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Inhibitory effect of menthol on expression of aspartyl proteinase 1 in fluconazole-resistant *Candida albicans*

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ABSTRACT

Introduction: Fluconazole-resistant *Candida albicans* is one of the biggest problems seen in clinical practices. One of the most common ways to resolve this problem is the use of natural pure compounds such as menthol. The aims of this study were to investigate the hyphal formation and gene expression profiling of fluconazole-resistant *C. albicans* treated by menthol.
Methods: Colonization of vaginal isolates of *C. albicans* was recognized and fluconazole-resistant yeasts detected by WHONET software. The relative minimum inhibitory concentrations (MICs) of menthol were determined by broth microdilution for fluconazole-resistant isolates. The potency of menthol to inhibit hyphal formation was exploited using a light microscope. A quantitative real-time RT- PCR was used to measure the expression of *SAP1* .
Results: Almost 100% of colonized vaginal isolates of *C. albicans* was found to be fluconazole-resistant. MIC_{90} for menthol in fluconazole-resistant isolates was 1.6 to 25 µg/mL. Furthemore, all isolates treated with menthol showed a significant reduction in hyphae and number of planktonic cells. In the fluconazole-resistance *C. albicans* cells treated with fluconazole, the expression levels of *SAP1* increased by 1.53- (2×MIC conc.) and 1.43-fold (1×MIC conc.). However, treatment with menthol down regulated the *SAP1* expression by 2.02- and 1.85-fold at concentrations of 2 × MIC and 1 × MIC, respectively ($P \leq 0.05$).
Conclusion: This study suggests that menthol might have potential applications in treatment of infections, due to fluconazole-resistance *C. albicans*. In addition, *SAP1* could be probable molecular target of menthol in *C. albicans*.

Implication for health policy/practice/research/medical education:
Menthol act as anticandidal agent and has the potential for therapy of candidiasis. These results can provide insights into the mechanism of menthol against *C. albicans*.
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Introduction

A common agent for opportunistic fungal infection of candidiasis is *Candida albicans*. In fact, candidiasis is a problem of growing clinical importance, particularly in individuals with immunologic (e.g., genetic susceptibility, cancer, organ transplant and human immunodeficiency virus infection) and non immunologic (e.g., pregnancy, neonates and childhood, burn, diabetes and nosocomial infections) predisposing conditions which contribute to enhanced susceptibility to infections (1-3).

Candida albicans has developed virulence factors with specific strategies to allow its successful host tissue colonization. Key virulence factors of *C. albicans* expressed or required for pathogenesis are secreted aspartyl proteinases (Saps), encoded by a 10-member gene family (*SAP1* to *SAP10*) (3-6). These enzymes with a signal peptide are secreted into the extracellular space. Sap9 and Sap10 are cell wall-associated proteases (7). Based on the amino acid sequence alignments and protein homology, the 10 *SAP* genes are classified into three subfamilies. The subfamilies consist of the *SAP1* to *SAP3*, *SAP4* to *SAP6*, *SAP9*, and *SAP10* genes (*SAP7* and *SAP8* are divergent) (5,6,8,9). The proteolytic activity of the Sap proteins could be involved in binding to the host cells and degradation of various host tissue barriers, immune response evasion, facilitate the nutrient availability for fungal growth from host protein digestion, adhesion and survival of fungal cells within host cells (10,11). Several studies have described differential expression of *SAP* family, *in vitro* and in *vivo,* during candidiasis (4-6). The

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expression pattern of these genes in the pathogenesis of *C. albicans* infection are associated with a number of other putative virulence factors of *C. albicans* including hypha formation, adhesion and phenotypic switching. Therefore, the expression of *SAP* genes varies at different stages of fungal growth and disease (3,5,6). Identification of *SAP1* gene was done by Hube et al (12) and characterization of the protein product by White et al (13). Sap1 enzyme related to yeast cell form is activiated in an acidic pH environment. Expression of *SAP1* is strain-specifc and it has been shown that Sap1 contribute significantly to mucosal infections (5,6,9,14). Sap1 is produced during growth in media containing proteins as the sole nitrogen source. Furthermore, *SAP1* mutants of *C. albicans* grew slowly after induction in proteinase-inducing medium and the production of their own proteinases were delayed compared with the parental wild type strain. The virulence of mutant strains lacking *SAP1* gene slightly reduced in animal models of candidiasis (14).

The widespread use of antifungal therapy has been associated with a marked increase in the incidence of treatment failures in candidiasis patients resulting from antifungal drug resistance with a serious threat to life (15,16). Recently, natural pure antifungal compounds derived from plants, such as menthol, have received a great deal of attention. Menthol ($C_{10}H_{20}O$) is a terpenoid compound of the *Mentha* genus in the Lamiaceae family. Results from several studies show that menthol possesses an antifungal activity (17-20). Menthol exerts its antifungal activity on the cell membrane permeability and the cell wall consistency, hence probably causing cell death or inhibiting the filamentation of *C. albicans* (18,21). The aim of the prresent study was to evaluate the antifungal effect of menthol against fluconazole-resistant *C. albicans* vaginal isolates in pregnant women. In particular, we performed antifungal susceptibility, hypha formation and gene expression profiling of *C. albicans* treated with menthol.

Materials and Methods

Microorganisms

Via vaginal swabs, 30 isolates of *C. albicans* were obtained from 100 healthy pregnant women who admitted in Ahvaz Imam Khomeini hospital affiliated to Jundishapur University of Medical Sciences, Ahvaz, Iran. Isolates were grown in Sabouraud Dextrose Agar (SDA, Biolife, Italy) at 37°C for 24 hours and identified by wet mount microscopy, Gram stain, macroscopic morphology on SDA and CHROMagar™ *Candida* (CHROMagar, France) and germ tube formation in human serum. Quality control strains of *C. albicans* ATCC 14053 and fluconazole-resistant *C. albicans* ATCC MYA-573 were also employed. Stock cultures of *C. albicans* were maintained at -75°C in Sabouraud Dextrose Broth (SDB, Biolife) containing 20% sterile glycerol. Before conducting the assays, each isolate was subcultured two or more times on SDA. Selected colonies (1 mm in dimater) were transferred to the sterile 0.85% NaCl. An optical density at 530 nm (OD530) for each isolate was adjusted to 0.08-0.10 using 0.85% NaCl sterile to give a standard inoculum of 1-5 $\times 10^6$ CFU/mL (22).

Antifungal susceptibility of fluconazole

The disk diffusion (CLSI M44-A2) and broth microdilution (CLSI M27-A3 and CLSI M27-S4) methods were used to test antifungal susceptibility of fluconazole against *C. albicans*. Results of susceptibility of isolates to fluconazole were analyzed using WHONET software (23).

Antifungal susceptibility of menthol

The susceptibility of menthol (M2772 Sigma-Aldrich Co. St. Louis, MO, USA) was measured according to the CLSI antifungal susceptibility method for yeasts (CLSI M27-A3 and CLSI M27-S4). After dissolving menthol in DMSO, one hundred µL of the two-fold dilution of menthol (range 0.0313–50 µg/mL) were prepared into 96-well plates in RPMI 1640 with L-glutamine and without bicarbonate (Sigma-Aldrich) supplemented with 2% glucose and MOPS buffer. *C. albicans* inoculum ($0.5–5 \times 10^3$ CFU/mL) was then inoculated into 96-well plates and incubated for 24 hours at 35°C. Growth (menthol-free) and sterility (medium alone) control wells were established on each test plate.

Relative MICs were measured at OD530 using a Stat Fax 303 Reader (Awareness Technology, Inc., USA). The lowest concentration in which ≥50% or 90% reduction of growth was evident considered to be a relative MIC. Fluconazole was used as a control (22,23).

Candida albicans hypha formation

Candida albicans ATCC MYA-573 was induced to form hypha formation according to the method described by Khodavandi et al (24). For hypha formation, 4 mL of menthol and fluconazole at 2× MIC and 1× MIC were prepared in 6-cm Petri dishes and 4 mL of *C. albicans* inoculum (1-5 $\times 10^6$ CFU/mL) was added on the Petri dishes. The dishes were incubated at 35°C for 90 minutes and the mixture was then incubated at 35°C for 16 hours with gentle shaking. The hyphae were briefly washed in PBS and observed with a light microscope (Leica, DMRA II, Germany).

Expression of *Candida albicans SAP1*

The expression assay of *SAP1* gene was conducted using relative quantitative Real time PCR (qRT-PCR) that was adapted from a previously described method (23). *C. albicans* ATCC MYA-573 treated with menthol and fluconazole was induced to form hypha formation. Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Hilden, Germany) containing DNase I in accordance

with manufacturer's instructions. RNA quality and quantity were determined by spectrophotometric in a NanoDrop® ND1000- spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and electrophoresis on a formaldehyde-denaturing agarose gel. The first strand of cDNA was synthesized using M-MuLV reverse transcriptase (Fermentas, USA) according to the manufacturer's instructions, using random hexamer oligonucleotides and total RNA (0.5 µg). The qRT-PCR was performed using SYBR™ Green qPCR Master Mix (Fermentas, EU) on a Bio-Rad MiniOpticonTM system (USA). The oligonucleotide primers used for qRT-PCR were listed in Table 1. The expression levels of *SAP1* gene were calculated by comparative Ct method ($2^{-\Delta Ct}$ formula) after normalization with beta *actin* gene (23,25).

Statistical analysis

All experiments were performed in duplicate or triplicate. Differences between specific means were analyzed by a one-way analysis of variance (ANOVA). Results are shown as the mean value ± standard deviation (SD). Values of $P<0.05$ were considered to be significant. The SPSS software (version 21; SPSS Inc., Chicago, IL) was used for statistical analysis.

Results

Clinical isolates of *C. albicans* were identified by morphological and biochemical characterization. Of 100 healthy women at the end of the pregnancy, 30 *C. albicans* were isolated and identified. The antifungal susceptibility of fluconazole on the growth of clinical isolates of *C. albicans* (using disk diffusion and broth microdilution methods) is reported in Table 2. Out of clinical isolates of *C. albicans*, 100% were found to be fluconazole-resistant. Kappa coefficient showed that there was a high degree of agreement between disk inhibition zone diameter and MICs for fluconazole.

The broth microdilution results obtained for the menthol compared with fluconazole against clinical isolates of *C. albicans* are reported in Table 3. Menthol induced a significant growth inhibition compared with fluconazole for *C. albicans*. The results showed a MIC_{90} of 1.6-25 µg/mL for fluconazole-resistance isolates of *C. albicans*.

Findings from potency of menthol to inhibit hypha formation of the *C. albicans* ATCC MYA-573 exhibited a significant reduction in hyphae and planktonic cells compared to the untreated control. Figure 1 shows the inhibitory property of menthol on *C. albicans* ATCC MYA-573 hyphal formation at concentrations of 2× MIC and 1× MIC after 16 hours. The effect of fluconazole was not dramatically decreased hyphae and planktonic cells of fluconazole-resistant *C. albicans* compared to the untreated control.

QRT-PCR was performed to investigate the effect of menthol on the expression of *SAP1* gene in fluconazole-resistance *C. albicans* cells (ATCC MYA-573). Box plots are shown in Figure 2. Fluconazole-resistance *C. albicans* cells showed significant changes in the expression levels of *SAP1* compared to the untreated control ($P \leq 0.05$). Treatment with fluconazole increased expression of *SAP1* by 1.53- and 1.43-fold at concentrations of 2 × MIC and 1 × MIC, respectively. In the fluconazole-resistance *C. albicans* cells treated with menthol the expression levels of *SAP1* were down regulated by 2.02- and 1.85-fold at concentrations of 2 × MIC and 1 × MIC, respectively ($P \leq 0.05$).

Discussion

Fluconazole resistance has been identified as the main cause of treatment failure of candidiasis (27,28). Considering a major clinical challenge of antifungal resistance, the antimicrobial potential of the natural products can be regarded as a precious resource. In this study, we showed that menthol displays a potential anti-*Candida* activity. The antifungal activities of menthol are

Table 1. Oligonucleotide primer sequences used for qRT-PCR

Primer	Orientation	Sequence	Reference
SAP1	Forward	5' TTTCATCGCTCTTGCTATTGCTT 3'	(6)
	Reverse	5' TGACATCAAAGTCTAAAGTGACAAAACC 3'	
ACT	Forward	5' GAGTTGCTCCAGAAGAACATCCAG 3'	(26)
	Reverse	5' TGAGTAACACCATCACCAGAATCC 3'	

Table 2. Results susceptibility of fluconazole using disk diffusion and broth microdilution methods against *Candida albicans* vaginal isolates in pregnant women

Susceptibility method	Antibiotic name	N	%R	%I	%S	%R 95% CI	Geom. Mean	MIC range µg/mL	Disk zone diameter (mm) distribution				MIC90 (µg/mL) distribution		
									9	10	11	12	16	32	64
Disk diffusion	Fluconazole	30	100	0	0	85.9-100	-	-	33.3%	20%	43.3%	3.3%	-	-	-
Broth microdilution	Fluconazole	30	100	0	0	85.9-100	27.857	8-64	-	-	-	-	40%	40%	20%

Table 3. Relative MIC (µg/mL) values of menthol against *Candida albicans* vaginal isolates in pregnant women

Isolates / Antifungals	Menthol		Fluconazole	
	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}
C. albicans ATCC 14053	6.50±0.04	1.60±0.02	4.00±0.08	1.00±0.06
C. albicans ATCC MYA-573	3.10±0.01	0.80±0.02	64.00±0.70	16.00±0.08
CI- 1	12.50±0.20	3.10±0.02	16.00±0.10	2.00±0.02
CI- 2	25.00±0.15	3.10±0.02	32.00±0.10	5.00±0.01
CI- 3	12.50±0.03	1.60±0.02	64.00±0.01	16.00±0.10
CI- 4	12.50±0.01	1.60±0.02	64.00±0.03	5.00±0.02
CI- 5	12.50±0.05	3.10±0.01	64.00±0.02	16.00±0.08
CI- 6	3.10±0.01	1.60±0.04	16.00±0.03	8.00±0.03
CI- 7	3.10±0.04	1.60±0.01	32.00±0.10	4.00±0.03
CI- 8	3.10±0.04	0.80±0.02	16.00±0.02	2.00±0.02
CI- 9	3.10±0.01	0.80±0.01	32.00±0.05	2.00±0.01
CI- 10	12.50±0.04	3.10±0.02	32.00±0.10	5.00±0.02
CI- 11	12.50±0.04	6.50±0.02	16.00±0.10	8.00±0.09
CI- 12	12.50±0.02	3.10±0.05	32.00±0.50	2.00±0.06
CI-13	3.10±0.04	0.80±0.02	16.00±0.40	2.00±0.05
CI- 14	1.60±0.01	0.80±0.02	32.00±0.30	2.00±0.01
CI- 15	12.50±0.05	1.60±0.04	64.00±0.50	10.00±0.10
CI- 16	12.50±0.01	1.60±0.02	32.00±0.20	5.00±0.06
CI- 17	25.00±0.01	6.50±0.02	16.00±0.05	4.00±0.05
CI- 18	12.50±0.01	3.10±0.02	64.00±0.04	2.00±0.03
CI- 19	6.50±0.01	3.10±0.05	16.00±0.03	1.00±0.07
CI- 20	25.00±0.02	12.50±0.02	32.00±0.10	2.00±0.06
CI- 21	12.50±0.04	3.10±0.04	16.00±0.09	1.00±0.02
CI- 22	12.50±0.04	1.60±0.04	16.00±0.30	4.00±0.02
CI- 23	6.50±0.02	1.60±0.01	32.00±0.10	4.00±0.02
CI- 24	3.10±0.04	0.80±0.02	16.00±0.06	1.00±0.04
CI-25	3.10±0.01	0.40±0.02	16.00±0.40	5.00±0.06
CI- 26	12.50±0.04	0.80±0.02	32.00±0.20	4.00±0.06
CI- 27	12.50±0.01	1.60±0.02	32.00±0.15	2.00±0.05
CI- 28	12.50±0.01	6.50±0.05	64.00±0.08	10.00±0.09
CI-29	25.00±0.04	3.10±0.04	32.00±0.05	2.00±0.06
CI- 30	12.50±0.02	1.60±0.02	16.00±0.06	2.00±0.04

CI: *C. albicans* vaginal isolates in pregnant women.

reported in research studies and within the context of existing literature (17-20).

The results presented in this paper showed 100% of resistancy in *C. albicans* vaginal isolates to fluconazole. These results are corroborated by the literature, citing fluconazole-resistant *C. albicans* (27-29). Fluconazole is a fungistatic rather than fungicidal medication and hence provides the opportunity for acquired resistance in certain fungi (29). Our data support the effects of menthol in fluconazole-resistant *C. albicans* vaginal isolates. Several lines of evidence demonstrate that natural pure compounds derived from plants are the most potent products to kill antifungal resistant *C. albicans* (23,30).

Our results showed that the antifungal activity of menthol on the fluconazole-resistant *C. albicans* is able to inhibit hyphal formation with a decreasing in the ability of *C. albicans* cells to adhere to host cell surfaces. Following hyphal formation, *C. albicans* hyphae secreted of hydrolases are thought to enhance the efficiency of nutrient uptake. In fact, Saps degrades host proteins, penetrates into the surrounding tissues and finally evades host immune defenses (31). However, the *SAP* genes play key roles in virulence of *C. albicans* required for infections (3-6). In the present study, the relative gene expression analyses were performed to investigate the possible effects of menthol at molecular level. The main changes in the gene expression induced by treatments with menthol were down regulated *SAP1*. These results are in agreement with those reported in the literature regarding the effects of antifungal agents on the expression of *SAP1* (24,30,32). Samaranayake et al. (33) indicated that the expression levels of *SAP1*, *SAP4*, *SAP6*, *SAP8–SAP10* genes were significantly higher in the *C. albicans* SC5314 biofilms than in the controls.

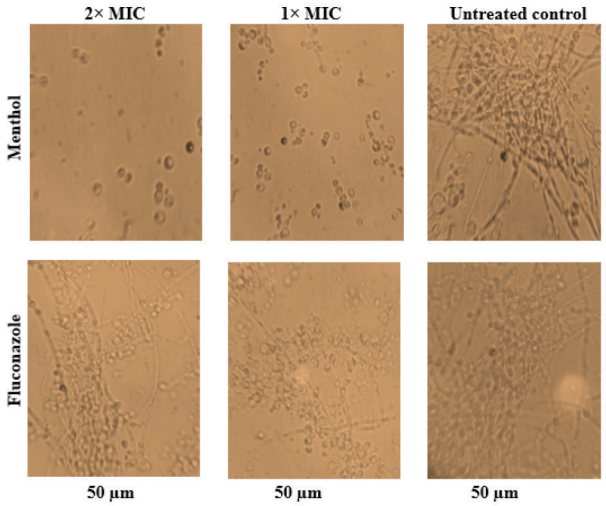

Figure 1. Effect of menthol and fluconazole at concentrations of 2× MIC and 1× MIC on *Candida albicans* ATCC MYA-573 hypha formation under light microscope at 40x magnification, Bar = 50 µm.

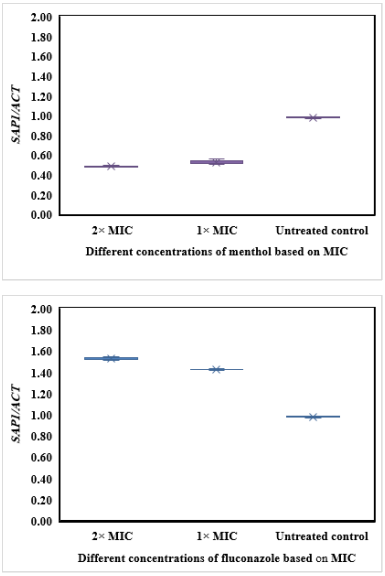

Figure 2. Box plots of *SAP1/ACT* ratio at different concentrations of menthol and fluconazole based on MIC in *Candida albicans* ATCC MYA-573.

In conclusion, our study demonstrated the efficacy of menthol on inhibition of growth, reduction of hyphal formation and down regulation *SAP1* against fluconazole-resistance *C. albicans*. As fluconazole-resistance *C. albicans* is one of the most alarming health issues, this study suggests that menthol may have potential applications in the treatment of infections due to these resistant fungi. In spite of our certainty that the best results are obtained by menthol, further investigation may be focused on the menthol's effcets on other significant genes contributing to the cell pathogenesis of *C. albicans*.

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Authors' contributions
SS carried out the experiments. AK designed the experiment, managed the literature searches and produced the initial draft. All authors read and approved the final version of the manuscript.

Conflict of interest
The authors declared no competing interests.

Ethical considerations
This study was approved by Research Ethics Committee of our institute (Ethical code 14930513952001). The study protocol conformed to the ethical guidelines of the 2008 Declaration of Helsinki.

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