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Evaluation of the Synergistic Interaction between *Satureja hortensis* and *Carum carvi* Essential Oils and Fluconazole against *Candida albicans*: In Vitro

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ABSTRACT

Backgrounds: Nowadays, excessive use of fungal drugs has led to the development of drug-resistant fungi, making it necessary to find natural and herbal antifungal agents. This in-vitro study aimed to evaluate the interactions of *Satureja hortensis* and *Carum carvi* essential oils together and each essential oil with fluconazole against *Candida albicans* ATCC-10231.

Materials & Methods: In this study, antifungal properties of different concentrations of *S. hortensis* (0.0244-1.56 µL/mL) and *C. carvi* (0.39-25 µL/mL) were investigated by broth-microdilution method based on CLSI M27-A3 and M27-S4 standard documents. The interactions of essential oils together and each essential oil with fluconazole were evaluated by checkerboard assay. Then using the ΣFIC index, the interaction results were interpreted.

Findings: *S. hortensis* essential oil showed higher antifungal activity than *C. carvi* essential oil. (MIC/MFC: *S. hortensis*: 1.56/3.12 µL/mL and *C. carvi*: 12.5/25 µL/mL). The interaction between *S. hortensis* essential oil and fluconazole was on the synergic and additive borderline (FICI=0.508), the interaction between *C. carvi* essential oil and fluconazole was additive (FICI=0.62), and *C. carvi* and *S. hortensis* essential oils showed no interaction together (FICI=2.015).

Conclusion: The essential oils of *S. hortensis* and *C. carvi* separately exhibited powerful antifungal activities. The use of *S. hortensis* essential oil at a very low concentration along with fluconazole caused an interaction very close to synergy and increased fluconazole antifungal activity. Therefore, *S. hortensis* is a potential candidate for combined use with fluconazole to treat *C. albicans* related diseases.

Keywords: *Satureja hortensis*, *Carum carvi*, *Candida albicans*, Fluconazole, Synergy.

CITATION LINKS

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Introduction

Candidiasis, as one of the most common opportunistic fungal infections, usually occurs as skin and mucosal infections. However, the importance of the pathogenesis of fungal infections is more apparent in immunocompromised patients with lethal systemic infections [1, 2]. *Candida albicans* is the most common species isolated from healthy or infected individuals, which accounts for the majority of candidiasis cases. However, in recent decades, the prevalence of non-albicans species such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei,* and *C. dubliniensis* has increased [2-6].

Antifungal drugs are less developed compared to antibiotics since it is hard to find a substance that is effective on eukaryotic fungal cells but is not toxic to host cells [7]. The most common antifungal drugs used to treat diseases caused by *C. albicans* are azoles (fluconazole and itraconazole) and polyenes (amphotericin B). Although, the treatment of fungal infections is associated with several challenges such as drug resistance, toxicity, and side effects [8], natural compounds are promising, and many of them are effective against *Candida* species [9]. Due to the mentioned challenges and economic considerations, research in the field of herbal medicines has increased in recent years [10, 11].

Carum carvi, also known as Persian cumin, is a biennial plant of the *Apiaceae* family, which is native to Europe, western Asia, and North Africa. The essential oil extracted from its fruit has antidiabetic, antifungal, anti-lipid, antioxidant, anti-inflammatory, antitumor, and antibacterial effects [12, 13].

Satureja hortensis or summer savory is a plant belonging to the savory species and the *Lamiaceae* family, which is traditionally used in the treatment of common cold and stomachache [14]. The essential oil extracted from the aerial parts of this plant has antioxidant, anti-inflammatory, antibacterial, and antifungal properties [15, 16].

Combination therapy is one of the ways to increase the effectiveness of drugs used to treat resistant infections [17]. The medical effects of some herbs and their synergistic effects together or in combination with common chemical drugs have been evaluated in some studies.

Objectives: The aim of this study was to evaluate the in-vitro interactions of *S. hortensis* and *C. carvi* essential oils together and each essential oil with fluconazole against *C. albicans* ATCC-10231 using the checkerboard test and the broth-microdilution method based on the Clinical and Laboratory Standards Institute (CLSI) M27-A3 and M27-S4 standard documents.

Materials and Methods

Essential oils preparation*: C. carvi* seeds were bought and ground using an electrical grinder. The preparation of *C. carvi* essential oil was done using a Clevenger at 70 °C for 3 hrs. The preparation procedure of *S. hortensis* essential oil was mostly identical to that of *C. carvi.* The only difference was that this oil was extracted from the aerial parts of *S. hortensis*. To prepare this plant for the essential oil extraction process, the pedicles and leaves of *S. hortensis* were dried at room temperature for 14 days with no direct sunlight or wind [18].

Essential oils decomposition: Gas chromatography (7890 A) and mass detector (5975 C) were used to decompose the prepared essential oils and identify their compositions. HP-5ms (phenyl methyl siloxane with 30 mm length, 0.25 mm width, and 0.25 µm thickness) capillary column was used as the column in this procedure, and helium (with 1 mL/min flow rate) was used as the carrier gas. The oven temperature was adjusted as follows. At first, the temperature was maintained at 40 °C for 5 min, then increased to 230 °C at a rate of 10 °C /min, and finally increased from 230 to 280 °C at a rate of 30 °C /min. Injector and detector temperatures were set at 240 °C.

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The compositions of the prepared essential oils were detected by comparing the retention indices (RIs) with each other using Wiley 7n and NIST (NIH software, second edition). At the end, the results were assessed according to the fragmentation pattern of the mass spectra available in authentic references [19].

Measuring minimum inhibitory concentration (MIC) of essential oils and fluconazole: *C. albicans* ATCC-10231 was tested for in-vitro susceptibility to essential oils and fluconazole using the broth microdilution method as described by CLSI (M27-A3 and M27-A4 standard documents) guidelines [20, 21]. According to CLSI, fluconazole (Sigma-Germany) was used in the concentration range of 0.125-64 µg/mL, and antifungal properties of different concentrations of *S. hortensis* (0.0244-1.56 µL/mL) and *C. carvi* (0.39-25 µL/mL) were investigated. The wells in columns 11 and 12 contained positive (no antifungal agent) and negative controls (no yeast), respectively. After 24 hrs of incubation, MIC values were determined as the lowest drug concentration that significantly reduced fungal growth compared to positive control levels. All the experiments were performed twice to increase the reliability of the results.

Determining minimum fungicidal concentration (MFC) of essential oils: After measuring MIC values, 10 µL of the contents of the wells treated with MIC concentrations and higher were removed and transferred to SDA (sabouraud dextrose agar, Pronadisa-Spain) media in petri dishes. Then the petri dishes were put in an incubator at 35±2 °C for 24 hrs. The lowest concentration of each essential oil that prevented the growth of *C. albicans* on SDA was considered as MFC [22].

Evaluating the synergic effect of essential oils and fluconazole: In this study, the checkerboard method was used to evaluate the synergic effect of essential oils and fluconazole. By repeating the experiments, the average results were used as the final data in the FICI formula to find the kind of interactions between essential oils and fluconazole [23].

$$FIC_A=\frac{MIC(A+B)}{MIC(A)} \quad FIC_B=\frac{MIC(A+B)}{MIC(B)} \quad FICI=FIC_A+FIC_B$$

Drawing the time-kill curve: The time-kill assay was used to evaluate the correlation between time and antifungal effect of essential oils and fluconazole. For this purpose, three solutions of essential oils and fluconazole in fungal suspension (5×10^5 cfu/mL in RPMI 1640) (Bio idea, Iran) were prepared with a final volume of 10 mL. The solutions were transferred to 25 mL flasks and incubated at 35±2 °C for 24 hrs. Then ten dilutions of each of the solutions in the flasks were prepared in 2 mL micro-tubes after 0, 2, 4, 8, 12, and 24 hrs (after making them). Then 30 µL of the solutions in the micro-tubes were transferred to SDA media in petri dishes by pour plate method. The petri dishes were incubated at 35±2 °C for 48 hrs, and then colony-counting was performed. By following this process for the dilutions prepared at each time interval and recording the data, a time-kill curve was drawn [24].

Findings

Essential oils composition: The analysis of the chemical composition of *C. carvi* essential oil revealed the presence of 19 compounds, constituting 96.9% of the total oil chemical composition. Limonene, ρ-cymene, cuminaldehyde, β –pinene, and α-phellandrene were the main components identified in *C. carvi* essential oil (Table 1).

The analysis of the chemical composition of *S. hortensis* essential oil also showed 14 components, constituting 96.1% of the total oil composition. Thymol, ɣ-terpinene, and limonene were the main components identified in the essential oil, followed by ρ-cymene and β-myrcene (Table 2).

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Table 1) Chemical composition of *Carum carvi*

Number	Component	Retention Time(min)	Percentage
1	Tnicyclene	12.080	0.591
2	α-pinene	12.490	1.422
3	β-pinene	15.739	12.29
4	β-myrcene	16.658	1.158
5	Octanol	17.195	0.566
6	α-phellandrene	19.156	10.897
7	ρ-cymene	22.182	21.91
8	o-cymene	30.937	3.35
9	Limonene	35.368	26.18
10	ρ-cymene-8-ol	36.408	0.388
11	Cuminaldehyde	38.367	16.72
12	Bornyle acetate	40.194	0.412
13	Cuminyl acetate	42.804	0.192
14	Eugenol	45.141	0.141
15	Methyl eugenol	46.343	0.063
16	Caryophyllene	47.879	0.245
17	Germacerene-D	48.240	0.121
18	ɣ-cadinene	50.786	0.096
19	Apiol	55.568	0.155

Table 2) Chemical composition of *Satureja hortensis*

Number	Component	Retention Time(min)	Percentage
1	α-phellandrene	12.156	1.555
2	α-pinene	12.461	0.866
3	β-pinene	15.100	0.812
4	β-myrcene	16.665	2.352
5	ρ-cymene	18.154	3.231
6	Limonene	19.119	7.23
7	ɣ-terpinene	22.548	26.89
8	Trans-anethol	34.084	0.104
9	Thymol	41.251	50.52
10	Carvacrol	42.740	0.260
11	β-elemene	51.117	1.308
12	Trans-caryophylene	52.926	0.141
13	Spathulenol	77.745	0.491
14	Caryophillen oxide	79.391	0.327

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Susceptibility testing and evaluating the possible synergism between the studied essential oils and fluconazole: Antifungal susceptibility test of *C. albicans* ATCC-10231 to essential oils demonstrated fungistatic and fungicidal activities for both essential oils, although these activities were stronger in *S. hortensis* essential oil than in *C. carvi* essential oil. The results of antifungal susceptibility testing are shown in Table 3.

In order to evaluate the synergic effect of the studied essential oils and fluconazole, *S. hortensis* essential oil was used in the range of 0.0244-1.56 µL/mL, *C. carvi* essential oil in the range of 0.39-25 µL/mL, and fluconazole powder in the range of 0.125-64 mg/mL considering the antifungal susceptibility test results (MIC and MFC values).

The analysis of the checkerboard test results revealed a synergic/additive interaction between *S. hortensis* essential oil and fluconazole, an additive interaction between *C. carvi* essential oil and fluconazole, and no interaction between *S. hortensis* and *C. carvi* essential oils ($p \leq .5$) (Table 3).

Time-kill assay: The time-kill curves drawn for the studied essential oils are shown in Figure 1. The time-kill curves showed that *S. hortensis* essential oil at MIC concentration and *C. carvi* essential oil at 2 MIC concentration exhibited a good and rapid fungicidal effect in less than 4 hrs. However, fluconazole at a concentration of 2 MIC (16 mg/mL) is a standard fungistatic medication with no fungicidal effect.

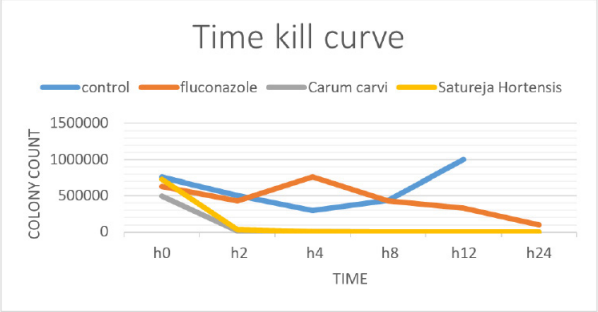

Figure 1) Time-kill plot showing the activity of fluconazole, *Carum carvi*, and *Satureja hortensis* against *C. albicans* ATCC 10231

Discussion

Table 3) Results of susceptibility and checkerboard tests

Combinations	MIC_a*	MIC_c*	FIC	FICI	Interaction
C.C-FLC*					
C.C*	12.5	6.25	0.5	0.62	Additive
FLC	8	1	0.12		
S.H-FLC					
S.H*	1.56	0.012	0.008	0.508	Additive to synergic
FLC	8	4	0.5		
C.C-S.H					
C.C	12.5	0.195	0.015	2.015	Indifferent
S.H	1.56	3.125	2		

*FICI: fractional inhibitory concentration index
*FLC: Flocunazole
*S.H: S.hortensis
*C.C: C.carvi
*MICa: MIC alone
*MICc: MIC combined

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The analysis of the chemical composition of *C. carvi* essential oil showed the presence of limonene (26.18%), ρ-cymene (21.91%), and cumin-aldehyde (16.72%) without carvone as the main components of the oil. A negative correlation was observed between the presence of carvone and limonene in *C. carvi* essential oil because limonene was the main component of caraway oil, while carvone could not be detected in this oil. This is consistent with the findings reported by Seidler et al. (2013) [13].

The analysis of the chemical composition of *S. hortensis* essential oil revealed the presence of thymol (50.52%), ɣ-terpinene (26.89%), and limonene (26.18%) as the main components of the oil, these results are approximately consistent with the findings of another study by Sharifzadeh,and colleagues (2016) [25], while Mihajilov et al. (2010) in their study reported that *S. hortensis* essential oil contained a large amount of carvacrol (67%), ɣ-terpinene (15.3%), and ρ-cymene (6.37%) [26]. Also, Mahboubi et al. (2011) reported thymol (28.2%), ρ-cymene (19.6%), ɣ-terpinene (16%), and carvacrol (11%) as the main components of *S. hortensis* essential oil [27]. Studies have shown that plants belonging to the *Lamiaceae* family have a good antifungal activity, but this activity may vary between different plants of the same family [28]. It indicates that herbs of the same species but from different geographical regions differ in the chemical composition of their essential oils [13]. The susceptibility test in the present study was done using the broth microdilution method, which is a more sensible technique with more reliable results compared to the disc-diffusion method.

Antifungal properties of *C. carvi* and *S. hortensis* essential oils have also been reported in previous studies. *C. carvi* essential oil in this study showed an acceptable fungicidal effect against *C. albicans* ATCC-10231 with MIC and MFC values of 12.5 and 25 µL/mL, respectively. The MIC value of *C. carvi* essential oil against *C. albicans* PFCC-50271 was reported as 10 µL/mL (1%) by Nasiri and colleagues (2014) [29]. This difference in MIC values could be due to the use of different *Candida* strains in these two studies.

S. hortensis essential oil in the present study showed a significant fungicidal effect against *C. albicans* ATCC-10231 with MIC and MFC values of 1.56 and 3.125 µL/mL, respectively. The MIC value of *S. hortensis* essential oil was reported as 0.048 µL/mL by Valizadeh et al. (2014) [18] and 0.125 µL/mL by Mahboubi et al. (2011) [27]. These differences in MIC values are mostly due to the diversity of the chemical composition of essential oils. As noted earlier, the inconsistency between the results of different studies could be attributed to several reasons, including different research methodologies, different types of microorganisms, and different chemical compositions depending on the origin of herbs and their geographical regions [28]. Therefore, finding the best extraction method with the highest antifungal effect requires more comparative studies. Also, considering that the essential oils of herbs from different geographical areas do not have the same components and antifungal effects; therefore, in order to commercialize herbal medicines, the active ingredients of their essential oils should be determined, and their efficiency criteria should be considered. Although no article has been published on the synergistic antifungal effects of *C. carvi* and *S. hortensis* essential oils, higher antifungal properties were observed in *S. hortensis* essential oil compared to *C. carvi* essential oil regardless of their genotypes. These results were validated by comparing the results reported in different articles [3, 18, 27, 30].

Excessive use of antibiotics has increased antibiotic resistance of pathogenic microorganisms to existing drugs [8]. Overcoming these resistant microorganisms needs the

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use of higher dosages of antibiotics, which may cause more side effects. As the main objective of this study, the interaction between synthetic and natural antifungals is an alternative way to reduce the required dosages and adverse side effects of antibiotics. To the best of our knowledge, no article has evaluated the interactions between *S. hortensis* and *C. carvi* essential oils and fluconazole. Therefore, this research is a pilot study in this field.

The present study results showed an interaction near to synergy between fluconazole and *S. hortensis* essential oil containing thymol as its main component, this result is confirmed by the findings of another study by Castro et al. (2015), indicating a synergic interaction between thymol and nystatin [31]. This study revealed an additive interaction between *C. carvi* essential oil and fluconazole. However, the interaction between cumin essential oil and fluconazole in Patil et al.'s (2015) study was reported to be synergy ($FICI_1$=0.31, $FICI_2$=0.19) [32]. Therefore, the wide differences between the chemical composition of *C. carvi* and cumin essential oils should be considered despite the similarity between these two plants.

In the time-kill assay, the fungicidal effect of *S. hortensis* essential oil occurred in less than 4 hrs, which could be due to the synergism between thymol and other components of *S. hortensis* essential oil. However, Ahmad et al. (2011) showed that thymol was almost ineffective at ½ MIC concentration, and its fungicidal effects at 1 and 2 MIC concentrations did not occur before 48 hrs [33].

It is necessary to conduct further studies on the antifungal effects of the essential oils of *S. hortensis* and *C. carvi* of different genotypes from different geographical regions against wild type and clinical species of *C. albicans*, either alone or in combination with fluconazole. It is also necessary to investigate their toxicity on human cells.

Conclusion

The findings of this pilot study show that *S. hortensis* and *C. carvi* essential oils studied in this research (grown in Zanjan/Iran) have powerful antifungal properties separately against *C. albicans* ATCC-10231, and the studied *S. hortensis* plant essential oil in combination with fluconazole powder is a potential candidate to reduce the required doses and increase the antifungal activity of fluconazole.

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Ethical permissions: All the methods used in vivo study were performed in accordance with relevant guidelines and regulations of the Ethics Committee of Zanjan University of Medical Sciences under IR.ZUMS.REC.1397.144 ethical code.

Author's contributions: Study concept and design: Dr. Neda Gholami, collection of data: Dr. Yasaman karbalaei, analysis and interpretation of data: Dr. Saeid Amanloo and Dr. Alireza Yazdineghad, drafting of the manuscript: Dr. Yasaman Karbalaei, critical revision of the manuscript for important intellectual content: Dr. Saeid Amanloo and Dr. Alireza Yazdineghad.

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