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Quorum-Sensing Quenching Compounds *Allium sativum*, *Allium hirtifolium* and *Allium cepa:* The Probable Quorum-Sensing Quenching Compounds against *Candida albicans*

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Quorum-sensing quenching activity of *Allium sativum*, *A. hirtifolium* and *A. cepa* were screened against *Candida albicans*. The morphological response and expression patterns of selected genes involved in quorum-sensing including transcriptional repressor gene, *TUP1* and hypha-specific genes *HWP1, ALS1* and *ALS3* were examined in *C. albicans* at different concentrations of tested extracts based on MICs. The data indicated that all extracts exerted antifungal effects through reducing the number of yeast form and inhibiting the transition from yeast to hyphae cells. Furthermore, the expression level of transcriptional repressor gene was up-regulated at different concentrations of tested extracts, which correlated with the qualitative and quantitative assessment of *C. albicans*. We find hypha-specific genes exhibited changes in expression at the time intervals of tested extracts exerted antifungal effects on *C. albicans*. These changes are in the opposite direction exhibited in transcriptional repressor gene. These are likely to be key genes in the quorum-sensing quenching in *C. albicans* during treated with tested extracts. Together, the results provide a valuable resource to quorum-sensing quenching mechanism in *C. albicans*. Given the capability of *A. sativum*, *A. hirtifolium* and *A. cepa* extracts to effects on *C. albicans* and differentially expression of transcriptional repressor and hypha-specific genes, it is suggested that the tested extracts probably have the potential to be to be used as quorum-sensing *quenchers*. The *A. sativum*, *A. hirtifolium* and *A. cepa* extracts can be used to develop powerful new therapeutic approaches.

Keywords: *ALS1; ALS3; Candida albicans*; *HWP1; TUP1*.

The polymorphic yeast *Candida albicans* is an opportunistic fungal pathogen found as part of the human commensal flora (Odds *et al*., 2003; Kruppa, 2008). Opportunistic fungal infections represent a serious threat, in an expanding population of immune- and medically compromised patients (Khodavandi *et al*., 2010). It is now well established that multiple virulence factors play critical role in the pathogenesis of *C. albicans*. These factors include transition from yeast to hyphae cells, molecules which mediate adhesion to and invasion into host cells, contact sensing and thigmotropism, biofilm formation, the ability to production of secreted hydrolytic enzymes (mainly proteinases and phospholipases), phenotypic switching (white-opaque transition) as well as evasion of host immune cells and a range of fitness attributes (Kruppa, 2008; Mayer *et al*., 2013; da Silva-Rocha *et al*., 2014).

Biofilm formation represents one of the many major virulence factors contributing to the pathogenesis of *C. albicans.* Biofilm formation occurs on both biological and inert surfaces through a series of steps: (i) the adhesion of yeast cells to the surface, (ii) the establishment of a microcolony along with the production of germ tubes, (iii) hyphal cells form a network with further

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maturation of the biofilm consisting of different cell types within an extracellular matrix, and (iv) newly formed yeast on the surface of the biofilm are released from the thick and stratified biofilm which allows further systemic dissemination of the microorganism (Kruppa, 2008; Khodavandi *et al*., 2011a; Nett, 2014). Many lines of evidence demonstrate that agglutinin like sequence (*ALS*) family and hyphal cell wall protein (*HWP1*) genes interfere with biofilm formation by *Candida* (Khodavandi *et al*., 2011a; Cuellar-Cruz *et al*., 2012).The *Candida* Tup1 protein is a transcription regulator that plays a role both in the regulation of the transition from yeast to hyphae cells and the transcription of a variety of virulence genes (Kebaara *et al*., 2008). It is relevant to highlight that the signal transduction pathways and quorum-sensing molecules play important role in biofilm development and further dissemination of infection (Kruppa, 2008; Han *et al*., 2011; Sharma and Jangid, 2013). *C. albicans* was found to produce a range of quorum-sensing molecules, including farnesol, tyrosol, farnesoic acid tryptophol, phenylethyl alcohol and morphogenic autoregulatory substance, although the precise mechanisms of action of these molecules remain unclear. Quorum-sensing has been described as a phenomenon contributing to morphogenic control in *C. albicans* through changes in cell density, which known as the inoculum effect. It was observed that *C. albicans* inoculated at cell densities below 10^6 cells/ml under certain conditions, will germinate into the hyphal form. However, if cells densities are greater than 10^6 cells/ml, little germination will occur and they remain predominantly in a yeast state (Kruppa, 2008; Han *et al*., 2011).

Recently, therapeutic approach for quorum-sensing quenching was pioneered by quorum-sensing inhibitors. There are three ways by which the quorum-sensing signaling can be stopped/hampered: (i) stop the production of signal molecules, (ii) the signal molecules can be degraded by enzyme(s), (iii) if the signal molecule is not allowed to bind to the receptor molecule, it will not act as a regulator to the gene promoter. In either case, it would disrupt the regulation circuit. Research over the past few years has demonstrated that quorum-sensing quenching mechanisms are widely conserved in organisms and can be used for developing and formulating a new generation of antimicrobials. Many medicinal and dietary plants are known to produce quorum-sensing quenching activity. Quorum-sensing quenching activity has been reported from the genus *Allium*, including *Allium sativum* (garlic) and *A. cepa* (onion) (Sharma and Jangid, 2013; Lade *et al*., 2014).

A. sativum, *A. hirtifolium* (shallot) and *A. cepa* have been used traditionally as the antimicrobial agents. It is demonstrated that *A. sativum*, *A. hirtifolium* and *A. cepa* extracts have been inhibitory activity against *C. albicans* planktonic cells and biofilms (Shuford *et al*., 2005; Palmeira-de-Oliveira *et al*., 2013; Vashisth *et al*., 2013; Khodavandi *et al*., 2014a). These results demonstrate the effects of fresh *A. sativum*, *A. hirtifolium* and *A. cepa* extracts as inhibitors of hyphae formation in *C. albicans* and suggest a possible link to quorum-sensing quenchers. The morphological response and expression patterns of selected genes involved in quorum-sensing including transcriptional repressor gene, *TUP1* and hypha-specific genes *HWP1*, *ALS1* and *ALS3* were examined in the presence or absence of *A. sativum*, *A. hirtifolium* and *A. cepa* extracts by relative quantitative RT-PCR analysis.

MATERIALS AND METHODS

Microorganisms

Ten clinical isolates of *Candida albicans* which were obtained from patients with systemic candidiasis and *C. albicans* ATCC 10231 as a reference quality-control strain was employed. All the isolates were maintained as sterile 20% (v/v) glycerol stocks and subcultured on Sabouraud dextrose agar with chloramphenicol (SDA, Difco Laboratories, Detroit, Michigan) at 35–37°C for 24–48 h to ensure viability and purity prior to testing.

Preparation of plant extracts

The bulbs of *Allium sativum*, *Allium hirtifolium* and *Allium cepa* were sourced from a Shiraz, Iran traditional medicine shop in mid-May and voucher specimens were confirmed by Department of Botany, University of Tehran. The plants were extracted according to the method described by Khodavandi *et al*. (2014a) with slight modifications. The fresh bulbs of *A. sativum*, *A. hirtifolium* and *A. cepa* were washed with sterile distilled water, sliced and dried in the oven for at least two days. The dried plant bulbs were crushed

to fine powder, passed through an 80 mesh sieve and stored in a sealed plastic bag. Subsequently, one g sample of the plant bulbs powder was mixed with 10 ml of sterile distilled water, ethanol or methanol to give a stock solution of 100 mg/ml (w/v). The extracts were incubated at room temperature for 30 min and finally the extracts had undergone a sequential extraction using soxhlet. The organic solvents were from the bulbs were filtered by Whatman No. 1 filter paper and the filtrate fractions were concentrated. Eventually, the different concentrations of tested extracts were made by dissolving in 5% DMSO and filter-sterilized (0.22-ìm durapore, Millipore).

Antimicrobial susceptibility tests

The antifungal disk diffusion susceptibility test was performed by following method of Clinical and Laboratory Standards Institute (CLSI- M_{44}-A) for yeast cell with slight modification. Briefly, *C. albicans* isolates were cultured on SDA and passaged at least twice to ensure viability and purity, and then incubated overnight at 37 ºC. Subsequently, five colonies that are more than 1mm in diameter of the overnight grown culture of *C. albicans* isolates were inoculated into 5 ml of sterile 0.85% saline and vortexed for 15 s. Cell densities were adjusted to1 $\times 10^6$ to 5×10^6 yeast cells/ml using spectrophotometer at 530 nm wavelengths to achieve the turbidity equivalent to 0.5 McFarland standards. Hundred µl of the cell suspension was poured on SDA and kept at room temperature for 15 min to dry. The prepared tested extracts was added at a final concentration of 50, 60, 70, 80, 90 and 100 mg/ml to paper discs and placed on the agar surface. Serial tenfold dilution was carried out to make different concentrations of plant extracts. Discs containing fluconazole (Sigma-Aldrich, St. Louis, MO, USA) with the concentration of 5 mg/ml were maintained as positive controls. The plate was incubated at 37°C for 24 h and observed for inhibition zone. Three technical replicates were performed for each test and experiment was repeated at least two times (Khodavandi *et al*., 2011a; 2014a, b).

In order to determine the MIC of the plant extracts against *C. albicans*, the extracts were subjected to broth dilution antifungal susceptibility test. The broth microdilution antifungal susceptibility test was carried out using CLSI reference method for yeast (M_{27}-A_3). The test was carried out using 96-well U-bottom tissue culture microplates containing 100 µl/well of (SDB, Fluka, Germany). Hundred ìL of the twofold dilution of the different concentrations of tested extracts were added to the wells. The antifungal agent, fluconazole was used as positive control. Yeast cells inocula were prepared (530 nm, abs 0.08–0.1) and diluted to reach a final concentration of 5×10^2 – 2.5×10^3 yeast cells/ml in the wells. Then the plates were incubated at 35°C for 24h. The endpoint was defined as the lowest concentration of each antifungal that caused 50% and 90% growth inhibition compared to control-growth (Khodavandi *et al*., 2011a; 2014a, b).

MFC values of tested extracts were determined for *C. albicans* isolates according to Canton *et al.* (2004). Briefly; MFCs were evaluated by transferring 0.1 ml from clear MIC wells following 24h of incubation onto Petri dishes containing SDA. The MFC was defined as the lowest extracts concentration that that killed e" 99.9% of yeast cells.

To evaluate the fungicidal effects of tested extracts against *C. albicans* time-kill assay was performed. Four ml of growing culture (10^6 cell/ml) of representative *C. albicans* isolates was added to the same volume of 1 × the MIC of extracts, and extract free medium was used as the growth control. Samples were obtained from each tube at predetermined time points of 0, 2, 6, 12, 24, 36 and 48 h for viable colony counts. The viable counts were determined using the serial dilution method after incubation at 35°C for 24 h. The lower limit of reproducibly quantifiable CFU according to these methods was 50 CFU/ml. All assays were conducted in in triplicates (Khodavandi *et al*., 2011b; 2014a, b).

Evaluation of quorum-sensing quenching activity

Quorum-sensing quenching activity of tested extracts on the *C. albicans* was performed by following method by Lim *et al*. (2009). Briefly, *C. albicans* ATCC 10231yeast cells were grown in Winge medium (0.4% glucose, 0.6% yeast extract) and placed into a *shaking incubator* at 25 °C overnight. The yeast cells were washed in phosphate buffer saline (pH 7.2) and adjusted to a cell density of 1×10^6 cells/ml in RPMI-1640 with L-glutamine (Sigma) supplemented with10% fetal bovine serum (Gibco, Invitrogen) and 100 U/ml

penicillin-streptomycin (Gibco, Invitrogen). The cultures were incubated at 37 °C with 5% CO_2 without and with aqueous plant tested extracts at different concentrations based on MIC (1/4× MIC, 1/2× MIC, 1× MIC and 2× MIC). The cultures were used for qualitative assay, as well as RNA extraction and quantitative RT-PCR analysis. We employed inverted light microscopy method as a qualitative assay to investigate the quorum-sensing quenching activity of tested extracts on the *C. albicans* at the time intervals of 0, 12 and 24 h.

RNA extraction and cDNA amplification

A suspension containing different concentrations of aqueous plant tested extracts and 1×10^6 cells/ml of *C. albicans* ATCC 10231were prepared as in the sample preparation for quorum-sensing quenching activity as explained above. The mixture was *subsequently* pelleted by *centrifugation at 3000 rpm* for 10 min and washed using PBS at least three times. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with slight modifications. RNA quality and quantity were determined by formaldehyde-denaturing agarose gel electrophoresis and Nano Drop Spectrophotometer ND-1000 (NanoDrop Technologies Inc., Wilmington, DE). Any genomic DNA contamination was removed using DNase I (Promega, USA). Total RNA (0.5 µg) was copied into complementary DNA (cDNA) with Moloney-Murine Leukemia Virus (MMLV) reverse transcriptase and random hexamers ((Fermentas, USA) according to the manufacturer's instructions. Each reaction was performed in three technical replications for each of three hyphal induction procedures with and without tested extracts.

Relative real time RT-PCR

C. albicans transcription regulator gene, *TUP1* and hypha-specific genes, *HWP1, ALS1 and ALS3* genes were amplified from the synthesized cDNA with primers as described in Table 1. Moreover, actin was established as a house-keeping gene to normalize the dissimilar RNA concentrations during RNA extraction. Semi-quantitative RT-PCR was conducted using ™SYBR Green qPCR Master Mix (Fermentas, EU) via Bio-Rad MiniOpticon™ system (USA). The cycling conditions included an initial step at 50æ%C for 2 min; holding at 95æ%C for 10 min, 40 cycles of denaturation at 95æ%C for 15 s and subsequently annealing at 60æ%C for 1 min. Eventually, the melting reaction was performed from 72–99æ%C. Relative gene expression was quantified by the Pfaffl method (Khodavandi *et al*., 2011a).

Statistical analysis

All experiments were performed in triplicate. Results are expressed as mean value ± standard deviations (S.D) of three replicates and analyzed by using the software SPSS 20.0 for windows (SPSS Inc. Chicago, IL, USA).

RESULTS

The results of preliminary disc diffusion assay screening on *A. sativum*, *A. hirtifolium* and *A.cepa* extracts revealed that tested extracts could able to show the strong inhibitory activity against *C. albicans* isolates comparable to standard antifungal agent. The antimicrobial activity (assessed in terms of inhibition zone) of the tested extracts was found to be more potent at a concentration of 100 mg/ml (Table 2). However, *A.*

Table 1. Oligonucleotide primers used for PCR

Gene	Primer sequence (5'→3')	PCR product size (bp)	Reference
TUP1	Forward: CTCTTGGCGACAGGTGCAG Reverse: GTGGTGACGCCGTCTTCGA	224	(Toyoda *et al*., 2004)
HWP1	Forward: TCAGTTCCACTCATGCAACCA Reverse: AGCACCGAAAGTCAATCTCATGT	99	(Khodavandi *et al*., 2011a)
ALS1	Forward: TTCTCATGAATCAGCATCCACAA Reverse: CAGAATTTTCACCCATACTTGGTTTC	53	(Green *et al*., 2005)
ALS3	Forward: AATGGTCCTTATGAATCACCATCTACTA Reverse: GAGTTTTCATCCATACTTGATTTCACAT	56	(Green *et al*., 2005)
ACT	Forward: GAGTTGCTCCAGAAGAACATCCAG Reverse: TGAGTAACACCATCACCAGAATCC	199	(Lim *et al*., 2009)

Table 2. Antimicrobial activities of different *Allium sativum*, *A. hirtifolium* and *A. cepa* extracts against isolates of *Candida albicans* at a concentration of 100 mg/ml of tested extracts

Isolates	Inhibition zone (mm)									
	A. sativum			*A. hirtifolium*			*A. cepa*			
	Ethanol extract	Methanol extract	Aqueous extract	Ethanol extract	Methanol extract	Aqueous extract	Ethanol extract	Methanol extract	Aqueous extract	Fluconazole
C. albicans ATCC 10231	17.15 ± 0.20	18.04 ± 0.11	17.12 ± 0.08	26.80 ± 0.01	25.00 ± 0.11	25.55 ± 0.90	17.03 ± 0.33	17.00 ± 0.10	16.03 ± 0.11	33.01 ± 0.01
CI-1	17.20 ± 0.18	18.34 ± 0.16	16.90 ± 0.04	25.12 ± 0.34	25.65 ± 0.74	24.15 ± 0.44	17.56 ± 0.54	16.05 ± 0.11	14.22 ± 0.09	33.11 ± 0.16
CI-2	17.15 ± 0.66	17.87 ± 0.68	16.70 ± 0.19	25.01 ± 0.21	24.85 ± 0.54	24.15 ± 0.44	16.32 ± 0.16	17.35 ± 0.24	15.15 ± 0.64	32.32 ± 0.51
CI-3	16.37 ± 0.35	17.11 ± 0.46	16.96 ± 0.19	26.01 ± 0.18	25.01 ± 0.78	26.11 ± 0.10	16.17 ± 0.16	16.71 ± 0.31	14.57 ± 0.24	33.00 ± 0.09
CI-4	17.29 ± 0.15	18.20 ± 0.33	15.80 ± 0.18	26.09 ± 0.30	25.19 ± 0.20	25.35 ± 0.30	17.05 ± 0.23	15.26 ± 0.44	13.28 ± 0.17	33.10 ± 0.11
CI-5	16.33 ± 0.30	18.10 ± 0.06	17.10 ± 0.19	25.25 ± 0.22	24.45 ± 0.16	24.05 ± 0.12	15.15 ± 0.11	16.85 ± 0.02	14.75 ± 0.17	33.00 ± 0.03
CI-6	16.62 ± 0.11	17.03 ±0.19	16.05 ± 0.24	26.18 ± 0.12	24.17 ± 0.14	26.14 ± 0.44	16.10 ± 0.11	17.11 ± 0.22	13.73 ± 0.45	32.02 ± 0.15
CI-7	17.26 ± 0.08	17.94 ± 0.73	17.03 ± 0.18	25.22± 0.12	25.20 ± 0.10	24.00 ± 0.90	15.36± 0.16	15.20 ± 0.20	14.35 ± 0.30	33.11 ± 0.11
CI-8	17.04 ± 0.25	18.41± 0.45	15.99 ± 0.18	25.65 ± 0.10	25.85 ± 0.80	25.35 ± 0.40	17.55 ± 0.18	16.05 ± 0.11	14.75 ± 0.68	33.01 ± 0.11
CI-9	16.30 ± 0.05	17.65 ± 0.10	16.35 ± 0.12	25.27 ± 0.68	24.23 ± 0.48	26.33 ± 0.18	16.36 ± 0.10	15.23 ± 0.11	14.55 ± 0.48	33.07 ± 0.22
CI-10	17.04 ± 0.40	17.76 ± 0.11	15.13 ± 0.25	26.45 ± 0.66	24.05 ± 0.16	27.55 ± 0.36	15.76 ± 0.26	15.11 ± 0.31	15.90 ± 0.66	32.22 ± 0.22

CI: Clinical isolates of *C. albicans*
Data are means ± standard deviation of three independent experiment

hirtifolium extract showed the strongest inhibitory zone against *C. albicans* isolates in comparison to *A. sativum* and *A.cepa.* The results obtained using three different solvent showed no significant differences of inhibition of tested extracts on the growth of *C. albicans* isolates. The MIC and MFC values of these tested extracts against *C. albicans* isolates are shown in Table 3. The MIC for all isolates was d" 25 mg/ml for tested extracts, with ranged from 5-15 mg/ml for *A. hirtifolium,* 11-25 mg/ml for *A. sativum* and *A.cepa*. These results indicated that the best antimicrobial activity was observed for *A. hirtifolium* extract with lowest MIC value 5-8 mg/ml, MFC value 11-14 mg/ml and inhibition zone 27.55 ± 0.36. The killing patterns of extracts tested against *C. albicans* isolates were shown in Fig. 1. All tested extracts significantly reduced the number of viable cells at different time intervals (*p*d" 0.0001).

Findings from the microscopic examination displayed quorum-sensing quenching activity of tested extracts on the *C. albicans* ATCC 10231 at the time intervals. *C. albicans* cells treated with *A. hirtifolium, A. sativum* and *A.cepa* extracts showed a significant reduction in the number of yeast form. In addition, the transition from a yeast cell to the hyphal form was reduced, while the untreated control was able to form hyphae and structured to show the pre-biofilm after 24 h incubation (Fig. 2).

Relative quantitative RT-PCR analysis of transcriptional repressor and hypha-specific genes were conducted in *C. albicans* ATCC 10231 exposed to *A. hirtifolium, A. sativum* and *A.cepa* extracts. The expression levels of the transcriptional repressor and hypha-specific genes were significantly different (*p*d" 0.0001) at all concentrations of tested extracts based on MIC (Fig. 3). In addition, patterns of *C. albicans* gene expression in response to quorum-sensing quenching activity of tested extracts were similar for hypha-specific genes *HWP1, ALS1* and *ALS3*. While, the expression of *TUP1* was different from the expression of *HWP1, ALS1* and *ALS3* as expected. The expression level of *HWP1, ALS1* and *ALS3* were significant (*p*d" 0.0001) down-regulated compared to untreated control. In the present study, the transcriptional repressor and hypha-specific genes demonstrated the highest fold change

Table 3. Relative MIC and MFC values of different *Allium sativum*, *A. hirtifolium* and *A. cepa* extracts against isolates of *Candida albicans*

	Tested extracts/Isolates		*C. albicans* ATCC10231	CI-1	CI-2	CI-3	CI-4	CI-5	CI-6	CI-7	CI-8	CI-9	CI-10
A. sativum[a]	Ethanolextract	MIC_{50}	12-15	11-14	11-14	11-14	12-15	12-15	11-14	11-14	12-15	12-15	11-14
		MIC_{90}	21-24	21-24	21-24	21-24	22-25	22-25	21-24	21-24	21-24	22-25	21-24
		MFC	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27
	Methanol extract	MIC_{50}	11-14	11-14	11-14	11-14	11-14	12-15	12-15	11-14	12-15	11-14	11-14
		MIC_{90}	21-24	21-24	21-24	21-24	21-24	22-25	22-25	21-24	22-25	21-24	21-24
		MFC	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27
	Aqueous extract	MIC_{50}	12-15	12-15	12-15	12-15	12-15	12-15	12-15	12-15	12-15	12-15	12-15
		MIC_{90}	22-25	22-25	22-25	22-25	22-25	22-25	22-25	22-25	22-25	22-25	22-25
		MFC	24-27	25-28	25-28	25-28	25-28	25-28	25-28	25-28	25-28	25-28	25-28
A. hirtifolium[a]	Ethanolextract	MIC_{50}	6-9	5-8	6-9	6-9	5-8	5-8	5-8	5-8	5-8	5-8	5-8
		MIC_{90}	11-14	10-13	11-14	11-14	10-13	10-13	10-13	10-13	10-13	10-13	10-13
		MFC	13-16	11-14	12-15	12-15	12-15	12-15	12-15	12-15	12-15	11-14	12-15
	Methanol extract	MIC_{50}	6-9	5-8	6-9	6-9	5-8	5-8	5-8	5-8	5-8	5-8	5-8
		MIC_{90}	11-14	10-13	11-14	12-15	10-13	10-13	10-13	10-13	10-13	10-13	11-14
		MFC	13-16	13-16	12-15	13-16	13-16	13-16	11-14	12-15	12-15	12-15	12-15
	Aqueous extract	MIC_{50}	5-8	5-8	6-9	6-9	6-9	6-9	5-8	6-9	5-8	6-9	6-9
		MIC_{90}	10-13	10-13	10-13	10-13	11-14	11-14	10-13	10-13	11-14	10-13	11-14
		MFC	12-15	13-16	13-16	12-15	12-15	12-15	13-16	13-16	13-16	13-16	13-16
A. cepa[a]	Ethanolextract	MIC_{50}	12-15	12-15	12-15	12-15	12-15	12-15	12-15	12-15	12-15	12-15	12-15
		MIC_{90}	22-25	21-24	22-25	21-24	22-25	22-25	22-25	22-25	21-24	21-24	21-24
		MFC	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27
	Methanol extract	MIC_{50}	12-15	11-14	12-15	11-14	11-14	12-15	12-15	11-14	11-14	11-14	12-15
		MIC_{90}	22-25	21-24	22-25	21-24	21-24	22-25	22-25	21-24	21-24	21-24	22-25
		MFC	25-28	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27	24-27
	Aqueous extract	MIC_{50}	12-15	12-15	12-15	12-15	12-15	12-15	12-15	12-15	12-15	12-15	12-15
		MIC_{90}	22-25	22-25	22-25	22-25	22-25	22-25	22-25	22-25	22-25	22-25	22-25
		MFC	25-28	25-28	25-28	25-28	25-28	25-28	25-28	25-28	25-28	25-28	25-28
Fluconazole[b]		MIC_{50}	0.5-2	0.5-2	4-8	1-4	1-4	1-4	1-4	4-8	4-8	1-4	1-4
		MIC_{90}	1-4	1-4	16-32	8-16	8-16	8-16	8-16	16-32	16-32	8-16	8-16
		MFC	8-16	8-16	32->64	16-32	16-32	16-32	16-32	32->64	32->64	16-32	16-32

[a]mg/mL;[b]ìg/mL;CI: Clinical isolates of *C. albicans*
Data are means ± standard deviation of three independent experiments

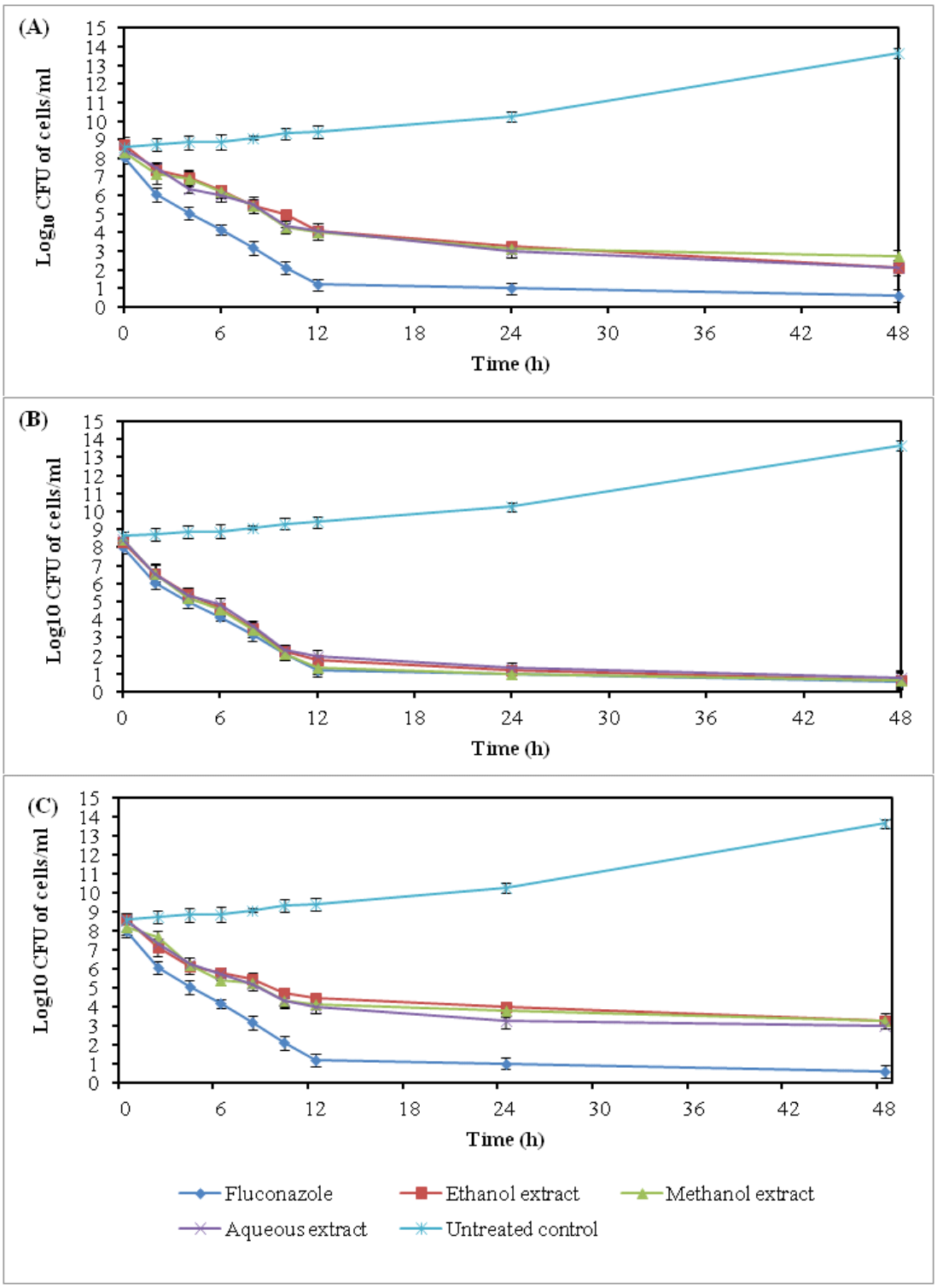


Fig. 1. Time dependent killing of sessile *Candida albicans* ATCC 10231 cells treated by different (A) *Allium sativum*, (B) *A. hirtifolium* and (C) *A. cepa* extracts

expression in *C. albicans* treated with *A. hirtifolium* compared to *A. sativum* and *A.cepa* extracts. The fold changes in terms of *HWP1* expression to untreated control for 2× MIC, 1× MIC, ½× MIC and ¼× MIC of *A. hirtifolium* were 5.56 ± 0.002, 2.44 ± 0.001, 1.70 ± 0.002 and 1.22 ± 0.004, respectively. Moreover, the fold changes in untreated control in terms of *ALS1* expression for 2× MIC, 1× MIC, ½× MIC and ¼× MIC of *A. hirtifolium* were 4.76 ± 0.005, 1.92 ± 0.002, 1.41 ± 0.004 and 1.14 ± 0.004, respectively. The *ALS3* mRNA was down-regulated 4.17 ± 0.001, 2.13 ± 0.004, 1.70 ± 0.001 and 1.14 ± 0.003 at *A. hirtifolium* concentrations of 2× MIC, 1× MIC, ½× MIC and ¼× MIC, respectively. While, the fold changes regarding to *TUP1* expression for 2× MIC, 1× MIC, ½× MIC and ¼× MIC of *A. hirtifolium* were 2.81 ± 0.001, 2.59 ± 0.002, 2.35 ± 0.00 and 2.00 ± 0.002, respectively. The authenticity of the PCR products was verified by DNA sequencing method using an outsourcing sequencing service. The sequences displayed high similarity analyzed via nucleotide BLAST in Gene Bank and confirmed in terms of homology to the related genes.

DISCUSSION

Quorum-sensing quenching activity has been a promising strategy to combat microbial infections. In this way it does not impose any selection pressure on the pathogen and, as such, a predominantly resistant population should be less likely to emerge. Plant and their derived metabolites have been extensively studied as quorum-sensing quenching approaches (Adonizio *et al*., 2006; Koh and Tham, 2011; Koh *et al*., 2013; Viswanathan *et al*., 2015). These data demonstrate that some species of the genus *Allium* play an important role in quorum-sensing quenching activity. These findings are consistent with previous observations demonstrate that quorum-sensing quenching activity could be induced by the genus *Allium* (Sharma and Jangid, 2013; Lade *et al*., 2014; Viswanathan *et al*., 2015).

The antimicrobial susceptibility results indicated that all *C. albicans* isolates were susceptible to plant extract tested. It is well known that the genus *Allium* have excellent inhibitory activity against *C. albicans* (Shuford *et al*., 2005;

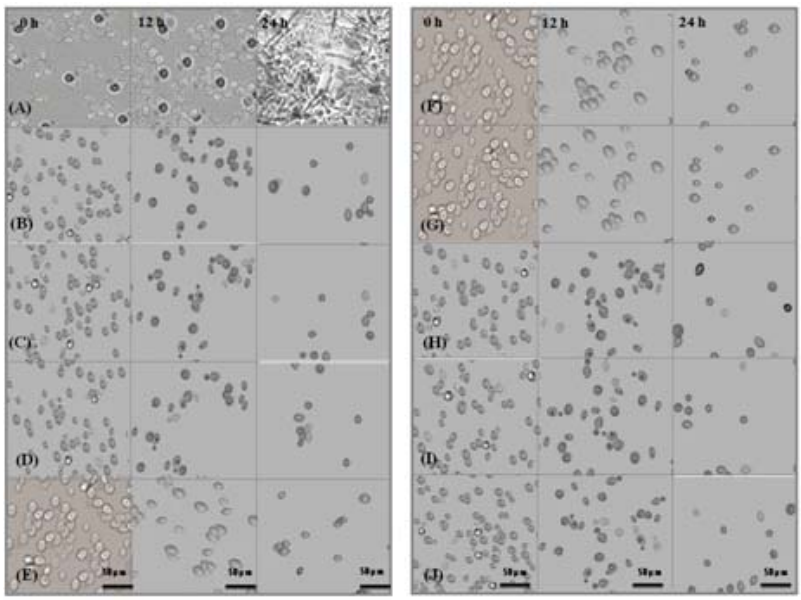
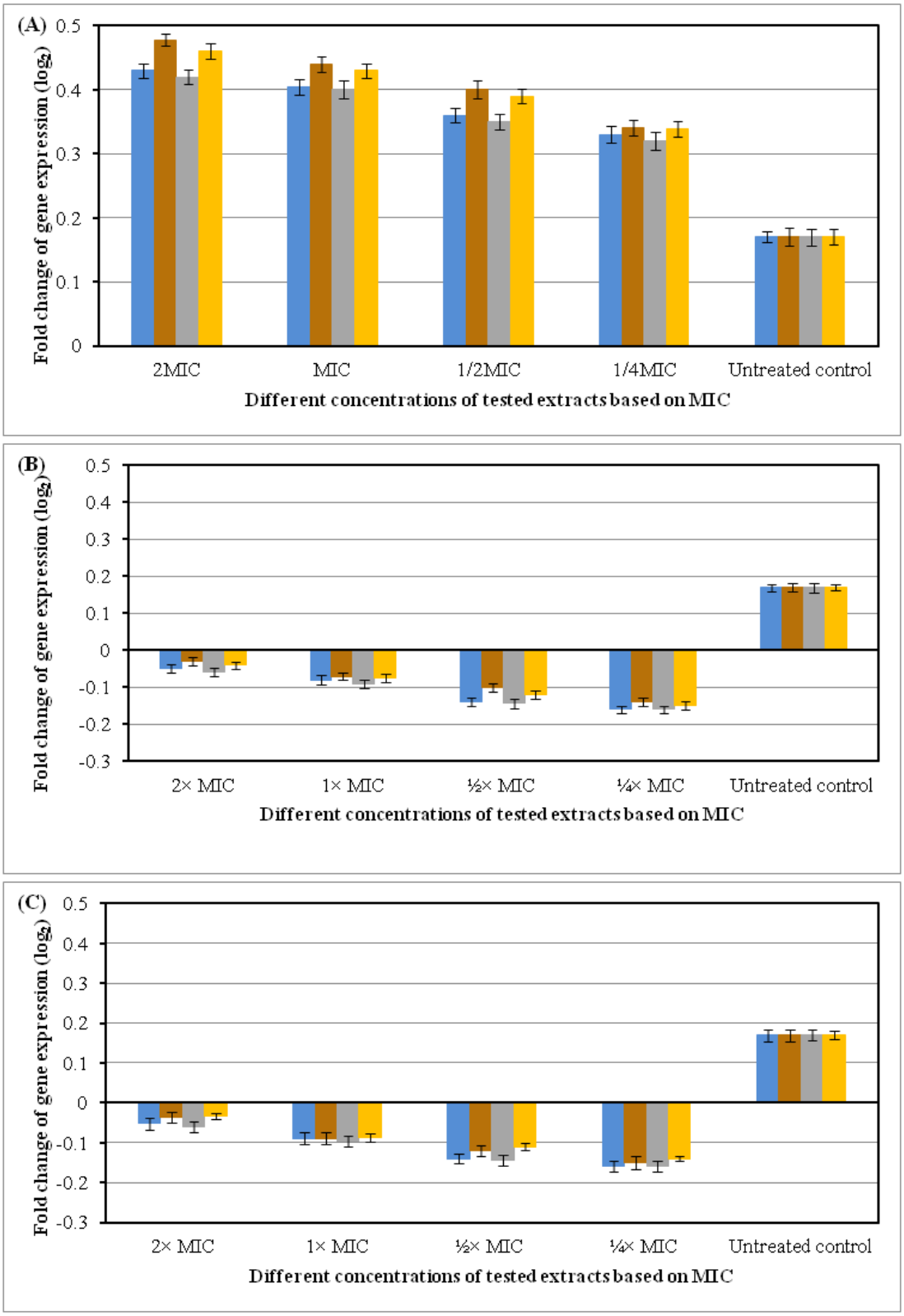


Fig. 2. Inverted light microscopic view of the quorum-sensing quenching activity of tested extracts on the *Candida albicans* ATCC 10231 at different time intervals. (A) Untreated control (B) *Allium sativum* ethanol extract (C) *A. sativum* methanol extract (D) *A. sativum* aqueous extract (E) *A. hirtifolium* ethanol extract (F) *A. hirtifolium* methanol extract (G) *A. hirtifolium* aqueous extract (H) *A. cepa* ethanol extract (I) *A. cepa* methanol extract (J) *A. cepa* aqueous extract. Magnification × 40, Bar = 50 µm


(A)
Fold change of gene expression (log2)
0.5
0.4
0.3
0.2
0.1
0
2MIC
MIC
1/2MIC
1/4MIC
Untreated control
Different concentrations of tested extracts based on MIC
(B)
Fold change of gene expression (log2)
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
2× MIC
1× MIC
½× MIC
¼× MIC
Untreated control
Different concentrations of tested extracts based on MIC
(C)
Fold change of gene expression (log2)
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
2× MIC
1× MIC
½× MIC
¼× MIC
Untreated control
Different concentrations of tested extracts based on MIC

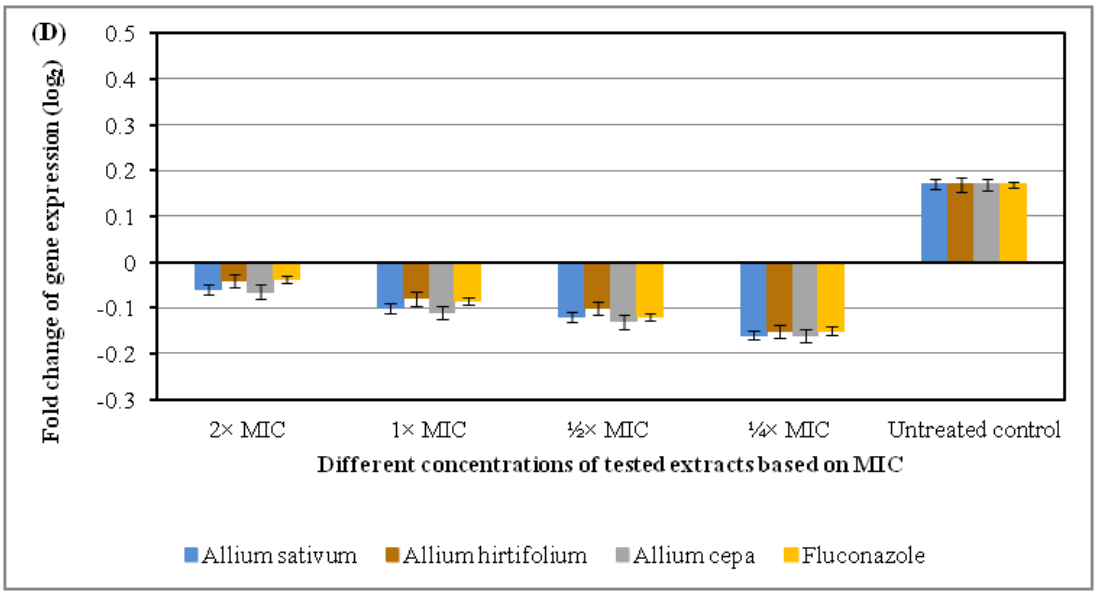


Fig. 3. Relative quantitation of (A) *TUP1*, (B) *HWP1*, (C) *ALS1* and (D) *ALS3* gene expression (normalized to house-keeping gene, actin) calculated with Log_2 in *Candida albicans* ATCC 10231 after 24 h of treatment with different concentrations of tested extracts by real time RT-PCR. Data are means of fold changes with standard deviation from three independent experiments amplified in triplicates

Palmeira-de-Oliveira *et al*., 2013; Vashisth *et al*., 2013; Khodavandi *et al*., 2014a). In this study, we analyzed the antifungal effects of *A. sativum*, *A. hirtifolium* and *A. cepa* extracts quantitatively and qualitatively by measuring the inhibition zone, minimum inhibitory concentration (MIC), minimal fungicidal concentration (MFC) and quorum-sensing quenching activity of *C. albicans*. In addition, we evaluated the expression analysis of selected genes related to quorum-sensing of *C. albicans* treated by *A. sativum*, *A. hirtifolium* and *A. cepa* extracts. The susceptibility profile exhibited for all *C. albicans* isolates to tested extracts. The genus *Allium* has been defined as the medications for many infectious diseases. It is demonstrated that the main bioactive components that originated from the genus *Allium* are sulfur-containing and non-sulfur components such as allicin and ajoene showing antifungal activities (Yoshida *et al*., 1987; Khodavandi *et al*., 2011a; Palmeira-de-Oliveira *et al*., 2013).

Quorum-sensing quenching activity of tested extracts on the *C. albicans* completely reduced the number of yeast form. This work demonstrated the ability of *A. hirtifolium*, *A. sativum* and *A.cepa* extracts to inhibit the transition from yeast to hyphae cells, a step essential to the virulence of *Candida*, suggesting that tested extracts could decrease the ability of *C. albicans* cells to cause disease. Similarly, Khodavandi *et al*. (2011c) found that the allicin damaged the cell surface of *C. albicans* resulting in the fungal load reduction and host survival time in alleviating systemic *C. albicans* infections.

In this study, the expression of *HWP1, ALS1 and ALS3*mRNA was found to be correlated with the transition from yeast to hyphae cells in *C. albicans* treated with *A. hirtifolium*, *A. sativum* and *A.cepa* extracts. The cells treated with tested extracts resulted in up-regulation of *TUP1* mRNA expression, which could result in relief of transcriptional repression at hypha-related gene promoters. The down-regulated hypha-specific genes *HWP1*, *ALS1* and *ALS3*, consistent with the morphological yeast-to-hyphal switch which completely reduced the number of yeast form and inhibit the transition from yeast to hyphae cells. In addition to signal transduction pathways, *C. albicans* yeast and filamentous growth are controlled by an assortment of quorum-sensing

molecules which trigger hyphal formation and biofilm development (Kruppa, 2008; Han *et al*., 2011; Sharma and Jangid, 2013). Previous investigations demonstrated that Tup1 plays an important role in the repression of filamentous growth and the expression of hypha-specific genes in *C. albicans* (Braun *et al*., 2000; Kebaara *et al*., 2008). In addition, some important genes in *C. albicans* encode adhesin and invasion and contribute to biofilm formation. *ALS* family is included of several glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins. Of the Als proteins, the hypha-associated adhesin Als1 and Als3 is especially important for adhesion. Another important adhesin of *C. albicans* is Hwp1, which is a hypha-associated GPI-linked protein. Tup1 acts negatively to regulate filamentous growth. *HWP1* and *ALS* formerly are activated by Efg1 and repressed by Tup1 (Hoyer *et al*., 2008; Bastidas *et al*., 2009; Mayer *et al*., 2013). Quorum-sensing quenching activity has been reported from *A. sativum*, and it has been found that *A. sativum* extract has a preference for the genes belonging to virulence and pathogenesis of *Pseudomonas aeruginosa* including like *LasA, LasB* (coding for elastase and protease), *rhlAB* encoding rhamnolipid, *chiC* (encoding for chitinase), as well *as aprA, phzA1B, phzS, phzC2D2E2F2G2, and PA1L* (Kalia, 2013).

CONCLUSION

Whether these events reflect the potential of *A. hirtifolium, A. sativum* and *A.cepa* extracts for quorum-sensing quenching activity in *C. albicans* which differentially expresses specific genes, requires further dissection. In summary, these results link the transcriptional repression gene to regulation of hypha-specific genes in a major human fungal pathogen. These functional clues will be combined with information from other approaches to further define of quorum-sensing quenching mechanisms. This might provide useful information to develop powerful new therapeutic approaches.

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REFERENCES

1. Adonizio, A.L., Downum, K., Bennett, B.C., Mathee, K. Anti-quorum sensing activity of medicinal plants in southern Florida. *J Ethnopharmacol* 2006; **105**: 427-435.
2. Bastidas, R.J., Heitman, J., Cardenas, M.E. The protein kinase Tor1 regulates adhesin gene expression in *Candida albicans*. *PLOS Pathog* 2009; 5, e1000294.
3. Braun, B.R., Head, W.S., Wang, M.X., Johnson, A.D. Identification and characterization of *TUP1*-regulated genes in *Candida albicans*. *Genetics* 2000; **156**: 31-44.
4. Canton, E., Peman, J., Gobernado, M., Viudes, A., Espinel-Ingroff, A. Patterns of amphotericin B killing kinetics against seven *Candida* species. *Antimicrob Agents Chemother* 2004; **48**: 2477-2482.
5. Cuellar-Cruz, M., Lopez-Romero, E., Villagomez-Castr, J.C., Ruiz-Baca, E. *Candida* species: new insights into biofilm formation. *Future Microbiol* 2012; **7**: 755-771.
6. da Silva-Rocha, W.P., Lemos, V.L.B., Svidizisnki, T.I.E., Milan, E.P., Chaves, G.M. *Candida* species distribution, genotyping and virulence factors of *Candida albicans* isolated from the oral cavity of kidney transplant recipients of two geographic regions of Brazil. *BMC Oral Health* 2014; **14**: 20.
7. Green, C.B., Zhao, X., Yeater, K.M.,Hoyer, L.L. Construction and real-time RT-PCR validation of *Candida albicans* PALS-GFP reporter strains and their use in flow cytometry analysis of *ALS* gene expression in budding and filamenting cells. *Microbiology* 2005; **151**: 1051-1060.
8. Han, T.L., Cannon, R.D., Villas-Bôas, S.G. The metabolic basis of *Candida albicans* morphogenesis and quorum sensing. *Fungal Genet Biol* 2011; **48**: 747-763.
9. Hoyer, L.L., Green, C.B., Oh, S.H., Zhao, X. Discovering the secrets of the *Candida albicans* agglutinin-like sequence (*ALS*) gene family-a sticky pursuit. *Med Mycol* 2008; **46**: 1-15.
10. Kalia, V.C. Quorum sensing inhibitors: an overview. *Biotechnol Adv* 2013; **31**: 224-245.
11. Kebaara, B.W., Langford, M.L., Navarathna, D.H.M.L.P., Dumitru, R., Nickerson, K.W., Atkin, A.L. *Candida albicans* Tup1 is involved in farnesol-mediated inhibition of filamentous-growth induction. *Eukaryotic cell* 2008; **7:** 980-987.
12. Khodavandi, A., Alizadeh, F., Aala, F., Sekawi, Z.,Chong, P.P. *In vitro* investigation of antifungal

activity of allicin alone and in combination with azoles against *Candida* species. *Mycopathologia* 2010; **169**: 287-295.

13. Khodavandi, A., Harmal, N.S., Alizadeh, F., Scully, O.J., Sidik, S.H.M., Othman, F., Sekawi, Z., Ng, K.P., Chong, P.P. Comparison between allicin and fluconazole in *Candida albicans* biofilm inhibition and in suppression of *HWP1* gene expression. *Phytomedicine* 2011a; **19**: 56-63.
14. Khodavandi, A., Alizadeh, F., Harmal, N.S., Sidik, S.M., Othman, F., Sekawi, Z.,Chong, P.P. Expression analysis of *SIR2* and SAPs1-4 gene expression in *Candida albicans* treated with allicin compared to fluconazole. *Tropical Biomedic* 2011b; **28**: 589-598.
15. Khodavandi, A., Alizadeh, F., Harmal, N.S., Sidik, S.H.M., Othman, F., Sekawi, Z., Farboodniaye Jahromi, M.A., Ng, K.P., Chong, P.P. Comparison between efficacy of allicin and fuconazole against *Candida albicans invitro* and in a systemic candidiasis mouse model. *FEMS Microbiol Lett* 2011c; **315**: 87–93.
16. Khodavandi, A., Alizadeh, F., Namvar, F., Rosfarizan, M., Chong, P.P. Anti-*Candida* potential of *Allium ascalonicum* Linn: antibiofilm activity and biomolecular mechanism of action. *J Pure Appl Microbiol* 2014a; **8**: 349-356.
17. Khodavandi, A., Alizadeh, F., Aghai Vanda, N., Karimi, G.,Chong, P.P. Possible mechanisms of the antifungal activity of fluconazole in combination with terbinafine against *Candida albicans*. *Pharm Biol* 2014b; **52**: 1505-1509.
18. Koh, K.H., Tham, F.Y. Screening of traditional Chinese medicinal plants for quorum-sensing inhibitors activity. *J Microbiol Immunol Infect* 2011; **44**: 144-148.
19. Koh, C.L., Sam, C.K., Yin, W.F., Tan, L.Y., Krishnan, T., Chong, Y.M., Chan, K.G. Plant-derived natural products as sources of anti-quorum sensing compounds. *Sensors* 2013; **13**: 6217-6228.
20. Kruppa, M. Quorum sensing and *Candida albicans*. *Mycoses* 2008; **52**: 1-10.
21. Lade, H., Paul, D., Kweon, J.H. Quorum quenching mediated approaches for control of membrane biofouling. *Inter J Biol Sci* 2014; **10**: 550-565.
22. Lim, C.S.Y., Wong, W.F., Rozita, R., Ng, K.P., Seow, H.F., Chong, P.P. 2-dodecanol (decyl methyl carbinol) inhibits hyphal formation and *SIR2* expression in *C. albicans*. *J Basic Microbiol* 2009; **49**: 579-583.
23. Mayer, F.L., Wilson, D., Hube, B. *Candida albicans* pathogenicity mechanisms.*Virulence* 2013; **4**: 119-128.
24. Nett, J.E. Future directions for anti-biofilm therapeutics targeting *Candida*. *Expert Rev Anti Infect Ther* 2014; **12**: 375-382.
25. Odds, F.C., Brown, A.J.P., Grow, N.A.R. Antifungal agents: mechanisms of action. *TRENDS Microbiol* 2003; **11**: 272-279.
26. Palmeira-de-Oliveira, A., Silva, B.M., Palmeira-de-Oliveira, R., Martinez-de-Oliveira, J., Salgueiro, L. Are plant extracts a potential therapeutic approach for genital infections? *Curr Med Chem* 2013; **20**: 2914-2928.
27. Sharma, R., Jangid, K. Fungal quorum sensing inhibitors. In: Quorum Sensing vs Quorum Quenching: A Battle with no End in Sight. -Kalia, V.C. (editor), 1th edition, New York, Springer, 2015, pp. 237-257.
28. Shuford, J.A., Steckelberg, J.M., Patel, R. Effects of fresh garlic extract on *Candida albicans* biofilms. *Antimicrob Agents Chemother* 2005; **49**: 473.
29. Toyoda, M., Cho, T., Kaminishi, H., Sudoh, M., Chibana, H. Transcriptional profiling of the early stages of germination in *Candida albicans* by real-time RT-PCR. *FEMS Yeast Res* 2004; **5**: 287-296.
30. Vashisth, P., Nikhil, K., Pemmaraju, S.C., Pruth, P.A., Mallick, V., Singh, H., Patel, A., Mishra, N.C., Singh, R.P., Pruthi, V. Antibiofilm activity of quercetin-encapsulated cytocompatible nanofibers against *Candida albicans*. *J Bioactive Compatible Polymers* 2013; **28**: 652-665.
31. Viswanathan, P., Rathinam, P., Suneeva, S.C. Plant quorum sensing inhibitors: food, medicinal plants, and others. In: Quorum Sensing vs Quorum Quenching: A Battle with no End in Sight. -Kalia, V.C. (editor), 1th edition, New York, Springer, 2015, pp. 269-281.
32. Yoshida, S., Kasuga, S., Hayashi, N., Ushiroguchi, T., Matsuura, H., Shizutoshi, N. Antifungal activity of ajoene derived from garlic. *Appl Environ Microbiol* 1987; **53**: 615-627.