



Antifungal activity of carvacrol in combination with fluconazole or amphotericin B against *Candida albicans*

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ABSTRACT

Aims: The aim of this study was to attempt to evaluate the antifungal activity of carvacrol in combination with fluconazole or amphotericin B against *Candida albicans*.

Methodology and results: Antifungal susceptibilities to carvacrol alone and in combination with fluconazole or amphotericin B were performed using the CLSI standard reference method against clinical isolates of *C. albicans* isolated from the immuno-compromised patients. Proteinase production assay and the expression of genes associated with secreted aspartyl proteinases synthesis (*SAP1* and *SAP2*) were carried out to evaluate the antifungal activity of carvacrol in combination with fluconazole or amphotericin B against *C. albicans*. The carvacrol alone and in combination with fluconazole and amphotericin B exhibited strong inhibitory activity. The carvacrol, exhibited significant synergy and partial synergy with fluconazole or amphotericin B against the test isolates. The data indicated that combination of carvacrol with fluconazole or amphotericin B exerted antifungal effects through reducing SAP enzyme activity. The expression levels of *SAP1* and *SAP2* genes were down-regulated by carvacrol, fluconazole and amphotericin B alone. After carvacrol was employed in combination with fluconazole or amphotericin B, the expression levels of *SAP1* and *SAP2* genes demonstrated the lower expression in comparison to carvacrol alone.

Conclusion, significance and impact of study: These results provide proof of concept for the implementation of carvacrol alone or in combination with fluconazole or amphotericin B inhibitors of *C. albicans*.

Keywords: Amphotericin B, Carvacrol, Fluconazole, *SAP1*, *SAP2*

INTRODUCTION

Human fungal infections claim an estimated 1.5 million lives every year. Although the major threats to health are bacterial, viral and parasitic infections, the clinical relevance of fungal infections has not been adequately recognized. Importantly, *Candida albicans* is a potentially deadly human pathogen in immune-competent and immune-compromised individuals, particularly in patients with hematological malignancies, organ transplant recipients, intensive care unit patients, preterm neonates and patients with various inherited or acquired immune deficiencies (Poulain 2013; Wirnsberger *et al.*, 2016; Kuchler *et al.*, 2016; Lionakis *et al.*, 2017).

The tissue-damaging hydrolytic enzymes such as secreted aspartyl proteinases (SAPs) are considered among the most important virulence factors, which is known to play a central role in the pathogenicity of *C. albicans*. *SAP1* to *SAP10* genes encode preproenzymes approximately 60 amino acids longer than the mature enzyme in *C. albicans* (Naglik *et al.*, 2003). Except for

SAP9 and *SAP10*, which remain bound to the cell wall, *SAP 1-8* secreted by *C. albicans* are released into the surrounding medium. SAPs are characterized by broad spectrum proteolytic ability and virulence properties and are differentially expressed at different stages and forms of *C. albicans* growth and disease. Different expression of *SAP* genes appears to be essential for mucosal and systemic infections. SAPs 1-3 required for adherence of *C. albicans* to the cell surface and tissue damage, whereas *SAP4-SAP6* are associated with systemic disease. Until recently, little is known about *SAP7* and *SAP8* expression pattern in *C. albicans* pathogenesis. *SAP7* and *SAP8* were the only genes expressed transiently during *C. albicans* infections. *SAP9*- and *SAP10*-encoded proteins maintain cell surface integrity, cell separation and also indirectly contribute to adhesion (Hube and Naglik, 2001; Naglik *et al.*, 2003; Borelli *et al.*, 2007; Cassone, 2013; Staniszewska *et al.*, 2014).

There are currently few antifungals in use include polyenes, azoles, allylamine and echinocandins, among which the oldest class of antifungal drugs is the polyenes

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such as amphotericin B is highly fungicidal and directly bind ergosterol and disrupt the fungal cell membrane. Azoles are the most common agent targeting the synthesis of fungal sterols and, in general, are fungistatic (Prasad *et al.*, 2016).

The ever-increasing rate of resistance of *C. albicans*, poses a serious threat to antifungal therapy, calling for urgent need in search of powerful tool in identifying new antifungal agents that could be used to treat the infections. In the other hand, most of the reported chemicals claimed to possess potential antifungal activities usually had strong potential of resistance reversion in antifungal-resistant *C. albicans* (Shao *et al.*, 2017). Therefore, finding safe natural products to enhance antifungal activity of synthetic antifungal agents can be taken into account as an alternative way to expand the antifungal bank.

Carvacrol $C_6H_3CH_3(OH)(C_3H_7)$ is a chemical compound of the essential oils of the *Lamiaceae* family. A monoterpene carvacrol is known to exert potent antimicrobial activity against several microorganisms. Studies have shown that carvacrol has antifungal activity against *C. albicans* (Vardar-Unlu *et al.*, 2010; Ahmad *et al.*, 2011; Chaillot *et al.*, 2015). The mode of action of the antifungal effect of carvacrol is related to fungal membrane instability by targeting and binding ergosterol (Lima *et al.*, 2013; Chaillot *et al.*, 2015). Some studies have reported the combination of oils with fluconazole or amphotericin B against *Candida* infections (Khan *et al.*, 2012).

In the present study, we investigated the antifungal activity of carvacrol against clinical isolates of *C. albicans* as well as a standard one with respect to growth inhibition, synergistic effect, proteinase production assay and the expression of genes associated with SAP synthesis in combination with the synthetic antifungal fluconazole or amphotericin B.

MATERIALS AND METHODS

Anti-microbial agents

Carvacrol, fluconazole, amphotericin B and chloramphenicol were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dimethyl sulfoxide (100%) was used to dissolve stock solutions of carvacrol, fluconazole and amphotericin B. These stock solutions were then diluted to the desired concentration based on CLSI M27-A3 (Clinical and Laboratory Standards Institute, 2008). The carvacrol and fluconazole or carvacrol and amphotericin B are mixed in 1 : 1 ratio.

Organisms

Candida albicans ATCC 14053 was provided as a reference quality control. Clinical isolates of *C. albicans* (Ca1-Ca10) were isolated from immuno-compromised patients in Yasooj, Iran. These isolates were preliminarily identified by microscopic and macroscopic morphology, germ tube formation, urease test. Further identification

was performed by plated on CHROMagar *Candida* medium (CHROMagar Company, France). PCR was performed using the universal fungal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The identity of the PCR products was confirmed by DNA sequencing method. The sequence similarity was analysed via nucleotide BLAST software (Evans and Richardson, 1989; Harmal *et al.*, 2012). All isolates were stored at -80 °C in SDB containing 300 µg/mL of chloramphenicol and sterile 20% (v/v) glycerol.

Preparation of inocula

Preparation of inocula for the broth microdilution testing was performed in accordance with CLSI M27-A3 with RPMI 1640 medium with L-glutamine (Sigma-Aldrich). For each study, after sub-culturing on Sabouraud Dextrose Broth (SDB, Merck Research Laboratories, Darmstadt, Germany). *Candida albicans* isolate were subcultured onto Sabouraud Dextrose Agar (SDA, Merck Research Laboratories) supplemented with chloramphenicol and passaged at least twice to ensure viability and purity, and incubated at 35 °C for 24 h. Fungal suspension was prepared based on CLSI M27-A3. Briefly, transferred five colonies more than 1 mm in diameter to 5 mL of sterile normal saline and the turbidity of each suspension was adjusted to a 0.5 McFarland standard at 530 nm. The inoculum suspension (a concentration of $1 - 5 \times 10^6$ yeast cells/mL) was diluted to reach a final inoculum of $0.5-5 \times 10^3$ yeast cells/mL. For each of the fungal isolates the viability of the yeast was > 97 % by viable pour plate counting method.

Antifungal susceptibility studies

The minimum inhibitory concentrations (MICs) of carvacrol alone and in combination with fluconazole or amphotericin B were determined with a broth microdilution test according to the reference method of the CLSI (M27-A3). The test was carried out using 96-well flat-bottom polystyrene microdilution plates containing 100 µL/well of the twofold dilution of the different concentrations of carvacrol (range 6.25-300 µg/mL) or fluconazole (range 0.03125-64 µg/mL) or amphotericin B (range 0.0313-16 µg/mL) alone and in combination in RPMI-1640 with L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) buffered to pH 7.0 with 0.165 M morpholinophosphonyl sulfate (MOPS)]. Yeast cells inocula were prepared (530 nm, abs 0.08-0.1) and diluted to reach a final concentration of $0.5-5 \times 10^3$ yeast cells/mL in the wells. Then the plates were incubated at 35 °C and MICs were measured at 530 nm from two independent experiments in three separate technical replicates using a Stat Fax 303 Reader (Awareness Technology, Inc., USA) after 24 h. The MIC₉₀ were defined as the lowest concentration of antifungal that caused 90% growth inhibition compared to control growth.

The fractional inhibitory concentration (FIC) index was equal to $(MIC_{\text{carvacrol}} \text{ in combination} / MIC_{\text{carvacrol}})$

alone) + (MIC_{fluconazole} or amphotericin B in combination/MIC_{fluconazole} or amphotericin B alone), in which synergism was interpreted as FIC \leq 0.5, partial synergism was defined when the FIC $>$ 0.5 but $<$ 1.0, additive when FIC = 1.0, indifferent when FIC $>$ 1.0 but $<$ 4.0, and antagonistic when FIC \geq 4.0 (Khodavandi *et al.*, 2010, 2014).

Proteinase production assay

Proteinase activity of *C. albicans* treated with carvacrol alone and in combination with fluconazole or amphotericin B were performed at different concentrations based on MIC (2x MIC, 1x MIC, 1/2x MIC and 1/4x MIC) by the following method: *C. albicans* was grown in 5 mL YCB + BSA medium (11.7 g/L Yeast Carbon Base (Difco Laboratories, Detroit, Michigan, USA), 10 g/L glucose; 5 g/L bovine serum albumin fraction V [Sigma-Aldrich]) and placed in a shaker-incubator at 200 rpm and 30 °C for 72 h. Proteolytic activity was also determined by measuring the increase in trichloroacetic acid soluble products absorbing at 280 nm in triplicate after 1 h incubation of the culture supernatant with BSA substrate at 37 °C. The specific activity was expressed as OD_{280 nm}/OD_{600 nm} values of the culture. The OD readings equal to or less than 0.02 were considered negative (Macdonald and Odds, 1980).

qRT-PCR analysis

The procedures of qRT-PCR analysis were described in a previous study of our group (Khodavandi *et al.*, 2011). Briefly, *C. albicans* ATCC strain (1-5 x 10⁶ yeast cells/mL) was treated with carvacrol alone and in combination with fluconazole or amphotericin B at different concentrations based on MIC (2x MIC, 1x MIC, 1/2x MIC and 1/4x MIC). 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 with RNase-free DNase I (Fermentas, USA). Single-stranded cDNA was synthesized from 0.5 µg extracted RNA using M-MuLV reverse transcriptase and random hexamer oligonucleotides (Fermentas, USA). *Candida albicans* SAP1 and SAP2 were amplified from the synthesized cDNA with primers as described in Table 1 by PCR using TMSYBR Green qPCR Master Mix (Fermentas, USA) in a Bio-Rad MiniOpticonTM system (USA), according to the manufacturer's protocol. Moreover, actin was established as a house-keeping gene to normalize the dissimilar RNA concentrations during RNA extraction. Relative gene expression was quantified by the Pfaffl method.

Table 1: The primers for qRT-PCR.

Primer	Orientation	Sequence	Length (bp)	Reference
SAP1	Forward	5' GTTGGTTTTGGTGGTGCTTC 3'	200	(Hosseini <i>et al.</i> , 2016)
	Reverse	5' GCAGCATTGGGAGAGTTGAG 3'		
SAP2	Forward	5' TGTGGATTCCAGGTACCACCA 3'	192	(Hosseini <i>et al.</i> , 2016)
	Reverse	5' GCAAATTCGGAAGCTGGA 3'		
ACT	Forward	5'GAGTTGCTCCAGAAGAACATCCAG 3'	199	(Khodavandi <i>et al.</i> , 2011)
	Reverse	5'TGAGTAACACCATCACCAGAATCC 3'		

Table 2: MIC₉₀ (µg/mL) and FIC values of carvacrol alone and in combination with fluconazole or amphotericin B against clinical isolates of *Candida albicans*.

Isolates / Antifungals	Carvacrol	Fluconazole	Amphotericin B	Carvacrol/ Fluconazole	Carvacrol/ Amphotericin B
	MIC ₉₀	MIC ₉₀	MIC ₉₀	MIC ₉₀ FIC (Interpretation)	MIC ₉₀ FIC (Interpretation)
<i>C. albicans</i> ATCC 14053	100	16	1	25/2 0.375 (SYN)	6.25/0.125 0.187 (SYN)
CI-1	150	16	1	50/4 0.583 (PSYN)	25/0.5 0.666 (PSYN)
CI-2	100	32	1	50/4 0.625 (PSYN)	6.25/0.125 0.187 (SYN)
CI-3	150	8	2	50/4 0.833 (PSYN)	25/0.5 0.417 (SYN)
CI-4	300	16	2	50/1 0.229 (SYN)	25/0.5 0.333 (SYN)
CI-5	200	16	4	50/4 0.50 (PSYN)	25/0.5 0.25 (SYN)
CI-6	150	16	2	50/4 0.583 (PSYN)	6.25/0.125 0.104 (SYN)
CI-7	200	32	4	100/8 0.75 (PSYN)	12.5/0.25 0.125 (SYN)
CI-8	250	32	4	200/16 1.30 (IND)	25/0.5 0.225 (SYN)
CI-9	200	16	1	100/8 1.00 (ADT)	12.5/0.25 0.312 (SYN)
CI-10	300	8	2	100/8 1.33 (IND)	25/0.5 0.333 (SYN)

CI: Clinical isolates of *C. albicans*; SYN, synergism; PSYN, partial synergism; ADT, additive; IND, indifferent.

Table 3: Results of proteinase production assay (Means OD_{280nm}/OD_{600nm} ± SD) of clinical isolates of *Candida albicans* treated with carvacrol alone and in combination with fluconazole or amphotericin B at different concentration based on MIC.

Clinical isolates of <i>C. albicans</i> /Concentration of antifungal agents	<i>C. albicans</i> ATCC 14053	CI-1	CI-2	CI-3	CI-4	CI-5	CI-6	CI-7	CI-8	CI-9	CI-10
Carvacrol											
2 × MIC	0.03 ± 0.04 ^a	0.03 ± 0.08 ^a	0.03 ± 0.01 ^a	0.03 ± 0.09 ^a	0.03 ± 0.05 ^a	0.03 ± 0.05 ^a	0.03 ± 0.01 ^a	0.03 ± 0.03 ^a	0.03 ± 0.09 ^a	0.03 ± 0.01 ^a	0.03 ± 0.01 ^a
1 × MIC	0.04 ± 0.03 ^b	0.03 ± 0.06 ^a	0.04 ± 0.06 ^b	0.03 ± 0.05 ^a	0.04 ± 0.02 ^b	0.03 ± 0.02 ^a	0.03 ± 0.05 ^a	0.04 ± 0.04 ^b	0.03 ± 0.01 ^a	0.04 ± 0.06 ^b	0.04 ± 0.01 ^b
½ × MIC	0.05 ± 0.05 ^c	0.04 ± 0.06 ^b	0.04 ± 0.08 ^b	0.04 ± 0.01 ^b	0.04 ± 0.09 ^b	0.04 ± 0.03 ^b	0.04 ± 0.07 ^b	0.05 ± 0.01 ^c	0.04 ± 0.02 ^b	0.05 ± 0.04 ^c	0.04 ± 0.01 ^b
¼ × MIC	0.05 ± 0.02 ^c	0.05 ± 0.06 ^c	0.05 ± 0.07 ^c	0.05 ± 0.07 ^c	0.05 ± 0.08 ^c	0.05 ± 0.05 ^c	0.04 ± 0.05 ^c	0.05 ± 0.03 ^c	0.04 ± 0.03 ^b	0.05 ± 0.02 ^c	0.05 ± 0.01 ^c
Untreated control	0.06 ± 0.01 ^d	0.06 ± 0.09 ^d	0.06 ± 0.02 ^d	0.06 ± 0.10 ^d	0.05 ± 0.05 ^c	0.06 ± 0.03 ^d	0.06 ± 0.08 ^c	0.06 ± 0.10 ^d	0.06 ± 0.04 ^c	0.06 ± 0.07 ^d	0.06 ± 0.08 ^d
Fluconazole											
2 × MIC	0.02 ± 0.01 ^a	0.02 ± 0.06 ^a	0.03 ± 0.03 ^a	0.02 ± 0.01 ^a	0.02 ± 0.04 ^a	0.02 ± 0.04 ^a	0.02 ± 0.02 ^a	0.02 ± 0.01 ^a	0.02 ± 0.04 ^a	0.02 ± 0.05 ^a	0.02 ± 0.03 ^a
1 × MIC	0.03 ± 0.02 ^b	0.02 ± 0.05 ^a	0.03 ± 0.01 ^a	0.03 ± 0.06 ^b	0.03 ± 0.01 ^b	0.02 ± 0.07 ^a	0.02 ± 0.07 ^a	0.03 ± 0.01 ^b	0.02 ± 0.04 ^a	0.03 ± 0.07 ^b	0.03 ± 0.04 ^b
½ × MIC	0.03 ± 0.09 ^b	0.03 ± 0.06 ^b	0.04 ± 0.03 ^b	0.04 ± 0.04 ^c	0.03 ± 0.09 ^b	0.03 ± 0.07 ^b	0.03 ± 0.04 ^b	0.03 ± 0.01 ^b	0.03 ± 0.05 ^b	0.03 ± 0.09 ^b	0.03 ± 0.06 ^b
¼ × MIC	0.04 ± 0.06 ^c	0.04 ± 0.05 ^c	0.04 ± 0.05 ^b	0.05 ± 0.02 ^d	0.04 ± 0.02 ^c	0.04 ± 0.01 ^c	0.04 ± 0.01 ^c	0.04 ± 0.01 ^c	0.05 ± 0.04 ^c	0.04 ± 0.09 ^c	0.05 ± 0.06 ^c
Untreated control	0.06 ± 0.01 ^d	0.06 ± 0.09 ^d	0.06 ± 0.02 ^c	0.06 ± 0.10 ^e	0.05 ± 0.05 ^d	0.06 ± 0.03 ^d	0.06 ± 0.08 ^d	0.06 ± 0.10 ^d	0.06 ± 0.04 ^d	0.06 ± 0.07 ^d	0.06 ± 0.08 ^d
Amphotericin B											
2 × MIC	0.02 ± 0.01 ^a	0.02 ± 0.01 ^a	0.02 ± 0.04 ^a	0.02 ± 0.05 ^a	0.02 ± 0.09 ^a	0.02 ± 0.01 ^a	0.02 ± 0.05 ^a	0.02 ± 0.01 ^a	0.02 ± 0.06 ^a	0.02 ± 0.06 ^a	0.02 ± 0.08 ^a
1 × MIC	0.03 ± 0.02 ^b	0.02 ± 0.01 ^a	0.03 ± 0.05 ^b	0.03 ± 0.04 ^b	0.02 ± 0.02 ^a	0.03 ± 0.02 ^b	0.03 ± 0.05 ^b	0.03 ± 0.01 ^b	0.02 ± 0.05 ^a	0.03 ± 0.02 ^b	0.03 ± 0.05 ^b
½ × MIC	0.04 ± 0.04 ^c	0.03 ± 0.01 ^b	0.03 ± 0.04 ^b	0.04 ± 0.04 ^c	0.04 ± 0.02 ^b	0.04 ± 0.01 ^c	0.03 ± 0.06 ^b	0.04 ± 0.01 ^c	0.03 ± 0.04 ^b	0.04 ± 0.01 ^c	0.03 ± 0.01 ^b
¼ × MIC	0.05 ± 0.02 ^d	0.04 ± 0.03 ^c	0.04 ± 0.05 ^c	0.04 ± 0.07 ^c	0.05 ± 0.02 ^c	0.04 ± 0.06 ^c	0.04 ± 0.02 ^c	0.04 ± 0.04 ^c	0.04 ± 0.08 ^c	0.04 ± 0.03 ^c	0.04 ± 0.02 ^c
Untreated control	0.06 ± 0.01 ^e	0.06 ± 0.09 ^d	0.06 ± 0.02 ^d	0.06 ± 0.10 ^d	0.05 ± 0.05 ^d	0.06 ± 0.03 ^d	0.06 ± 0.08 ^d	0.06 ± 0.10 ^d	0.06 ± 0.04 ^d	0.06 ± 0.07 ^d	0.06 ± 0.08 ^d
Carvacrol/Fluconazole											
2 × MIC	0.01 ± 0.02 ^a	0.02 ± 0.01 ^a	0.02 ± 0.02 ^a	0.02 ± 0.01 ^a	0.02 ± 0.01 ^a	0.02 ± 0.02 ^a	0.02 ± 0.02 ^a	0.02 ± 0.01 ^a	0.02 ± 0.00 ^a	0.01 ± 0.05 ^a	0.02 ± 0.01 ^a
1 × MIC	0.02 ± 0.02 ^b	0.02 ± 0.03 ^b	0.02 ± 0.02 ^a	0.02 ± 0.01 ^a	0.02 ± 0.01 ^a	0.02 ± 0.00 ^a	0.02 ± 0.01 ^a	0.02 ± 0.01 ^a	0.02 ± 0.01 ^a	0.02 ± 0.00 ^a	0.02 ± 0.00 ^a
½ × MIC	0.02 ± 0.02 ^b	0.03 ± 0.04 ^c	0.02 ± 0.03 ^a	0.03 ± 0.03 ^b	0.02 ± 0.01 ^a	0.02 ± 0.01 ^a	0.02 ± 0.00 ^a	0.03 ± 0.02 ^b	0.03 ± 0.05 ^b	0.03 ± 0.04 ^b	0.02 ± 0.07 ^a
¼ × MIC	0.02 ± 0.02 ^b	0.03 ± 0.03 ^c	0.02 ± 0.01 ^a	0.03 ± 0.02 ^b	0.03 ± 0.02 ^b	0.03 ± 0.02 ^b	0.03 ± 0.04 ^b	0.03 ± 0.01 ^b	0.03 ± 0.02 ^b	0.03 ± 0.01 ^b	0.03 ± 0.00 ^b
Untreated control	0.06 ± 0.01 ^c	0.06 ± 0.09 ^d	0.06 ± 0.02 ^b	0.06 ± 0.10 ^c	0.05 ± 0.05 ^c	0.06 ± 0.03 ^c	0.06 ± 0.08 ^c	0.06 ± 0.10 ^c	0.06 ± 0.04 ^c	0.06 ± 0.07 ^c	0.06 ± 0.08 ^c
Carvacrol/Amphotericin B											
2 × MIC	0.01 ± 0.01 ^a	0.02 ± 0.02 ^a	0.02 ± 0.01 ^a	0.02 ± 0.01 ^a	0.01 ± 0.04 ^a	0.02 ± 0.01 ^a	0.02 ± 0.01 ^a	0.02 ± 0.03 ^a	0.02 ± 0.02 ^a	0.01 ± 0.00 ^a	0.02 ± 0.00 ^a
1 × MIC	0.02 ± 0.02 ^a	0.02 ± 0.02 ^a	0.02 ± 0.02 ^a	0.02 ± 0.03 ^a	0.02 ± 0.01 ^b	0.02 ± 0.01 ^a	0.02 ± 0.01 ^a	0.02 ± 0.01 ^a	0.02 ± 0.02 ^a	0.02 ± 0.00 ^b	0.02 ± 0.00 ^a
½ × MIC	0.03 ± 0.02 ^b	0.02 ± 0.01 ^a	0.02 ± 0.05 ^a	0.02 ± 0.05 ^a	0.02 ± 0.01 ^b	0.03 ± 0.01 ^b	0.02 ± 0.01 ^a	0.03 ± 0.02 ^b	0.02 ± 0.05 ^a	0.02 ± 0.03 ^b	0.02 ± 0.00 ^a
¼ × MIC	0.03 ± 0.02 ^b	0.02 ± 0.01 ^a	0.03 ± 0.03 ^b	0.03 ± 0.01 ^b	0.03 ± 0.04 ^c	0.03 ± 0.04 ^b	0.02 ± 0.01 ^a	0.03 ± 0.03 ^b	0.03 ± 0.02 ^b	0.02 ± 0.03 ^b	0.03 ± 0.03 ^b
Untreated control	0.06 ± 0.01 ^c	0.06 ± 0.09 ^b	0.06 ± 0.02 ^c	0.06 ± 0.10 ^c	0.05 ± 0.05 ^d	0.06 ± 0.03 ^c	0.06 ± 0.08 ^b	0.06 ± 0.10 ^c	0.06 ± 0.04 ^c	0.06 ± 0.07 ^c	0.06 ± 0.08 ^c

CI: Clinical isolates of *C. albicans*. ^{a-e}Means ± S.D in each treatment and column with different superscript differ significantly (Tukey's HSD, *p* < 0.05). The results were performed in three independent experiments.

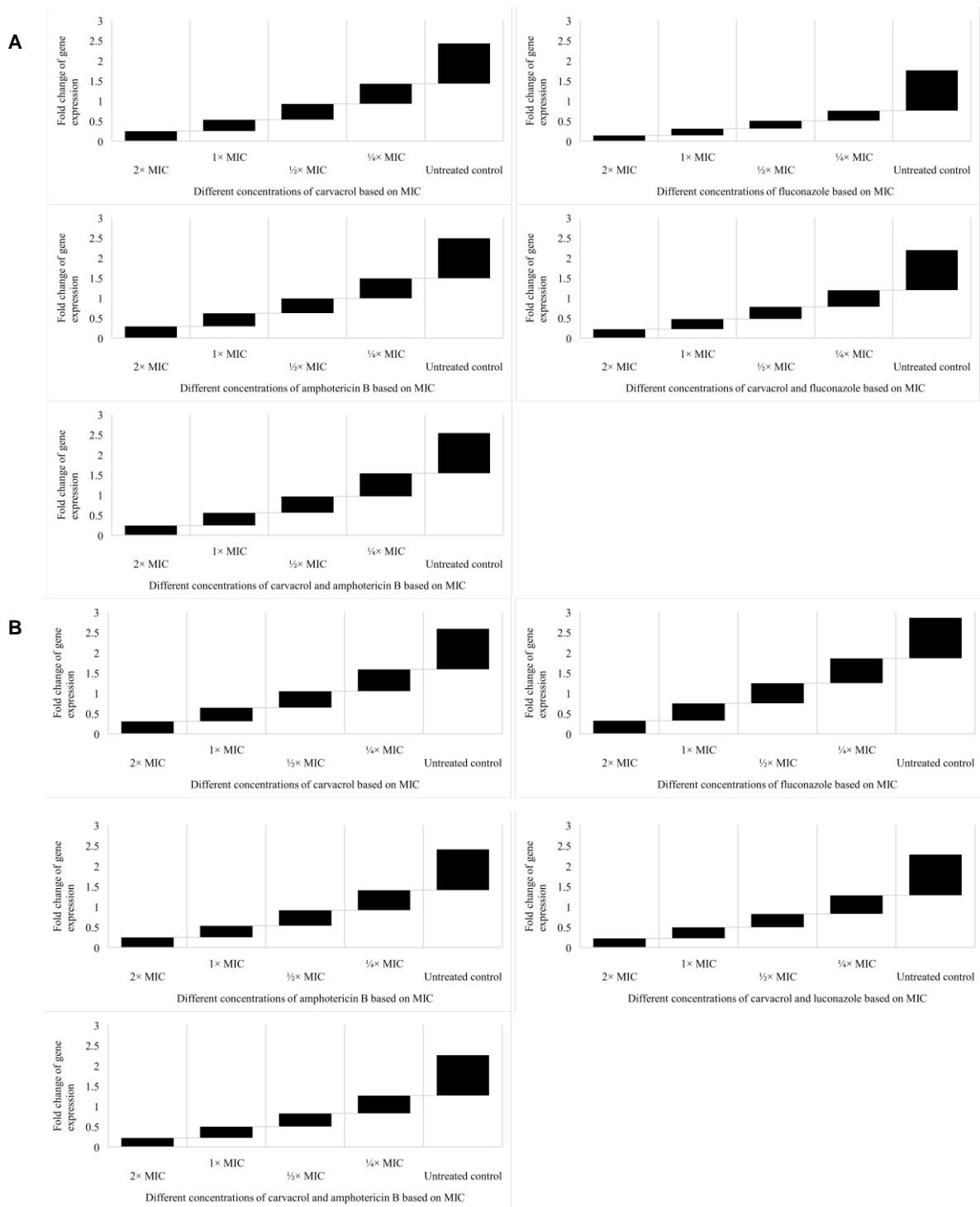


Figure 1: qRT-PCR analysis of *SAP1* (A) and *SAP2* (B) genes expression in *Candida albicans* ATCC 14053 treated with carvacrol in combination with fluconazole or amphotericin B at different concentrations based on MIC compared with the untreated control.

The clinical isolates of *C. albicans* treated with carvacrol alone and in combination with fluconazole or amphotericin B showed decreased proteolytic activity compared to untreated control (Tukey's HSD, $p < 0.05$; Table 3). Compared with carvacrol used alone, the proteolytic activity of carvacrol was reduced 1.5–3-fold in concomitant use with fluconazole or amphotericin B.

Impacts of carvacrol alone and in combination with fluconazole or amphotericin B on gene expressions

In the present study, *C. albicans* ATCC was treated with different concentrations of carvacrol alone and in combination with fluconazole or amphotericin at different concentrations based on MIC (2x MIC, 1x MIC, 1/2x MIC and 1/4x MIC). Compared with the untreated control, the expression of *SAP1* was down-regulated by 4.00-, 3.52-, 2.54- and 1.99-fold and *SAP2* down-regulated by 3.26-, 2.96-, 2.47- and 1.86-fold after 2x MIC, 1x MIC, 1/2x MIC and 1/4x MIC carvacrol treatment, respectively ($p < 0.05$). When *C. albicans* exposed to 2x MIC, 1x MIC, 1/2x MIC and 1/4x MIC of fluconazole the expression of *SAP1* was down-regulated by 6.99-, 5.95-, 5.08- and 3.97-fold and *SAP2* down-regulated by 3.06-, 2.35-, 2.01- and 1.64-fold, respectively ($p < 0.05$). The expression level of *SAP1* was down-regulated by 3.40-, 3.05-, 2.71- and 1.99-fold and *SAP2* down-regulated by 3.97-, 3.55-, 2.63- and 2.03-fold after 2x MIC, 1x MIC, 1/2x MIC and 1/4x MIC amphotericin B treatment, respectively ($p < 0.05$). Under the combined application of 2x MIC, 1x MIC, 1/2x MIC and 1/4x MIC of carvacrol and fluconazole, it could be observed that the expression of *SAP1* was down-regulated by 4.46-, 3.94-, 3.32- and 2.39-fold and *SAP2* down-regulated by 4.41-, 3.69-, 3.05- and 2.19-fold, respectively ($p < 0.05$). The expression of *SAP1* was down-regulated by 4.12-, 3.19-, 2.46- and 1.74-fold and *SAP2* down-regulated by 4.48-, 3.60-, 3.07- and 2.29-fold, after 2x MIC, 1x MIC, 1/2x MIC and 1/4x MIC carvacrol and amphotericin B treatment, respectively ($p < 0.05$, Figure 1). In addition, patterns of *C. albicans* gene expression were similar in response to antifungal activity of carvacrol alone and in combination with fluconazole or amphotericin B. Under the combined treatment of carvacrol with fluconazole or amphotericin B, the expression levels of *SAP1* and *SAP2* genes demonstrated the higher fold change of expression in comparison to carvacrol alone.

DISCUSSION

Invasive fungal infections have increased in different immuno-compromised populations. Currently, an increase in the number of *Candida* that are resistant to synthetic antifungal drugs is recognized worldwide; therefore, high throughput screening has emerged as a powerful tool in identifying new antifungal agents that could be used to treat the infections caused by resistant pathogens. Antifungal agents with these characteristics and favourable safety and drug interaction profile would be attractive to evaluate the role of combination therapy for treatment of infections that are difficult to treat (Sardi *et*

al., 2013; Haque *et al.*, 2016; Lionakis *et al.*, 2017). Carvacrol was reported to possess antimicrobial activity against bacteria and also show a certain inhibition on *C. albicans* (Vardar-Unlu *et al.*, 2010; Ahmad *et al.*, 2011; Chaillot *et al.*, 2015).

In this study, we made a rigorous test on the antifungal activity of carvacrol alone and in combination with fluconazole or amphotericin B. Carvacrol alone displayed anti-*C. albicans* effect compared with fluconazole or amphotericin B. The synergism of carvacrol with fluconazole or amphotericin B against clinical isolates of *C. albicans* indicated the strong potential of carvacrol to promote the therapy of fluconazole or amphotericin B. Our results are in partial agreement with Doke *et al.* (2014), who demonstrated that, carvacrol and fluconazole in combination effectively inhibits *C. albicans* biofilm. Khan *et al.* (2012) demonstrated that, the oils of *Cymbopogon martini*, i.e., citral and cinnamaldehyde especially eugenol, exhibited significant synergy with fluconazole or amphotericin B, against multi-drug resistant isolates of *C. albicans*. The combination of carvacrol with cinnamaldehyde (Ye *et al.*, 2013), nisin (Periago and Moezelaar, 2001) and ampicillin and nitrofurantoin (Zhang *et al.*, 2011) showed good synergistic antibacterial effect against bacterial strains.

In this study, the proteolytic activity was reduced in *C. albicans* treated with carvacrol in concomitant with fluconazole or amphotericin B. In addition, *SAP1* and *SAP2* genes associated with SAP synthesis were analyzed by qRT-PCR. As demonstrated, *SAP1* and *SAP2*, both of which were down-regulated in *C. albicans* cells treated with carvacrol alone and in combination with fluconazole or amphotericin B. After carvacrol was employed in combination with fluconazole or amphotericin B, the expression levels of *SAP1* and *SAP2* genes demonstrated the higher fold change of expression in comparison to carvacrol alone, inferring that the *C. albicans* would produce lower SAPs. Our results are in partial agreement with Hosseini *et al.*, (2016), who demonstrated that, carvacrol is effective in reduction of *SAP1-3* expression with a particular effect against fluconazole-resistant *C. albicans* isolates.

In this study, we confirmed that carvacrol could be a candidate of synergist with fluconazole or amphotericin B against clinical isolates of *C. albicans*. Whether these events reflect the potential of carvacrol alone and in combination with fluconazole or amphotericin B for inhibition of SAP synthesis in *C. albicans* which differentially expresses specific gene requires further studies.

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