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Phytochemical analysis and antifungal activity of *Satureja hortensis* and *Zataria multiflora* essential oils

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Abstract

Over the last few decades, the rising incidence of fungal infections has become a significant and growing threat to healthcare systems worldwide. *Candida* species, particularly *C. albicans*, and *C. tropicalis*, constitute zoonotic, opportunistic pathogens transmissible from animals to humans, potentially driving the emergence of novel infectious diseases and underscoring the urgent need for innovative antifungal agents, and treatment strategies. This study was conducted to evaluate the antifungal effects of two natural products, *Satureja hortensis* and *Zataria multiflora* essential oils, against four pathogenic fungal microorganisms: *Candida* species, including *C. albicans*, *C. dubliniensis*, and *C. tropicalis*, as well as *Penicillium chrysogenum*. The extraction of essential oils (EOs) was performed using a Clevenger-type apparatus. The chemical constituents of *S. hortensis* EO were analyzed using gas chromatography-mass spectrometry (GC-MS). Finally, to determine the minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of *S. hortensis* and *Z. multiflora*, the broth microdilution method was employed. Findings from this investigation demonstrated that the main compound of *S. hortensis* was Thymol (36.51%). The MIC range was from 0.039 - 0.156 µL/mL for *Z. multiflora* and from 0.019 - 0.312 µL/mL for *S. hortensis* Bioss. Corresponding MFC values ranged from 0.039- 0.625 µL/mL for *S. hortensis*, and 0.078- 1.25 µL/mL for *Z. multiflora* Boiss. These results suggest that the studied EOs may represent promising candidates for the development of new agents to treat cutaneous and mucosal Candida infections.

Introduction

Fungi are important disease agents because particular species cause infections in humans, animals, and plants, ranging from superficial to life-threatening. Molds (such as *Aspergillus*) and yeasts (like *Candida*) represent major fungal groups that differ in form and pathogenic mechanisms, but both include clinically relevant species. *Candida* spp. are predominant etiological agents of fungal infections in humans (1). Among yeasts, *Candida albicans* (*C.*

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albicans) is the most frequently documented species and is associated with numerous pathological conditions; however, species such as *C. tropicalis*, *C. krusei*, and *C. glabrata* are also common (1). *Candida* spp. have emerged as a leading nosocomial pathogen in intensive care units, with infections rising alarmingly. Members of the genus are commensals in the digestive tract, upper airway, and genital regions of various animals, where they can act as opportunistic pathogens. Among these, *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and newly identified strains such as *C. auris* show significant zoonotic potential (2, 3). These species can serve as reservoirs in wild and farmed animals, enabling transmission to humans via direct contact, environmental contamination, or contaminated materials. This zoonotic aspect is particularly relevant for immunocompromised individuals, as transmission can lead to systemic candidiasis. Environmental changes and antimicrobial resistance further exacerbate risk, underscoring the need to monitor animal populations to mitigate threats to human health. Pathogenic molds, including members of *Fusarium*, *Aspergillus*, and *Penicillium* genera, are notable factors in food spoilage and foodborne diseases. These fungi can contaminate food at various stages, including cultivation, harvest, transport, and storage, and may develop antifungal resistance either intrinsically or during treatment (4-6).

The use of natural compounds, especially plant essential oils (EOs), has risen as an alternative to antifungal drugs (7). EOs are volatile, natural products comprising diverse aromatic compounds, including sesquiterpenes, monoterpenes, and their oxygenated derivatives (aldehydes, alcohols, ethers, esters, ketones, oxides, phenols) (8). Composition varies with geography, climate, season, and plant development. Their lipophilicity allows integration into fungal membranes, increasing permeability, promoting efflux of intracellular constituents, and inactivating enzymes (9).

The genus *Satureja*, a member of the Lamiaceae family, comprises over 30 aromatic species, primarily found in the Mediterranean region (10). In Iran, *Satureja* comprises 12 species, eight of which are endemic, concentrated in the northern, northwestern, and western regions. Many *Satureja* spp. exhibit aromatic and medicinal properties, which are valuable for various applications (11). The antifungal properties of *S. hortensis* (*S. hortensis*) EO have been linked to Thymol and Carvacrol, which disrupt fungal cell membranes, compromise membrane integrity, and inhibit biofilm formation, contributing to antifungal activity via cell membrane deformation and cell death (12). *Zataria multiflora* (*Z. multiflora*), a Thyme-like plant, is commonly found in southern and central Iran, Afghanistan, and Pakistan (13). It contains high levels of Thymol and Carvacrol. These compounds disrupt fungal membranes, permeabilize them, and compromise cellular homeostasis, leading to cell death (14, 15). Additionally, they inhibit *Candida* biofilm formation, thereby reducing pathogenicity and antifungal resistance (16).

Despite studies on *S. hortensis* antifungal activity, phytochemical and antifungal data for *S. hortensis* from Ardabil Province, Northwest Iran, are lacking. This study evaluates the inhibitory effects of the EOs of *S. hortensis* and *Z. multiflora* Boiss, against four *Candida* species (*C. albicans*, *C. dubliniensis, C. tropicalis, C. krusei*) and one mold (*P. chrysogenum*), using minimal fungicidal concentrations (MFC) and minimal inhibitory concentrations (MIC).

Materials and Methods

Collection of plants and preparation of EOs

The EO of *Z. multiflora* Boiss, derived via steam distillation, was acquired from the Iranian Institute of Medicinal Plants in Karaj, Alborz province, Iran. The *S. hortensis* plant was collected in spring (May) from Ardabil province and then dried, and subsequently specimens were recognized by the Herbarium of the Faculty of Pharmacy at Tabriz

University of Medical Sciences, Tabriz, Iran. The EO of *S. hortensis* aerial parts was obtained through a Clevenger apparatus and a three-hour hydrodistillation process. The acquired oil was dried employing anhydrous $Na_2 SO_4$ before storage at 4 °C for subsequent evaluation.*GC-MS analysis of S. hortensis essential oil*

The extracted EO of *S. hortensis* was analyzed using GC. The Data were collected using an Agilent 6890 UK chromatograph equipped with an HP-5MS capillary column measuring 30 mm in length, 0.25 mm in internal diameter, and featuring a film thickness of 0.25 µm. The analytical conditions included an initial temperature of 50 °C, with 5 °C/min temperature ramps followed by a 240 °C/min ascent to reach 300 °C (maintained for 3 minutes). The injector functioned at 290 °C. Helium was utilized as the carrier gas, with a flow rate maintained at 0.8 mL/min for the split ratio. The obtained findings were confirmed through GC-MS analysis of EO using an Agilent 6890 gas chromatograph coupled with an Agilent 5973 mass-selective detector, both from Agilent UK, under identical analytical conditions and using the same capillary column. The settings of the electron-ionization mode, operated at 70 eV, shaped the MS configuration (17).

Tested Microorganisms

The antifungal activities of EOs were assessed against four *Candida* species, including *C. albicans* ATCC 11006, *C. tropicalis* ATCC 750, *C. dubliniensis* CD36, and *C. krusei* PTCC 5295, along with *Penicillium chrysogenum* ATCC 11709. These fungal strains were obtained from the mycology laboratory at the Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran.

Evaluation of antifungal activity of S. hortensis and Z. multiflora Boiss EOs

The MICs and MFCs of EOs were determined using the broth microdilution technique, as specified in the protocols established by the Clinical and Laboratory Standards Institute (CLSI, M27-A3). Sodium bicarbonate-free and L-glutamine-containing RPMI 1640 (Roswell Park Memorial Institute medium) (Sigma, Germany) was used as the broth medium to prepare the microdilution plates. By adding 0.165 mol/L of MOPS (morpholino propanesulfonic acid) (Sigma, Germany), the medium was adjusted to a pH of 7.0. In the microdilution assay, 100 µL of stock concentration (10 µL/mL) of each EO, which was diluted using DMSO 10% (Merck Company, Germany) solution and sterilized using a 0.45 µm filter, was introduced to the first well, which contained 100 µL of medium. Then, two-fold serial dilutions were performed for EOs across wells 1 to 10, resulting in concentrations ranging from 5 µL/mL to 0.009 µL/mL. Fluconazole was employed as the standard antifungal medication compound in this study. A stock solution at a concentration of 128 µg/mL was prepared, and, similar to the EO preparation procedure, two-fold dilutions were conducted directly within the microplate to achieve final concentrations ranging from 64 to 0.125 µg/mL in the wells (1-10). The growth control well (well 11) was inoculated with 100 µL of the inoculum suspension into 100 µL of sterile medium devoid of antifungal agents. The final well (well 12) was a sterility control, containing only medium. To monitor for potential contamination of the EOs, a control well containing 100 µL of the diluted EO and 100 µL of broth medium was included.

The fungal suspension preparation was initiated with vortex mixing, followed by standardization of the McFarland standard transmittance to 0.5 at a wavelength of 530 nm. The inoculum solution was prepared to contain a concentration between 0.5×10^3 and 2.5×10^3 cells/mL. For *Penicillium*, the final inoculum yielded was 10^4 cells/mL. MICs were assessed visually after 24 hours of incubation at 35°C for *Candida* species and 48 hours at 30°C for *Penicillium*. The occurrence or absence of cellular proliferation was documented, and the proliferation observed in each experimental well was evaluated in comparison to that in the control well (18). The MFC is defined as the lowest EO concentration that inhibits observable proliferation. A 10 µL aliquot from each non-growing MIC well

was subcultured in triplicate onto Sabouraud dextrose agar (Biolife Italiana, Italy) and incubated for 48 hours. The MFC was established as the lowest EO concentration at which no colonies were detected in any of the wells.

Statistical analysis

The results are presented as the mean ± SD of three replicates and were analyzed employing SPSS software (Version 27). Statistical significance among groups was evaluated using one-way ANOVA, followed by Duncan's multiple comparison test. A *p-value* of less than 0.05 ($P< 0.05$) was considered indicative of statistical significance.

Results

Chemical compositions of S. hortensis EO

The composition of *S. hortensis* EO was determined utilizing GC-MS. The constituents of *S. hortensis* EO, and chromatogram are shown in Table 1 and Figure 1. The GC-MS analysis of the desiccated aboveground portions of *S. hortensis* EO identified 21 compounds, representing 97% of the total EO, and was characterized by a large amount of Thymol (36.51%).

Table 1. Chemical compositions of *S. hortensis* essential oil

No	*S. hortensis* EO		
	Compounds	Retention time	Area (%)
1	α-Thujene	4.59	1.84
2	α-pinene	4.74	1.4
3	Camphene	5	0.45
4	2-β-pinene	5.49	0.77
5	β-Myrcene	5.68	1.96
6	δ-3-Carene	5.96	0.32
7	1-phellandrene	6.05	0.27
8	α-Terpinene	6.2	1.65
9	ρ-Cymene	6.59	25.36
10	γ-Terpinene	7.13	13.05
11	Styrene	7.49	0.41
12	Linalool	7.61	0.23
13	3-Cyclohexen-1-ol	9.21	1.79
14	Carvacrol methyl ether	10.53	3.69
15	Thymol	11.98	36.51
16	Carvacrol	12.08	4.75
17	Caryophyllene	14.1	1.2
18	Ledene	15.71	0.24
19	B-bisabolene	15.92	0.28
20	Spathulenol	17.52	0.39
21	Caryophyllene oxide	17.64	0.44
Total	-	-	97

Discussion

Scientific investigation of plants used in traditional medicine is crucial for discovering new antimicrobial compounds. Furthermore, the renewed interest in natural therapies and rising consumer demand for safe and effective natural products necessitate quantitative data on plant oils and extracts (19). GC-MS analysis of the *S. hortensis* aerial parts EO indicated that Thymol was the most abundant compound (36.51%). Other significant constituents included ρ-cymene and γ-terpinene, while Carvacrol and Carvacrol methyl ether were present in lower concentrations. Only three compounds were present in amounts greater

than 10% (74.92% of compositions). These GC-MS results align with previous studies that also reported Thymol as the primary component of this EO (20, 21).

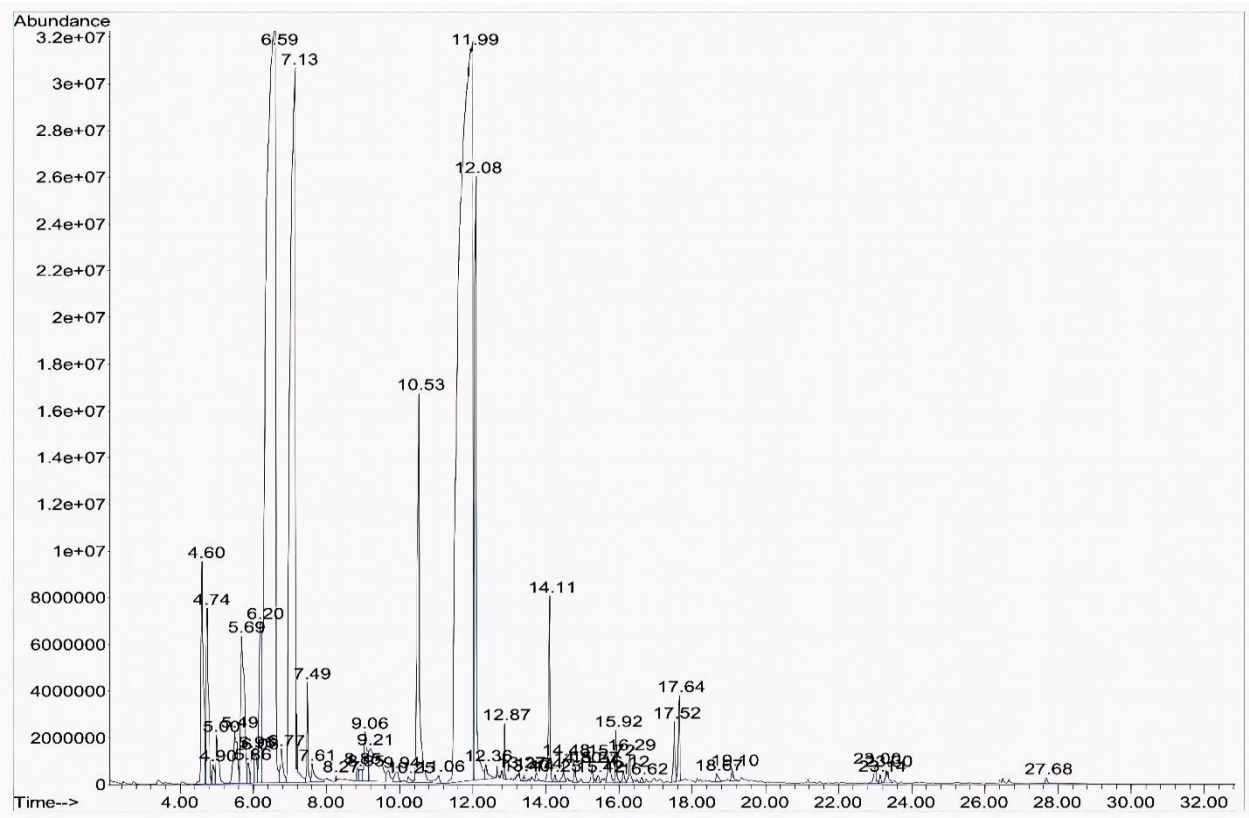

Fig. 1. Chromatogram of *Satureja hortensis* essential oil. *Antifungal activity of S. hortensis and Z. multiflora Boiss EOs*

In the broth microdilution method, the MIC ranges for *S. hortensis* and *Z. multiflora* Boiss EOs against fungal species were 0.019- 0.312 µL/mL and 0.039- 0.156 µL/mL, respectively. Corresponding MFC values spanned 0.039- 0.625 µL/mL for *S. hortensis* and 0.078- 1.25 µL/mL for *Z. multiflora* Boiss. Table 2 presents the MIC and MFC values of *S. hortensis* and *Z. multiflora* Boiss EOs against *C. albicans*, *C. tropicalis*, *C. kruzei*, *C. dubliniensis*, and *P. chrysogenum.*

Table 2. The MIC and MFC of *S. hortensis* and *Z. multiflora* Boiss EOs (µL/mL) and Fluconazole (µg/mL) against different fungal species (Mean ± SD)

Microorganisms	MIC or MBC	Plant EOs		Fluconazole
		S. hortensis	*Z. multiflora* Boiss	
C. albicans	MIC	0.039 ± 0^{b}	0.156 ± 0^{c}	2
	MFC	0.039 ± 0^{a}	0.41 ± 0.18^{b}	-
C. dubliniensis	MIC	0.019 ± 0^{a}	0.039 ± 0^{a}	0.125
	MFC	0.078 ± 0^{b}	0.078 ± 0^{a}	-
C. tropicalis	MIC	0.039 ± 0^{b}	0.156 ± 0^{c}	0.5
	MFC	0.078 ± 0^{b}	0.625 ± 0^{c}	-
C. kruzei	MIC	0.039 ± 0^{b}	0.156 ± 0^{c}	32
	MFC	0.078 ± 0^{b}	1.25 ± 0^{d}	-
P. chrysogenum	MIC	0.312 ± 0^{c}	0.1 ± 0.045^{b}	-
	MFC	0.625 ± 0^{c}	0.156 ± 0^{a}	-

Different small letters in each column (a-d) indicate statistically significant difference ($p < 0.05$).

In a similar study, GC-MS analysis of *S. hortensis* EO highlighted a distinct regional difference in the dominant compounds. Plants collected in western Turkey exhibited Carvacrol as the primary component, while those from eastern Turkey showed Thymol as the principal constituent (22). The content and composition of EOs are significantly affected by the research methodology used. Sefidcon et al. found that drying methods and extraction techniques influenced the chemical compounds and the yield of *S. hortensis* EO in plants farmed in Iran. They concluded that oven-drying at a temperature of 45 °C, followed by hydrodistillation, was optimal for achieving rapid drying, a high oil yield, and a high percentage of Carvacrol in the aerial parts of *S. hortensis* (23).

In a study conducted by Keykhosravy et al. (2020), Carvacrol was identified as the primary constituent of *Z. multiflora* Boiss EO (24). The main components of *Z. multiflora* Boiss EO are phenolic monoterpenes, Thymol and Carvacrol, which are synthesized from γ-terpinene via a biosynthetic pathway that uses p-cymene as an intermediate. Thymol and Carvacrol-containing oils commonly also contain these biosynthetic precursors (25). Similar studies have revealed that Carvacrol is the most abundant component of *Z. multiflora* Boiss EO (26, 27). In contrast to the present investigation, several other studies detected Thymol as the main compound of *Z. multiflora* Boiss EO. The discrepancies and heterogeneity in previous reports concerning the chemical profile of *Z. multiflora Boiss* EO (28, 29) can be attributed to various factors, including the plant's developmental stage, climate and seasonal variations, and different geographical conditions.

The findings from this research align with prior studies that have reported the antifungal potential of *S. hortensis* and *Z. multiflora* Boiss EOs rich in phenolic compounds such as Thymol and Carvacrol (20, 27). The observed activity is likely attributed to the high concentration of these phenolic monoterpenes, which are known to disrupt fungal cell membranes and inhibit ergosterol biosynthesis (12, 30).

The antifungal activity of EOs from *S. hortensis* and *Z. multiflora* Boiss was evaluated against *Candida* species and *P. chrysogenum*, aligning with the objectives to assess their inhibitory potentials compared to fluconazole, and hypothesizing that effective alternatives could be found due to compounds like thymol disrupting fungal membranes. Results showed MIC ranges of 0.019–0.312 µL/mL for *S. hortensis* and 0.039–0.156 µL/mL for *Z. multiflora*, with MFCs indicating fungicidal action, *S. hortensis* outperforming against *Candida spp*., and *Z. multiflora* against *P. chrysogenum*. Fluconazole was effective (MIC 0.125–2 µg/mL) against susceptible strains but less so against *C. krusei* (MIC 32 µg/mL), reflecting the inherent resistance of this species.

Our findings demonstrate that *S. hortensis* EO exhibits significant antifungal properties, as evidenced by the low MIC and MFC values observed against *C. albicans, C. tropicalis, C. dubliniensis,* and *C. kruzei* in the broth microdilution method. Therefore, the antifungal activities of the examined EOs were found to be more effective than those of fluconazole. These results are encouraging regarding the potential use of these EOs as alternatives to antifungal medications in the future; however, further studies are necessary to assess their in vivo effects.

The species exhibiting the highest susceptibility was *C. dubliniensis*, which demonstrated a MIC of 0.019, followed by *C. albicans, C. tropicalis, and C. kruzei,* which presented MIC values between 0.039 and 0.078. *P. chrysogenum* was less susceptible, exhibiting higher MIC and MFC at concentrations of 0.312 and 0.625, respectively. The antifungal activity of *S. hortensis* EO appears to be variable. While an in vitro study reported no effect for *S. hortensis* EO in a MIC agar dilution

assay (21), another study found a MIC of 0.2 µL/mL against *C. albicans* (22). The relatively higher resistance observed in *P. chrysogenum* compared to *Candida* species might be attributed to differences in cell wall composition and inherent resistance mechanisms, as also seen by researchers in the past (31, 32).

The findings of the present study demonstrate that *Z. multiflora* Boiss EO possesses significant antifungal properties against *P. chrysogenum* compared to *S. hortensis* EO. Interestingly, *Z. multiflora* Boiss EO exhibited higher antifungal potency against *C. dubliniensis* compared to the other tested microorganisms, with the MIC value of 0.039 µL/mL. This higher potency against this specific fungal pathogen suggests potential therapeutic applications that warrant further exploration. These results align with prior studies that have documented the antifungal activity of *Z. multiflora* Boiss (33, 34).

According to the findings of this study, the EO derived from *S. hortensis* exhibits more potent and extensive antifungal activity than that of *Z. multiflora* Boiss against *Candida* species. The findings have practical implications for the development of novel antifungal agents. The increasing prevalence of antifungal resistance necessitates the exploration of alternative therapeutic strategies, and EOs of plants such as *S. hortensis* and *Z. multiflora* Boiss could serve as a promising candidate for further investigation. Further research should focus on characterizing the active antifungal components within these EOs, as well as evaluating their efficacy in vivo.

Conclusion

This study highlights the potent antifungal activity of EOs from *S. hortensis* and *Z. multiflora*, primarily attributed to their dominant compounds Thymol and Carvacrol, respectively, against key pathogenic fungi, including *Candida* species and *P. chrysogenum*. The observed low MIC and MFC values underscore their potential as natural alternatives for combating the escalating threat of fungal infections, particularly in cutaneous and mucosal contexts.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethical approval

This study was approved by The Ethics Committee of the Sarab Faculty of Medical Sciences (Ethic number: IR.SARAB.REC.1403.002).

Artificial Intelligence Statement

The researchers recognize the application of artificial intelligence resources, such as Grammarly's paraphrasing feature, Perplexity AI, Grammarly tool, and Grok3, in refining the scholarly excellence of the manuscript, which was predominantly authored by the contributors themselves. Furthermore, the platform "https://imgupscaler.ai/" was employed to elevate the resolution of visual elements.

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