Tropical Biomedicine 28(3): 589–598 (2011)

Expression analysis of *SIR2* and SAPs1-4 gene expression in *Candida albicans* treated with allicin compared to fluconazole

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Received 6 March 2011; received in revised form 21 May 2011; accepted 29 May 2011

Abstract. One of the main factors for virulence of fungus such as *Candida albicans* is the ability to change its morphology from yeast to hyphae. Allicin, one of the volatile sulfur-oil compounds from freshly crushed garlic, has a variety of antifungal activities. In this study, the effect of allicin on growth and hyphae production in *C. albicans* as compared to fluconazole, an antifungal drug was investigated using survival time *in vitro* and microscopic image at different time intervals. Additionally, the expression of selected genes involved in hyphae formation and development such as *SIR2* and *SAP1-4* was evaluated by semi-quantitative RT-PCR and relative real time RT-PCR. Allicin was shown to down-regulate the expression of *SIR2* (5.54 fold), similar to fluconazole (3.48 fold) at 2x MIC concentrations. Interestingly, allicin had no effect on SAPs1-4 expression, whereas fluconazole was able to suppress *SAP4* expression. Our findings showed that allicin was effective in suppressing hyphae development of *C. albicans* to an extent that is sometimes equal or more than fluconazole. Moreover, allicin and fluconazole seemed to share a common anti-*Candida* mechanism through inhibition of *SIR2* gene, while fluconazole appeared to also exert its fungistatic effect through another pathway that involved *SAP4* suppression.

INTRODUCTION

*Candida albicans*is one of the opportunistic fungi causing candidiasis in humans. Normally, *Candida* could be found in mucosal tissues such as human vagina as a commensal microorganism, but changes in some basic environmental factors such as pH or nutrient composition are able to induce infection by *Candida* (Calderone & Fonzi, 2001). It has been demonstrated that one of the main factors for virulence of *C. albicans* is yeast-to-hyphal-phase dimorphic transition. On the other hand, the ability of *Candida* to attach on surfaces such as epithelial cells, and release proteinases is another strategy for pathogenicity (Odds, 1987). Previous authors reported that *C. albicans* could display different morphologies such as budding form (blastospore), pseudohyphae or true hyphae depending on the environmental conditions as described above. This transition phenomenon (bud-filament) has been termed 'phenotypic

switching'. The silent information regulatory gene (*SIR2*) was indicated as one of the significant genes which are interfering in this event (Perez-Martin *et al.*, 1999; Calderone & Fonzi, 2001). Moreover, secreted aspartyl proteinases (*SAP*) are additional virulence factors of *C. albicans* that were shown to play major roles in the pathogenicity particularly in host tissue damage and are encoded by 10 *SAP* genes (White & Agabian, 1995). It has also been shown that there is some correlation between expression of *SAP* genes and hyphae production. Indeed, the interruption of transcriptional factors which control yeast-to-hyphae transition also has an effect on the expression of hyphae-independent genes such as the proteinase genes *SAP1* and *SAP3* (Korting *et al.*, 2003).

Allicin (diallylthiosulfinate), one of the sulfur and volatile compounds from garlic has been shown to possess a broad spectrum of antifungal activity against *Candida* species, *Aspergillus* species, and *Trichophyton* spesies (Yamada & Azuma, 1977; Ankri & Mirelman, 1999). Previous reports demonstrated that the target of some anti-*Candida* drugs such as azoles was disruption of ergosterol resulting in the formation of cell membrane (Odds *et al.*, 2003). It was suggested that the mechanisms of action of garlic and some derivatives might be the destruction of cell membrane integrity, inhibition of growth and induction of oxidative stress in *C. albicans* (Lemar *et al.*, 2002, 2005; Low *et al.*, 2008) but, the main protein or cellular targets of allicin on *C. albicans* is not well understood. Our previous study using crude garlic extract has shown that garlic possessed anti-*Candida* property and was able to suppress hyphae formation with down-regulation of *SIR2* gene. However, it was not known whether allicin, a main bioactive component of garlic was responsible for this observed effect. Hence, in this study, we endeavored to investigate the effect of allicin on the morphology switching phenomenon in *C. albicans* and to compare the effect of allicin against fluconazole on not only *SIR2*, but also *SAP1*, *SAP2*, *SAP3* and *SAP4* gene expression.

MATERIALS AND METHODS

Antifungal Agents

Allicin was purchased from Alexis Biochemicals Co. (purity ≥ 98%, Batch No. ALX-350-329, San Diego, USA) and dissolved at 1 mg/mL in methanol/water/formic acid (60:40:0.1), then stored at -20 to -80ºC until use. Fluconazole was purchased from Sigma Chemicals Co. (St. Louis, Mo., USA). The stock solution was prepared as explained earlier in our previous reports (Khodavandi *et al.*, 2010, 2011).

Treatment of *C. albicans* ATCC 14053 with different MIC concentrations of antifungal agents for gene expression analysis

The minimal inhibitory concentration (MICs) of allicin and also fluconazole as a standard anti-*Candida* drug was determined against *C. albicans* ATCC 14053 using broth microdilution method according to the Clinical Laboratory Standards Institute (CLSI, 2002) M27 A2 document as described in our previous reports (Khodavandi *et al.*, 2010, 2011).

A suspension of *C. albicans* ATCC 14053 containing 1 x 10^6 cells/mL in RPMI 1640 (with L-glutamine) was mixed with different dilutions of antifungal agents based on MIC concentrations (¼x MIC, ½x MIC, 1x MIC and 2x MIC) as explained earlier in our previous reports (Khodavandi *et al.*, 2010, 2011) and then incubated at 35ºC for 24 h with brief shaking. The formation of hyphae (with 1x MIC concentrations of antifungals) was examined under light microscopy at 40 x magnification at different time intervals (0, 12 and 24 h of incubation).

Total RNA was extracted using RNeasy mini kit (Qiagen, Germany) with slight modifications for yeast. According to the manufacturer's operating instructions for yeast cells, 2 mL of sorbitol lysis buffer (1 M sorbitol and 0.1 M EDTA pH7.4) was added to 5-10 mL of each treated samples. Then 50 U lyticase/zymolyase (ICN Chemicals, USA) and 10 µL of β-Mercaptoethanol respectively were added to lyse the yeast cell wall and generate spheroplasts. The extracted RNA was treated with 1 U DNase I (Promega, UK)

to remove genomic DNA. RNA quality was checked by 1.2% (w/v) formaldehyde-denaturing agarose gel electrophoresis at 70 V for 45 min and the concentrations and absorbance ratio of RNA was also measured for purity estimation using the Nanodrop ND-1000 spectrophotometer. The ratios of A 260/280 and A 260/230 for all samples were above 2.0 in nuclease free water.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) for comparing *SIR2* and *SAP1-4* gene expression levels

According to the manufacturer's protocol, single-stranded cDNA was synthesized from approximately 0.5-1 µg extracted RNA using Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase and random hexamer oligonucleotides (Fermentas, USA). The reverse transcription of the RNA was carried out at least in triplicates.*Candida albicans SIR2* and *SAP1-4* genes were amplified from the synthesized cDNA. The primer sequences were designed via Primer3 and Primer Premier 5 software (Table 1). β-actin gene was also amplified as a housekeeping gene and internal control to normalize the incongruity of RNA concentrations during extraction of total RNA using RNeasy mini kit. Moreover, a negative control without M-MuLV reverse transcriptase was included for each sample to ensure that the PCR products originated from cDNA. The amplification conditions were 94ºC for 5 min, and 26 cycles of 94ºC for 45 s, 56.8ºC for 45 s, and 72ºC for 50 s; followed by 72ºC for 10 min (Low *et al*., 2008). PCR products were analyzed by gel electrophoresis and visualised via AlphaImager HP imaging system. On the other hand, the intensity of PCR products amplified from target and housekeeping genes was quantitated by comparing to known molecular weight DNA markers (Fermentas, USA) using AlphaImager software.

Real-time RT-PCR validation of gene expression analysis

Relative real-time RT-PCR reactions to confirm the semi quantitative RT-PCR results were performed using ™SYBR Green qPCR Master Mix (Fermentas, EU) in a Rotor Gene-3000 real-time cycler (Corbett, Australia), according to the manufacturer's protocol. The cycling conditions included holding at 95ºC for 15 min, 35 cycles of denaturation at 94ºC for 15 s, annealing at 56.8ºC for 20 s and elongation stage at 72ºC for 20 s. Finally, the melting reaction was at 72-99ºC (Lim *et al*., 2009). The mathematical method calculation of relative quantification was determined by conventional method based on concentration of PCR products as follows:

Table 1. Oligonucleotide primers used for PCR

Primer (length in bp)	Orientation	Sequence	Reference
SAP1 (325)	Forward	5' CTTGTGATAAACCTCGTCCTG 3'	This study
	Reverse	5' GCATTCTTGGCAATAACTCC 3'	
SAP2 (345)	Forward	5' ATGCTGCCACGGGACAAA 3'	This study
	Reverse	5' CGGAAGCTGGAACGGAAA 3'	
SAP3 (252)	Forward	5' TGCTACTCCAACAACTTTCA 3'	This study
	Reverse	5' GAATCAGGAACCCATAAATC 3'	
SAP4 (201)	Forward	5' GAGTGTTCTTGCTTTCGCTTTA 3'	This study
	Reverse	5' TTGCCCACATCATTTCTACC 3'	
SIR2 (180)	Forward	5' ACGAGCAGGATTGAAACTGGAA 3'	(Low *et al*., 2008)
	Reverse	5' CCAAAATGGATTGGTGCTTGTT 3'	
ACT (516)[a]	Forward	5' ACCGAAGCTCCAATGAATCCAAAATCC 3'	(Low *et al*., 2008)
	Reverse	5' GTTTGGTCAATACCAGCAGCTTCCAAA 3'	
ACT (199)[b]	Forward	5' GAGTTGCTCCAGAAGAACATCCAG 3'	(Lim *et al*., 2009)
	Reverse	5' TGAGTAACACCATCACCAGAATCC 3'	

[a]Housekeeping gene used in semi quantitative RT-PCR.
[b]Housekeeping gene used in relative real time RT-PCR.

Fold change in target gene expression =

$$\frac{\text{Ratio of target gene expression (experiment/untreated control)}}{\text{Ratio of reference gene expression (experiment/untreated control)}}$$

In this experiment, the calculation of data for relative real-time RT-PCR was based on the Standard Curve method (Larionov *et al.*, 2005). The fold changes were also calculated using the Pfaffl method (Pfaffl, 2001) (data not shown) and these were found to correlate closely with the values calculated using the former method.

The identity of the PCR products was confirmed by DNA sequencing method using an outsourcing sequencing service (1st BASE, Malaysia). The sequence similarity was analyzed via nucleotide Blast software against the nonredundant GenBank database in the NCBI website and verified (data not shown).

For statistical analysis all data was examined in terms of normality and then one way analysis of variance (ANOVA) was carried out. P values of < 0.05 were considered significant. Statistical analysis was performed using SPSS version 17 software (SPSS Inc., Chicago, IL). All experiments were carried out at least in triplicates.

RESULTS

Growth and morphology of *C. albicans* cells treated with allicin

Figure 1 shows the light microscopy pictures from *C. albicans* ATCC 14053 treated with the antifungal agents at different time intervals. In comparison to the untreated growth control, both the *C. albicans* cells treated with either allicin or fluconazole showed a significant reduction ($p < 0.001$) in the number of cells over time after 2, 4, 6, 8,

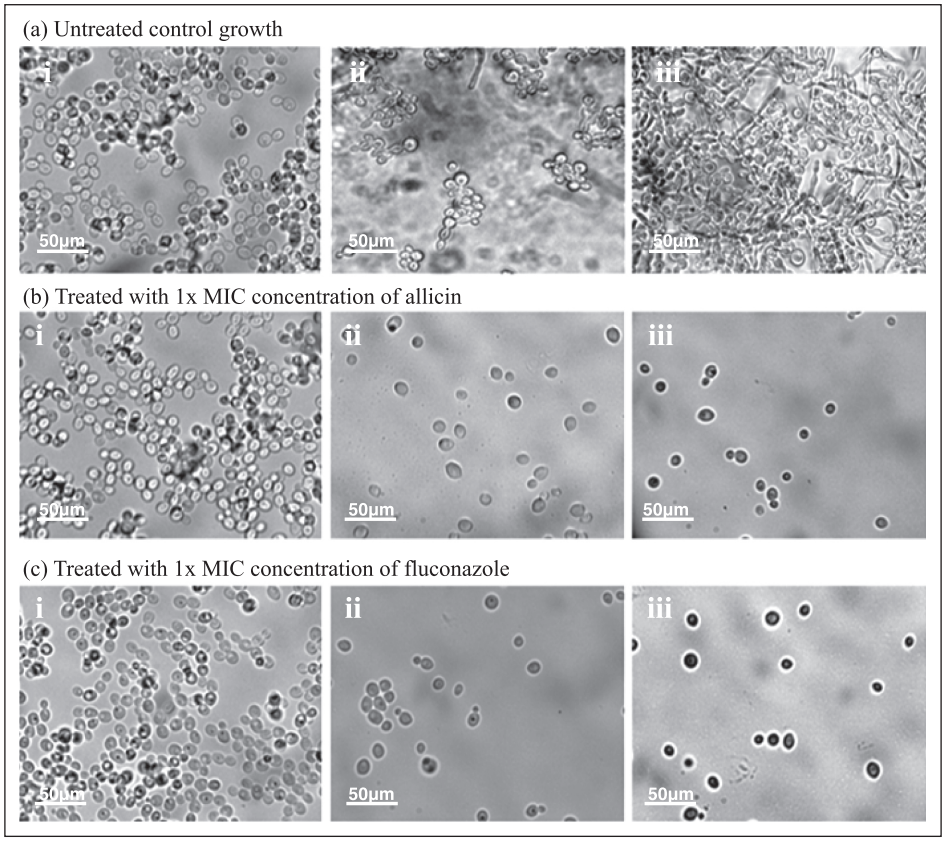

Figure 1. Microscopic view of *Candida albicans* ATCC 14053 hyphae production after 0 h (i), 12 h (ii) and 24 h (iii) incubation at 35°C. Magnification x 40

12 and 24 hours. In addition, the treated cells appeared to have lost their ability to switch morphology, remaining as yeast form compared to the growth control which produced germ tubes and filamentous hyphae.

Relative quantitative RT-PCR of *SAP1-4* and *SIR2*

Approximately 1 x 10^6 cells/mL of *C. albicans* ATCC 14053 were treated with allicin and fluconazole at different folds of MIC concentrations and incubated for 24 h at 35°C, then harvested for RNA extraction. The cell cultures were prepared in triplicates for greater credibility of results. The RNA extracted from treated and untreated cells were converted to cDNA. Results of representative agarose gel electrophoresis of the RT-PCR products for *SAP1-4* and *SIR2* genes are shown in Figures 2 and 3, respectively. β-actin used as a housekeeping gene showed relatively stable expression across different samples, but small variations of its expression level was observed in some samples. This is due to the different initial

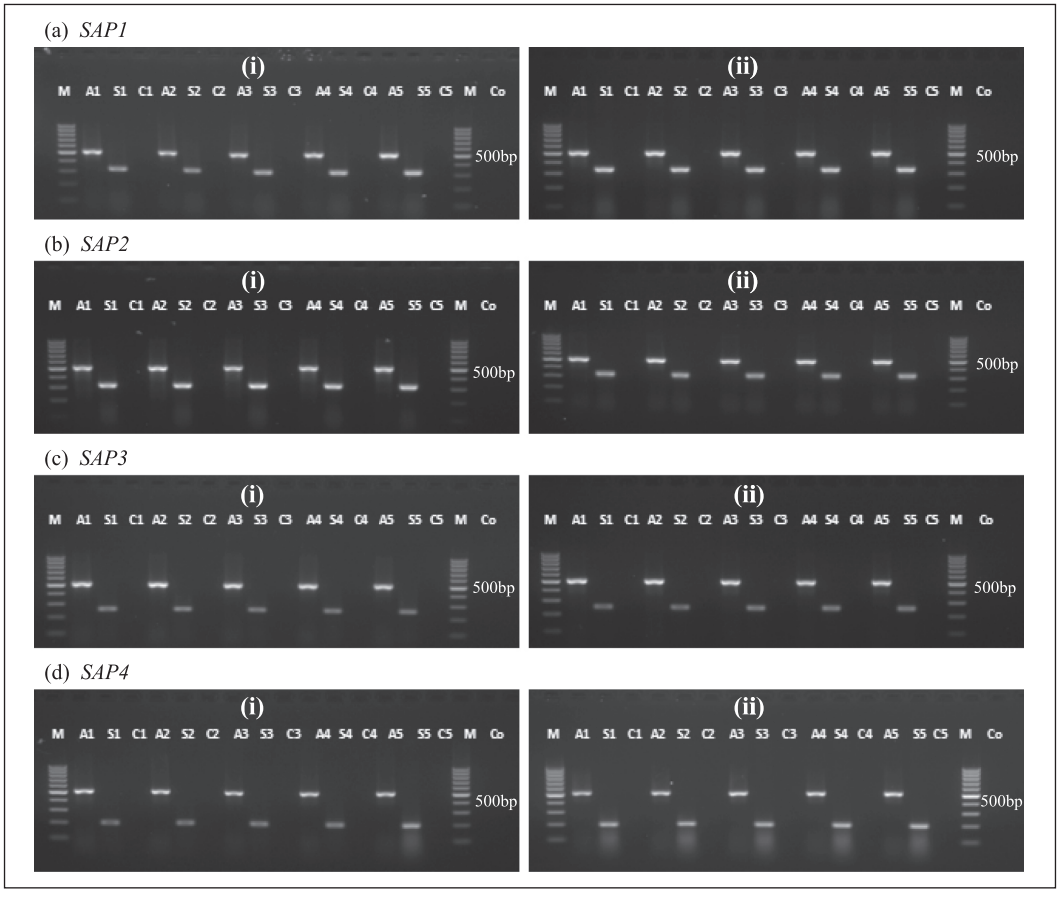

Figure 2. Gel electrophoresis of semi-quantitative RT-PCR products of *SAP* genes from *C. albicans* ATCC 14053 treated with allicin (i) and fluconazole (ii). M: 100 bp DNA Ladder, A1: *Actin* with 2x MIC concentration of antifungals, S1: *SAP* with 2x MIC concentration of antifungals, C1: Internal control without M-MuLV reverse transcriptase, A2: *Actin* with 1× MIC concentration of antifungals, S2: *SAP* with 1x MIC concentration of antifungals, C2: Internal control without M-MuLV reverse transcriptase, A3: *Actin* with ½x MIC concentration of antifungals, S3: *SAP* with ½x MIC concentration of antifungals, C3: Internal control without M-MuLV reverse transcriptase, A4: *Actin* with ¼x MIC concentration of antifungals, S4: *SAP* with ¼x MIC concentration of antifungals, C4: Internal control without M-MuLV reverse transcriptase, A5: *Actin* without antifungals (untreated control), S5: *SAP* without antifungals (untreated control), C5: Internal control without M-MuLV reverse transcriptase, Co: Control negative for PCR

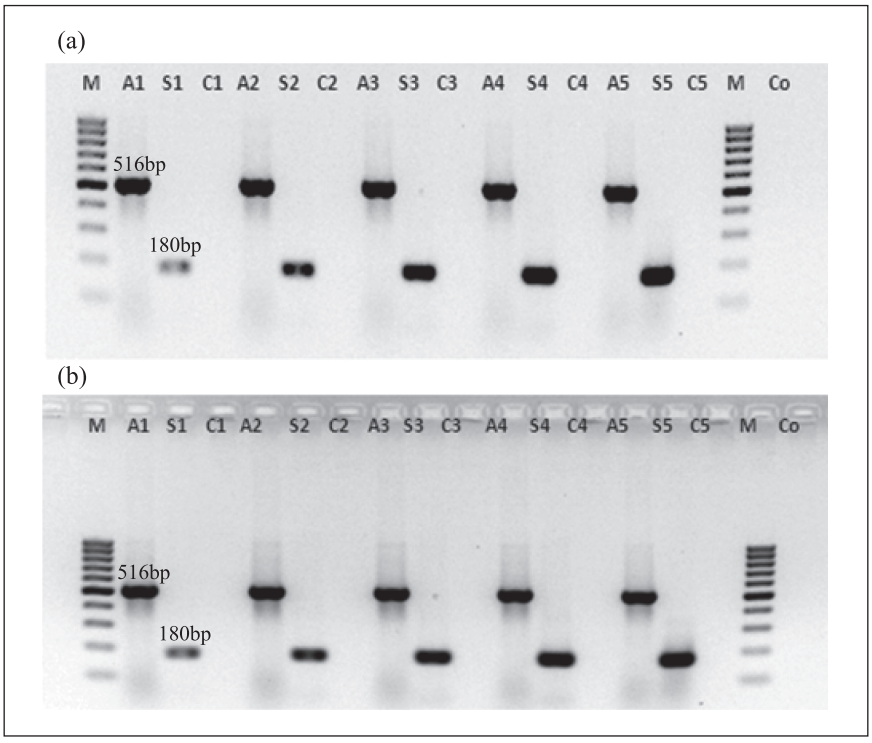

Figure 3. Gel electrophoresis of semi-quantitative RT-PCR products of *SIR2* gene from *C. albicans* ATCC 14053 treated with allicin (a) and fluconazole (b). M: 100 bp DNA Ladder, A1: *Actin* with 2x MIC concentration of antifungals, S1: *SIR2* with 2x MIC concentration of antifungals, C1: Internal control without M-MuLV reverse transcriptase, A2: *Actin* with 1x MIC concentration of antifungals, S2: *SIR2* with 1x MIC concentration of antifungals, C2: Internal control without M-MuLV reverse transcriptase, A3: *Actin* with ½x MIC concentration of antifungals, S3: *SIR2* with ½x MIC concentration of antifungals, C3: Internal control without M-MuLV reverse transcriptase, A4: *Actin* with ¼x MIC concentration of antifungals, S4: *SIR2* with ¼x MIC concentration of antifungals, C4: Internal control without M-MuLV reverse transcriptase, A5: *Actin* without antifungals (untreated control), S5: *SIR2* without antifungals (untreated control), C5: Internal control without M-MuLV reverse transcriptase, Co: Control negative for PCR

amounts of RNA obtained from each sample and also the inefficiency of cDNA synthesis, but this discrepancy could be compensated after normalization of the target gene to the housekeeping gene in each run (Low *et al.*, 2008). The relative quantification of *SAP1-4* and *SIR2* gene expression ratio normalized to actin indicated that some but not all of the genes tested had significant differences in gene expression between the antifungal-exposed cells and the untreated control. The *SIR2* mRNA was down-regulated 5.54, 3.44, 1.91 and 1.34 folds (data not shown) at allicin concentrations of 2x MIC, 1x MIC, ½x MIC and ¼x MIC respectively. Similarly, the *SIR2* mRNA was down-regulated 3.48, 2.30, 1.36 and 0.87 folds (data not shown) at fluconazole concentrations of 2x MIC, 1x MIC, ½x MIC and ¼x MIC respectively. On the other hand, our data showed that *SAP4* mRNA was down-regulated only for fluconazole treated samples. Meanwhile, no significant reduction in gene expression compared to untreated control was observed for *SAPs* 1-3 (Fig. 4). The significant reduction of *SIR2* expression in *Candida* treated with allicin was confirmed via relative real-time RT-PCR. Figure 5 shows the relative quantification of *SIR2* expression treated with the antifungal agents. *SIR2* was down-regulated 5.25

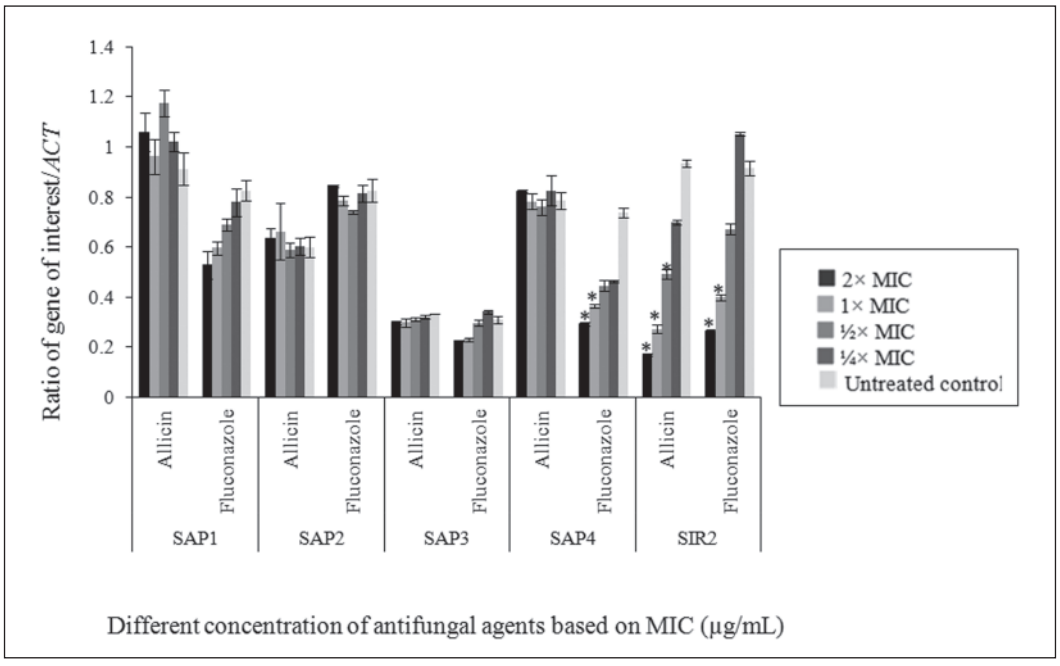

Figure 4. Relative quantitation of *SAP*s and *SIR2* expressions (normalized to housekeeping gene, actin) in *Candida albicans* ATCC 14053 after 24 h of treatment with different concentrations of antifungal agents, (*) means significant reduction of gene expression to untreated control at level $p < 0.0001$. Data are means the ratio of gene expression with standard error from three independent experiments amplified in triplicates

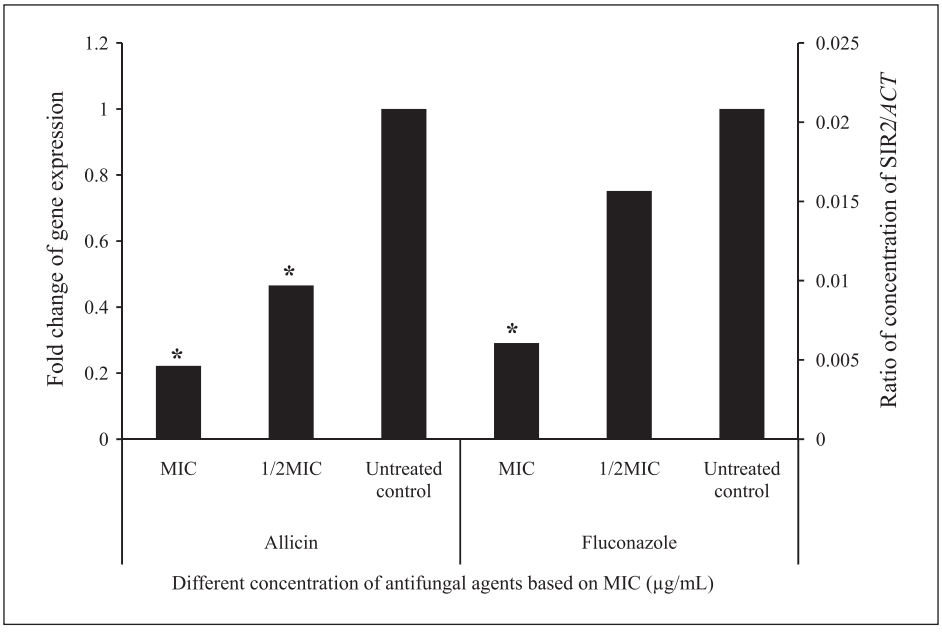

Figure 5. Relative quantitation of *SIR2* expression (normalized to housekeeping gene, actin) in *Candida albicans* ATCC 14053 after 24 h of treatment with different concentrations of antifungal agents by real time RT-PCR, (*) means significant reduction of gene expression to untreated control at level $p < 0.0001$. Data are means of fold changes with standard error from three independent experiments amplified in triplicates

and 1.91 fold (data not shown) at allicin concentrations of 1x MIC and ½x MIC respectively and 3.5 and 1.05 fold at fluconazole concentrations of 1x MIC and ½x MIC respectively.

DISCUSSION

Among the important opportunistic fungi, the yeast species *C.albicans* is the main agent of candidiasis in human. It has been demonstrated that many of the *Candida*-specific genes could encode some catabolic proteins which are essential for the survival of the microorganism as a commensal or a pathogen. In summary, there are some virulence factors in *Candida* including adhesion, hyphae production, phenotypic switching and formation of some extracellular hydrolytic enzymes (Calderone & Fonzi, 2001). The initiation step of candidal infection is adherence of *Candida* to host cells. On the other hand, *Candida albicans* could also attach on other surfaces such as medical devices and form biofilm which is resistant to antifungal agents and increase the infection (Yan-Liang, 2003). Some reports showed that secreted aspartyl proteinases (SAPs) family included 10 different genes (*SAP1-10*) which can destroy many human proteins such as hemoglobin, albumin, secretory immunoglobulin A and keratin (White & Agabian, 1995; Perez-Martin *et al.*, 1999; Yan-Liang, 2003). It has been shown that *SAP1-3* can be expressed by planktonic form while expression of *SAP4-6* is limited to hyphae form (Yan-Liang, 2003). Moreover, it is mentioned that the reversible ability to switch between planktonic and hyphae or pseudohyphae form is the most important virulence factor in *C. albicans* and affected via many physical and chemical agents such as temperature, pH and the composition of the nutrient in the environment (Odds, 1987; Antony *et al.*, 2007). In fact, the basic mechanism of phenotypic switching in the virulence of *C. albicans*is not well understood, although some of the probable mechanisms may include chromosomal rearrangements and also a *SIR2*-like regulation in yeast such as *Saccharomyces cerevisiae* and *C. albicans* (Odds, 1987; Calderone & Fonzi, 2001). It was reported that following deficiencies in the *SIR2* gene in *Candida*, the high-frequency switching phenotype could be observed (Odds, 1987; Fu *et al.*, 2008). On the other hand, the up-regulated expression of *SIR2* in *C. albicans* was associated with true hyphae evolvement in serum-induced hyphal growth conditions (Lemar*et al.*, 2007).

Inhibition of growth of human pathogenic microorganisms and suppression or reduction of expression of virulence genes with some antimicrobial agents are effective strategies for the control of infectious diseases. Mostly, for treatment of systemic fungal infections, azoles such as fluconazole are used, but one of the biggest problems faced in clinical practice is the emergence of resistant strains for most of these drugs currently used (Odds, 1987). Hence, alternative or new therapeutic strategies are necessary. Several previous reports demonstrated that allicin showed antifungal activity against *Aspergillus* species, *Trichophyton* species and *Candida* species (Yamada & Azuma, 1977; Ankri & Mirelman, 1999; Khodavandi *et al.*, 2010, 2011). It was discovered that after crushing of fresh garlic cloves, allin (precursor of allicin) can convert to allicin (diallylthiosulfinate) through the action of allinase (Ankri & Mirelman, 1999). Although the mechanism of action of allicin is not well understood, it was suggested that garlic and some derivatives such as diallyl disulfide (DADS) could destroy the cell membrane integrity, inhibit the growth and produce oxidative stress by depleting glutathione in *C. albicans* (Lemar *et al.*, 2002, 2005, 2007; Low *et al.*, 2008). In this study, we have used fluconazole as a standard anticandidal drug for comparison to allicin. In the former study, fresh garlic extract at 80-100 mg/mL was found to be effective against *C. albicans*, and semi-quantitative RT-PCR results revealed that *SIR2* expression was down-regulated (1.2-2.5 folds respectively) (Low *et al.*, 2008). In the present study, we sought to probe further on whether the commercial pure compound derived from garlic, allicin, was the agent responsible for the hyphae suppression. We also attempted

to expand our study to compare the effects of allicin against that of fluconazole on not just *SIR2* but also *SAPs1-4*, using real-time RT-PCR, which is a more reliable and sensitive method than the gel-based semi-quantitative RT-PCR. The present finding in this study also showed that allicin could reduce the expression of *SIR2* 1.34-5.54 folds measured via semi quantitative RT-PCR ($p < 0.0001$) and 1.91-5.25 folds when quantified via relative real-time RT-PCR ($p < 0.0001$) in comparison to untreated control at different MIC concentrations of allicin against *C. albicans*. In parallel, fluconazole-treated samples showed down-regulation of expression of *SIR2* at 0.87-3.48 (semi-quantitative RT-PCR) ($p < 0.01$) and 1.05-3.5 (relative real-time RT-PCR) ($p < 0.05$) folds versus untreated control at different concentrations of the drug based on MIC against *C. albicans* (Fig. 3-5). Hence, it can be concluded that allicin was effective in inhibiting hyphae production of *C. albicans* to an extent that is sometimes equal or more than fluconazole. Most of these abilities are probably related to SH-modifying potential in allicin, because the activated disulfide bond of this molecule can react with thiol-containing compounds such as some proteins (Miron *et al.*, 2004). It is indicated also, in a living body, allicin is not stable and converts immediately to some other products such as ajoene, diallyl disulfide and diallyltrisulfide (Davis, 2005). Nonetheless, hitherto little is known about the molecular mechanisms of action of allicin against *Candida*. SAPs have been known to play a significant role in virulence of *C. albicans* and are closely associated with hyphae development (Korting *et al.*, 2003). Thus it was hypothesized that allicin could reduce the expression of *SAP1-4*, but our RT-PCR results did not demonstrate any significant decrease in SAPs expression in allicin-treated *C. albicans* (Fig. 2, 4). Therefore, the hyphal suppression by allicin seems to be unrelated to *SAP* genes. In contrast, fluconazole in concentrations of 2x MIC and 1x MIC was found to reduce the expression of *SAP4* significantly, suggesting that fluconazole acts through a different pathway compared to allicin. Interestingly, fluconazole in a high concentration resulted in an initial decrease in *SAP1* and *SAP3* expression, but the decrease was however not significant when compared to untreated control (Fig. 4).

In conclusion, our finding showed that allicin, the bioactive compound from garlic, could inhibit the growth and suppress hyphae formation in *C. albicans* through a pathway that involves reduction of *SIR2* expression with increasing concentrations of allicin. Currently, it is unconfirmed whether allicin is transformed into other products by its interaction with certain proteins within *C. albicans* cells. Further work would be performed in order to investigate the effect of ajoene and other by-products of allicin catalysis on *C. albicans* growth in order to elucidate the molecular mechanisms of action of garlic against *C. albicans*.

Acknowledgments. We thank Dr Mohammad Ali Farbodniay Jahromi, senior researcher of Fars Technological and Environmental Research Center, Shiraz-Iran, for helpful guidance and collaboration. Funding was partially provided by Science Fund (02-01-04-SF0761) from the Ministry of Science, Technology and Innovation of Malaysia.

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