 2). All isolates were also susceptible when treated by amphotericin B and MICs were ranged from 0.0125-1 $\mu\text{g/mL}$ (Table 2). The combined results from both disc diffusion agar and broth microdilution tests have demonstrated that Shallot extracts have the slightly poorer efficacy than amphotericin B in terms of *Candida* growth inhibition. These results also seemed to suggest that amphotericin B and Shallot-alcoholic extracts were comparable in their abilities to inhibit the growth of the yeast cells, while Shallot-aqueous extract was not (Fig. 1). This may be due to presence of its content of phenolic and sulfur compounds (like Garlic). On the other hand, the organic compounds in Shallot like flavonols such as quercetin, and sulfur compounds such as diallyl disulphide could be more soluble in the alcohol²⁴. Interestingly the total content of these compounds in Shallot extract were higher than in Garlic and this is the main reason that Shallot is more powerful than Garlic to inhibit microorganisms²⁵.

Clearly, *Amaryllidaceae* is a famous family in Plantae kingdom including different genus and species such as Garlic, Onion and Shallot. The presence of sulfur compounds in these plants is characterized by their high potential bioactivities. Most of these properties are linked to redox

Table 3. XTT assay results for biofilm-associated *C. albicans* ATCC 14053 treated with Shallot-hexane extract

Concentration of Shallot-hexane extract	Means absorbance at 490 nm \pm SD
Untreated control	3.904 \pm 0.0048 ^a
1/4 \times MIC	0.011 \pm 0.0014 ^b
1/2 \times MIC	0.008 \pm 0.0013 ^b
1 \times MIC	0.008 \pm 0.0013 ^b
2 \times MIC	0.006 \pm 0.0004 ^b

*a,b: Mean \pm standard deviation in a column with different superscript differ significantly ($p < 0.05$) using Duncan test. The results were performed in three independent experiments.

processes, metal binding and catalytic reactions^{24,26}. Allicin originated from Garlic is the best example to show the strong antimicrobial activities. Indeed, the main biological abilities of garlic and its organo-sulfur derivatives such as allicin are characterized with high potential of selectivity to thiols (S-H). The disulfide-S-oxides bond can react with low molecular weight protein contained thiol such as glutathione, which may result in the inhibition of function in the cells²⁶⁻²⁸. Additionally, it is also demonstrated that allicin was effective in inhibiting biofilm production of *C. albicans* to an extent that is sometimes equal or more than fluconazole as a standard drug¹⁷.

The biological activities of Shallot may be also due to presences of sulphur compounds although it is not well understood. It is demonstrated that the saponins such as alliospiroside A and B could be the predominant antifungal compounds in the Shallot-alcohol extract. Indeed alliospiroside A has potential to antifungal activity against a wide range of fungi¹⁸. The microscopic observation in the present study demonstrated that Shallot-alcohol extract could inhibit to form biofilm in *C. albicans* in early stage (Fig. 2). Our data from gene expression study also showed that *HWPI* was down-regulated in *C. albicans* after treatment with Shallot-alcohol extract and ranged from 1.8, 3.1, 4.3 and 5.5 folds in 1/4 \times MIC, 1/2 \times MIC, 1 \times MIC and 2 \times MIC concentrations, respectively (Fig. 3). Interestingly, it seems Shallot-alcohol extract and allicin use the common target to inhibit biofilm in *C. albicans*

The data in this study provides a better understanding of the probable mechanisms of action of Shallot on *Candida*. Preliminary tests such as disc diffusion agar assay, broth microdilution assay and also time kill study conducted in this study as documented earlier have shown that all clinical and ATCC isolates of *Candida* species tested were sensitive to Shallot. Mostly, the previous reports have not been able to uncover the molecular evidence in terms of gene expression analysis. On the other hand, there are very little studies that investigated the activity of Shallot as an anticandidal agent *in vivo*. The gene expression results were supported by phenotypic observation of the biofilm disruption through light microscopy and XTT assay. Additionally, *HWPI* is introduced as a possible target of Shallot in *C. albicans* by this study. Further investigation will be needed to find the probable targets of Shallot and its bioactive compounds such as alliospiroside A and B on the hyphae, cell membrane, biofilm and other significant virulence factors of *C. albicans* using different techniques such as real time PCR and microarray.

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