

# *Satureja khuzistanica* Jamzad essential oil and its anti-candidal activities against clinical isolates of *Candida albicans* isolated from women with candidiasis

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## Abstract

*Satureja khuzistanica* Jamzad is known as antiseptic and analgesic agent in folk medicine. The aim of this investigation was to evaluate the anti-candidal activity of *S. khuzistanica* aerial parts essential oil against clinical isolates of *Candida albicans*, which were isolated from women with chronic recurrent candidiasis. For this purpose, the chemical composition of hydro-distilled essential oil was determined by GC and GC-MS analysis. Then, the anti-candidal activity of essential oil and its main component (carvacrol) were determined. Carvacrol (94.1%) was the main component of essential oil, followed by  $\beta$ -bisabolene, *p*-cymene and  $\gamma$ -terpinene. *S. khuzistanica* essential oil had strong anti-candidal activity against clinical isolates of *C. albicans* via inhibition of germ tube formation and induction the huge punctures in the cytoplasmic structures. The cell membranes were intact in presence of essential oil or carvacrol. *S. khuzistanica* essential oil as the main source of carvacrol can be used for treatment of *C. albicans* related infections.

**Keywords:** *Satureja khuzistanica*, essential oil, *Candida albicans*, Germ tube, Carvacrol

## Actividad anti-*Candida* de los aceites esenciales del Jamzad *Satureja khuzistanica* contra aislados clínicos de *Candida albicans* obtenidos de mujeres con candidiasis

## Resumen

*Satureja khuzistanica* Jamzad es conocido como analgésico y antiséptico en la medicina tradicional. El objetivo de esta investigación fue evaluar el efecto anti-*Candida* de los aceites esenciales obtenidos de las partes aéreas de *S. khuzistanica* sobre aislados clínicos de *Candida albicans*, obtenidos de mujeres con candidiasis crónica recurrente. Para este propósito la composición química de aceites esenciales hidrodestilados fueron determinados por análisis GC y GC-MS. Luego la actividad anti-candidasica de los aceites esenciales y de su componente principal (carvacrol) fue determinando. Carvacrol (94.1%) fue el principal compuesto del aceite esencial seguido por  $\beta$ -bisaboleno, *p*-cimeno and  $\gamma$ -terpineno. El aceite esencial de *S. khuzistanica* tuvo fuerte actividad anti-candida contra aislados clínicos de *C. albicans* via la inhibición de tubo germinal y la inducción de estructuras puntiformes en la membrana citoplásmica. Las membranas celulares quedaron intactas en presencia del aceite esencial o del carvacrol. El aceite esencial de *S. khuzistanica* como fuente principal de carvacrol podría ser usado como tratamiento de infecciones relacionadas con *Candida albicans*.

**Palabras claves:** *Satureja khuzistanica*, aceites esenciales, *Candida albicans*, tubo germinal (prueba), Carvacrol

## Introduction

*Candida albicans* as the most important cause of fungal infections in immune compromised patients can be the reason for different infections from simple mucosal infections to the lethal forms<sup>1</sup>. Candidiasis is one of the most common fungal infections, which is caused by *Candida* species espe-

cially by *C. albicans*. Topical azole drugs are usually used for treatment. Continuous use of these drugs has been associated with adverse effects<sup>2</sup> and development of resistant *C. albicans* isolates<sup>3,4</sup>. Drug resistant *C. albicans* isolates and the adverse effects of current treatments, have encouraged the scientists to find the new natural herbal antifungal agents in different systems of traditional medicines<sup>5-9</sup>.

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*S. khuzistanica* has been traditionally used as an antiseptic and analgesic agent<sup>10</sup>. Some biological activities of *S. khuzistanica* such as immune stimulatory action<sup>11</sup>, neuro-protective effect<sup>12</sup>, improving the fertility in rats<sup>13</sup>, inhibitory effects against diabetic nephropathy<sup>14</sup>, protective effects against toxicity of malathion<sup>15</sup> and cyclophosphamide<sup>16</sup>, increasing the opioid analgesic tolerance to morphine<sup>17</sup>, efficacy in treatment of IBD (inflammatory bowel Disease)<sup>12</sup>, antioxidant<sup>18</sup>, anti-diabetic<sup>19,20</sup>, antinociceptive<sup>17</sup>, and anti-parasitic effects<sup>21,22</sup> were confirmed in different investigations.

The antifungal activity of *S. khuzistanica* essential oil against *Cryptococcus neoformans*<sup>23</sup>, *Aspergillus flavus*, *A. niger*, *Penicillium* sp., *Fusarium* sp., *Alternaria* sp., *Rhizopus* sp. and *Mucor* sp<sup>24</sup> were reported. In this study, we evaluated the anti-candidal activity of *S. khuzistanica* essential oil against clinical *C. albicans*, which were isolated from vaginal samples of woman suffering from recurrent candidiasis. *S. khuzistanica* essential oil related mechanisms were also determined by evaluating its ability to germ tube formation and observing the structures of cell by scanning electron microscope (SEM) and transmission electron microscopy (TEM) in presence of essential oil and carvacrol.

## Materials and methods

### Plant materials, extraction of essential oil and GC, GC-MS analysis

*Satureja khuzistanica* aerial parts at full flowering stage were collected from Lorestan Province, Iran in June 2014 and were authenticated under herbarium number of 168-1 by Agricultural Department of Medicinal Plant Research Center of Barij, Kashan, Iran by Dr. H. Hosseini. The dried aerial parts of *S. khuzistanica* were grinded and subjected to hydro-distillation by Clevenger type apparatus for 3 h. The yellow essential oil was separated and kept in dark vial at cold place (4 °C) until the analysis.

The chemical composition of *S. khuzistanica* essential oil was analyzed using Gas chromatography (GC) and Gas chromatography-Mass Spectra (GC-MS). The GC and GC-MS analyses were conducted on Agilent technology (HP) 6890 with capillary column of HP-1MS (30 m × 0.25 mm, film thickness 0.25 µm) and Agilent technology (HP) 6890 that was coupled with 5973 network mass selective detector system, respectively. The oven temperature program was initiated at 40 °C, held for 1 min, then raised up to 230 °C at a rate of 3 °C/min, held for 10 min. Helium was used as carrier gas at a flow rate of 1.0 ml/min with a split ratio equal to 1/50 injector. The detector and injector temperatures were 250 and 230 °C, respectively. Components of essential oil were identified by comparison with Retention Indices (RI) relative to homologous series of n-alkanes (injected in conditions equal to sample) and by computer search using libraries of Wiley 275.L and Wiley 7n.1, as well as comparison of the fragmentation pattern of the mass spectra with data published in the literature<sup>25</sup>.

### Microbial strains

This study was conducted on 30 clinical isolates of *C. albicans* that were isolated from vaginal samples of women suffering from candidiasis. *C. albicans* isolates were identified according to the results of positive germ tube formation, growth on chloramphenicol sabouraud dextrose agar, chlamyospore formation, color of colony, carbohydrate absorption and fermentation tests. *C. albicans* ATCC 10231 was used as control strains.

### Anti-candidal activities evaluation of essential oil

By using the CLSI methods including disc diffusion (M44-A2) and micro broth dilution (M27-A3) assays, the anti-candidal activities of essential oil, and synthetic carvacrol (Merck) were determined on clinical isolates of *C. albicans*. The turbidity of each *C. albicans* suspension was adjusted to 85% at 530 nm ( $1 \times 10^6$  CFU/ml) by spectrophotometer. Suspensions were cultured on Sabouraud dextrose agar using a sterile cotton swab. Subsequently, filter discs (6 mm in diameter) (Padtan Teb Co, Tehran, Iran) were saturated with different concentrations of essential oil (0.5, 0.75, 1, 1.5 µl) and synthetic carvacrol (0.473, 0.945 µl). Clotrimazole, fluconazole and amphotericin B discs (Rosco Diagnostica A/S, Taastrupgaardsvej 30, DK-2630, Taastrup) were used as positive controls. The plates were incubated at 37 °C for 48 h. The inhibition zone diameters were determined and reported in millimeter ± Standard Deviation (mm±SD)<sup>26</sup>.

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of essential oil, clotrimazole and carvacrol were determined by micro broth dilution assay. The compounds were two-fold serially diluted. 100 µl of dilutions were poured in 96-well micro titer plates. MOPS-buffered RPMI 1640 was used as broth media. 100 µl of diluted candidal suspensions ( $10^4$  CFU/ml) was added to each well and incubated at 35±2 °C. MIC values were defined as the lowest concentrations of compounds that inhibited the growth after 48 h. MFC values were the first concentrations that showed no growth on Sabouraud dextrose agar<sup>27</sup>. The experiments were replicated three times and means±standard deviation (SD) were used for drawing the graphs and analysis by Graphpad prism 6. ONE-Way ANOVA test was used to compare the difference between compounds with  $p_{\text{values}}$  at level of 0.05.

### Germ tube inhibition test

For germ tube inhibition assay, cell suspension from overnight cultured Sabouraud dextrose agar with *C. albicans* ATCC 10231 was prepared and the turbidity was adjusted to obtain a density of  $1.0 \times 10^6$  CFU/ml. *S. khuzistanica* essential oil and carvacrol were diluted in DMSO. Then, 10 µl of diluted essential oil or carvacrol were added to 990 µl of yeast suspension to obtain the concentrations equal to 1/8, 1/4, 1/2 and 1 times MIC value. Control suspension (without essential oil or carvacrol) was included as positive control. After treatment, 0.5 ml of sheep serum was added to yeast sediment, the tube was incubated for 2-3 h in a water bath with temperature of 35±2 °C. A drop of this suspension was placed on Neobar slide. The germ tube formation was evaluated and the results of three independent experiments were presented as means±SD<sup>28</sup>.

### Observation of treated *C. albicans* cells by TEM and SEM

For investigating the effect of *S. khuzistanica* essential oil and carvacrol in liquid phase by TEM, *C. albicans* cell suspension ( $1 \times 10^6$  CFU/ml) was inoculated in RPMI containing the MIC value of *S. Khuzistanica* essential oil or carvacrol (mixed with 0.5% Tween 80). The flasks were incubated at 30 °C and 180 rpm for 24 h. The treated *C. albicans* cells and control cells were separated from the media by centrifugation at 8000 rpm for 10 min. The treated *C. albicans* cells were fixed with 2.5% glutaraldehyde in phosphate buffer for 30 min and washed three times in 0.1 M phosphate buffer solution (pH 7.2). Each suspension was serially dehydrated with ethanol 25%, 50%, 75%, 90%, and 100%, respectively. Then, the cells were lyophilized. The pellet was post-fixed in 1% osmium tetroxide for 30 min, washed with phosphate buffer solution (pH 7.2), serially dehydrated in ethanol and embedded in Epon–Araldite resin for making the blocks of the cells pellet. Ultra-thin sections of the cells were stained with gold-palladium and observed under a Philips transmission electron microscope (EM 208) at 100 KV and direct magnification of  $180,000 \times^{29}$ .

For SEM, a thin serially dehydrated film of cells with ethanol was smeared on a glass cover slip. Samples were stored in desiccators until they were coated with gold-palladium sputter for 200-seconds (Nano Structured coating co. Iran). SEM micrographs were performed using TESCAN VEGA3S electron microscope, at 20 KV.

### Results

#### Chemical Composition of *S. khuzistanica* essential oil

The extraction yield for essential oil was 4.9% (v/w). The GC and GC-MS analysis of *S. khuzistanica* essential oil showed the presence of 34 different components, representing 98.4% of total essential oil composition. Carvacrol (94.1%),  $\beta$ -bisabolene (1.7%) were the main components of *S. khuzistanica* essential oil, followed by *p*-cymene (0.44%) and  $\gamma$ -terpinene (0.34%) (Table 1).

#### Antifungal activity of *S. khuzistanica* essential oil and carvacrol

In disc diffusion method, different concentrations of *S. khuzistanica* essential oil (0.5, 0.75, 1 and 1.5  $\mu$ l/disc) and carvacrol (0.48, 0.95  $\mu$ l/disc) showed that the inhibition zone diameters (mm) of essential oil or carvacrol were increased dose dependently. 1.5  $\mu$ l/disc of *S. khuzistanica* essential oil had higher inhibition zone diameters on clinical isolates of *C. albicans* than that of ketoconazole (10  $\mu$ g/disc), and clotrimazole (10  $\mu$ g/disc). The inhibition zone diameters of 1  $\mu$ l/disc *S. khuzistanica* essential oil was higher than amphotericin B (50 U/disc) but was lower than ketoconazole (10  $\mu$ g/disc), and clotrimazole (10  $\mu$ g/disc). The inhibition zone diameter of 0.75  $\mu$ l/disc *S. khuzistanica* essential oil was equal to amphotericin B. The comparison of inhibition zone diameters of *S. khuzistanica* essential oil showed that there is no significant difference between 0.47  $\mu$ l/disc carvacrol (equal to amount of carvacrol in 0.5  $\mu$ l *S. khuzistanica* oil) and 0.5  $\mu$ l/disc essential oil, but the inhibition zone diameter of 0.945  $\mu$ l/disc carvacrol (equal to amount of carvacrol in 1  $\mu$ l essential oil) was higher than that of 1  $\mu$ l/disc *S. khuzistanica* essential oil (Figure 1).

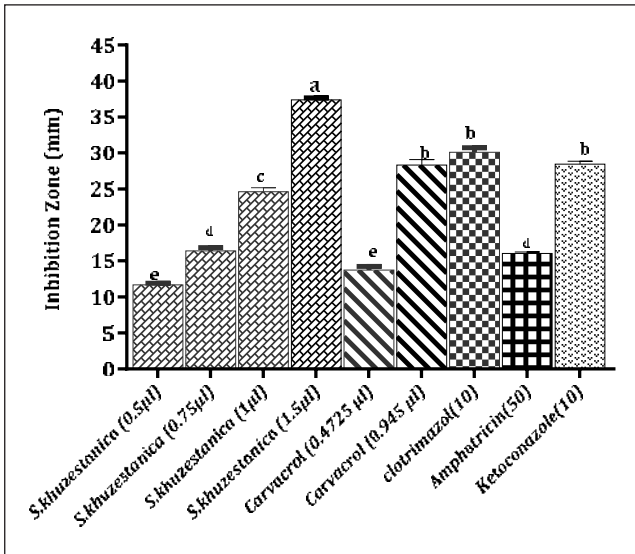
**Table 1.** GC-MS analysis of *Satureja khuzistanica* essential oil

compound	RI	percent
furfural	737	0.01
$\alpha$ -thujene	837	0.04
$\alpha$ -pinene	843	0.04
comphene	858	0.01
sabinene	886	0.01
$\beta$ -pinene	888	0.01
2,3-dehydro-1,8-cineole	905	0.07
$\beta$ -myrcene	906	0.05
$\alpha$ -phellandrene	917	0.05
$\delta$ -3-carene	922	0.01
$\alpha$ -terpinene	929	0.20
<i>p</i> -cymene	938	0.44
$\beta$ -phellandrene	941	0.10
1,8-cineole	943	0.01
$\gamma$ -terpinene	971	0.34
cis-sabinene hydrate	981	0.01
$\alpha$ -terpinolene	999	0.05
linalool	1013	0.10
4-terpineol	1081	0.02
carvacrol methyl ether	1142	0.05
thymol	1200	0.04
Carvacrol	1257	94.1
4-hydroxy-3-(1-methylethyl) benzaldehyde	1275	0.04
Eugenol	1295	0.20
carvacryl acetate	1303	0.02
trans-caryophyllene	1327	0.34
$\alpha$ -bergamotene	1336	0.10
neryl acetone	1350	0.10
$\alpha$ -farnesene	1352	0.02
$\gamma$ -curcumene	1366	0.02
cuparene	1369	0.03
2-phenylethyl ester	1375	0.03
3-Isopropyl-1,2-dimethoxybenzene	1377	0.02
$\beta$ -Bisabolene	1387	1.70
$\alpha$ -Cedrene	1388	0.02

RI=Retention Index

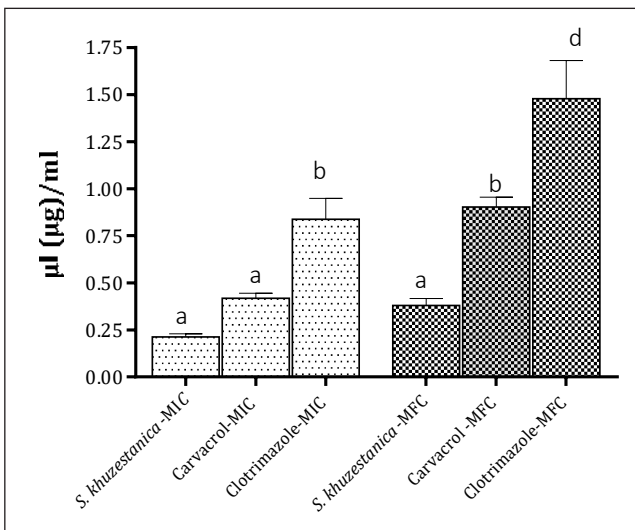
The antifungal activity evaluation of *S. khuzistanica* essential oil and carvacrol by micro broth dilution assay by assessing their MIC and MFC values showed insignificant difference between the MIC and MFC values of *S. khuzistanica* essential oil and MIC value of carvacrol ( $P > 0.05$ ). Furthermore, the MFC value of carvacrol had significant difference with its MIC value (Figure 2).

Evaluation the inhibitory effects of essential oil and carvacrol on germ tube formation exhibited that the germ tube inhibitory effect of *S. khuzistanica* essential oil was stronger than that of carvacrol (Figure 3).



**Figure 1.** The anti-candidal activity evaluations of compounds against clinical isolates of *C. albicans* by disc diffusion method. Significant at the level of 0.05 and the higher inhibition zone diameter to the lower level were shown by the letter a, b, c, d, e.

In order to observe the caused cytological changes by *S. khuzistanica* essential oil and carvacrol, the prepared samples were observed by SEM and TEM. Figure 4 exhibits the untreated (control), treated with essential oil or carvacrol, respectively. In SEM observation, minimal changes were observed on the structure of treated cells. Some bubbles and notches were observed on cell membranes of treated *C. albicans* cells. In TEM images, significant ultra-structural modifications were observed in response to *S. khuzistanica* essential oil and carvacrol in cytoplasm structures. Huge punctures were observed in treated cells with *S. khuzistanica* essential oil and carvacrol. In fact, *S. khuzistanica* essential oil and carvacrol formed huge pits in cytoplasm in comparison with untreated *C. albicans* cells.

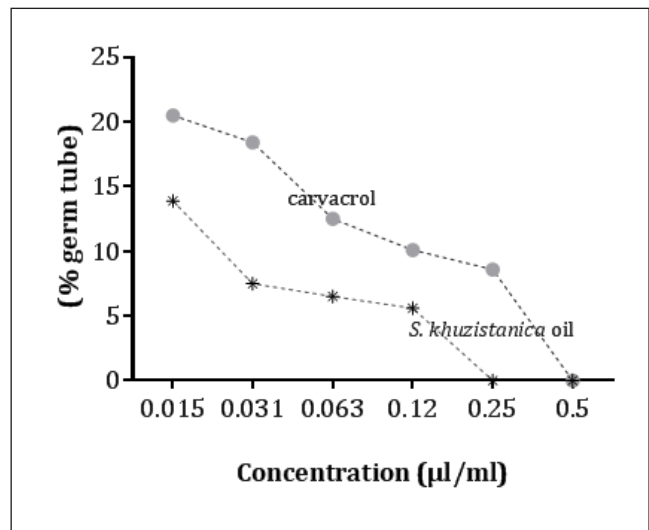


**Figure 2.** The antifungal activity of *S. khuzestanica* essential oil and carvacrol (µl/ml) in comparison with clotrimazole (µg/ml) against clinical isolates of *C. albicans* by micro broth dilution assay. Significant at the level of 0.05 and the lower value to higher level were shown by the letter a, b, c, d, respectively.

## Discussion

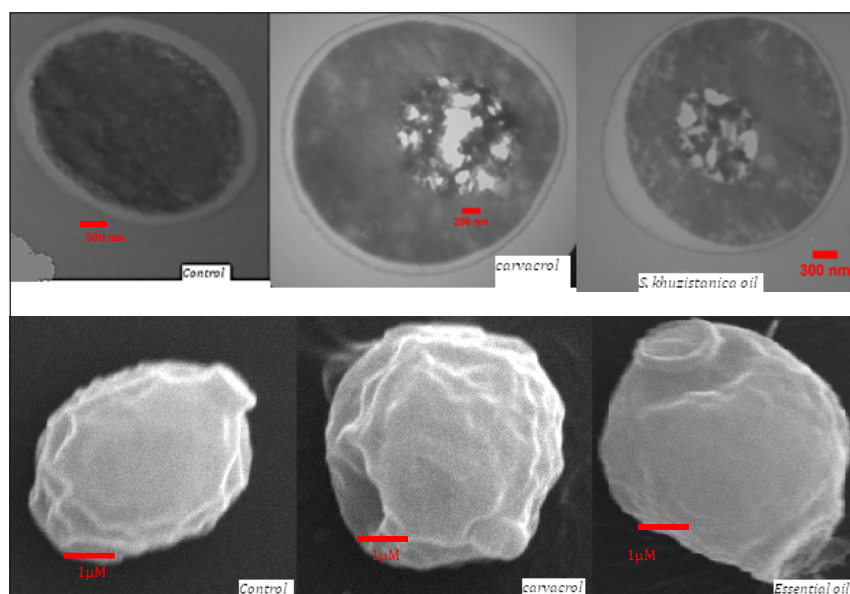
*C. albicans* is the cause of superficial or life threatening systemic infections. In two different kinds of infections, the pathogenicity of *C. albicans* is determined by some virulence factors such as germ tube formation. Germ tube formation by *C. albicans* has been reported as critical factor in attachment and pathogenesis of its strains<sup>30</sup>. Development of novel therapies against surface colonization of *C. albicans* by *Aloe vera* extract<sup>31</sup>, *Ferulago capillaris* essential oil<sup>32</sup>, and *Rosmarinus officinalis* essential oil<sup>33</sup> has been the subject of some investigations. According to the results of this study, *S. khuzistanica* essential oil showed significant anti-candidal effects on clinical *C. albicans* and inhibitory effects on germ tube formation. The germ tube inhibitory effects of *S. khuzistanica* essential oil was related to its main component "carvacrol" according to the results of our study. Due to the higher germ tube inhibitory effect of *S. khuzistanica* essential oil than that of carvacrol, it can be concluded that the other minor compounds of essential oil along with carvacrol may involve in germ tube inhibitory effects of *S. khuzistanica* essential oil. For example, *p*-cymene alone as no efficient antimicrobial agent, can enhance the antimicrobial activity of carvacrol<sup>34</sup>.

The results of anti-candidal activity evaluation of carvacrol on clinical isolates of *C. albicans* was different by two methods. In disc diffusion method, the inhibition zone diameter of carvacrol was higher than *S. khuzistanica* essential oil. In broth dilution assay, carvacrol showed the lower anti-candidal effects against *C. albicans*. Although, carvacrol is a known antifungal agent<sup>35,36</sup>, but the quota of carvacrol in *S. khuzistanica* essential oil with higher than 90% carvacrol (94.1%) has not been the subject of any investigation. Due to lower anti-candidal activity of pure carvacrol than that of *S. khuzistanica* essential oil with 94.1% carvacrol, other minor components of essential oil may exhibit synergistic effects with carvacrol.



**Figure 3.** The inhibitory effects of *S. khuzestanica* essential oil and carvacrol on germ tube formation





**Figure 4.** Observation of treated *C. albicans* cells with *S. khuzistanica* essential oil, carvacrol as compared with untreated *C. albicans* cells by Transmission electron microscopy (TEM) (line 1) and scanning electron microscope (SEM) (line 2)

Investigation on cellular structures of *C. albicans* by TEM and SEM images showed that cytoplasmic membranes of *C. albicans* cells were intact in presence of *S. khuzistanica* essential oil and carvacrol, while some huge punctures were observed in cell cytoplasm in TEM images. Although, the fungicidal activity of carvacrol through the disruption of ergosterol synthesis has been reported<sup>37</sup>; but our results showed the cell's membranes were intact in presence of carvacrol or essential oil. Therefore, the anti-candidal activity of *S. khuzistanica* essential oil was related to changes in the cytoplasmic structures of *C. albicans* cells. The results of microscopic observations were in conformity with the results of anti-candidal activities.

Our results have provided the new evidences implicating that *S. khuzistanica* essential oil had promising anti-candidal activity against clinical isolates of *C. albicans* by inhibition of germ tube formation and destruction of cytoplasmic structures. Furthermore, it needs further formulations for evaluating the efficacy of *S. khuzistanica* essential oil against *C. albicans* related infections in animal models and human studies.

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Ethical responsibilities

**Protection of human and animal subjects.** This research do not use animal nor human material or data.

**Confidentiality of data.** Not applicable

### Conflict of interest statement

We declare that we have no conflict of interest.

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