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Vol. 7(30), pp. 2271-2274, 10 August, 2013
DOI: 10.5897/JMPR12.659
ISSN 1996-0875 ©2013 Academic Journals
http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Antifungal activity of *Satureja hortensis* alcoholic extract against *Aspergillus* and *Candida* species

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Accepted 27 August, 2012

Pharmacological and biological investigations have justified the traditional application of *Satureja hortensis* as a natural source of compounds for food conservation, as well as in the treatment of ailments including inflammatory diseases, cramps, muscle pains, nausea, indigestion, diarrhea, and infectious diseases. Regarding to the low number of reports of *Satureja hortensis* antifungal activities, we focused on the inhibitory and fungicidal effects of the Satureja alcoholic extract on *Candida* and *Aspergillus* species. The subjects included patients with clinical isolates of *Candida* and *Aspergillus* obtained from UMSU University Hospital, Urmia, Iran. A total of 51 clinical isolates, including 31(60.8%) *Candida albicans* and 20(39.2%) *Aspergillus* species, respectively, were obtained. McFarland 0.5 solutions of the fungal isolates were treated with alcoholic (ethanol) extract of *S. hortensis* using 1-tube serial dilutions (MIC) and 2- agar serial dilutions (MFC). The Inhibitory range for *Candida* isolates was shown to be 1/20 to 1/320. Findings of MIC and MFC on *Aspergillus* isolates showed that the lowest effective concentration was detected at the dilution point of 1/80 (MIC=12.5 mg/ml). Also, we got the result of 24 hours as the best incubation time of treatment for all the *Aspergillus* isolates tested. Our findings showed that the alcoholic extract of *S. hortensis* is able to inhibit growth of some *Candida* and *Aspergillus* clinical isolates *in vitro* and also killed them in higher concentrations.

Key words: Antifungal, *Satureja hortensis*, *Aspergillus*, *Candida*.

INTRODUCTION

Pharmacological and biological investigations have justified the traditional application of *Satureja hortensis* as a natural source of compounds for food conservation, as well as in the treatment of ailments including inflammatory diseases, cramps, muscle pains, nausea, indigestion, diarrhea, and infectious diseases due to its antispasmodic, anti-diarrheal, antioxidant, antibacterial and antifungal properties as reported in literature (Adiguzel et al., 2007; Dikbas et al., 2008; Güllüce et al., 2003; Hajhashemi et al., 2000; Heydari et al., 2011; Mihajilov-Krstev et al., 2010; Razzaghi-Abyaneh et al., 2008; Şahin et al., 2003). The antimicrobial activity of *S. hortensis* extract has also been reported in previous studies.

Some studies have evaluated the antimicrobial and antioxidant activities of the essential oil obtained, using a Clevenger distillation apparatus, water soluble (polar) and water insoluble (non polar) sub fractions of the methanol extracts from aerial parts of *S. hortensis* plants, and methanol extract from calli established from the seeds

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using Gamborg's B5 basal media supplemented with indole-3-butyric acid (1.0 ppm), 6-benzylaminopurine (N(6)-benzyladenine) (1.0 ppm), and sucrose (2.5%) (Dikbas et al., 2008). The antimicrobial test results showed that the essential oil of *S. hortensis* had great potential antimicrobial activities against all 23 bacteria and 15 fungi and yeast species tested (Güllüce et al., 2003). The essential oil and methanol extract of *S. hortensis* were also evaluated for antifungal activity against *Aspergillus flavus in vitro* on Petri plates and liquid culture, and under storage conditions. The oil showed strong antifungal activity based on the inhibition zone and minimal inhibitory concentration values against the pathogen on Petri plates assays (Dikbas et al., 2008). Other studies reveal a new biological activity for *S. hortensis* L. as strong inhibition of aflatoxin production by *Aspergillus parasiticus*. Carvacrol and thymol, and the effective constituents of *S. hortensis* L., may be useful in controlling aflatoxin contamination of susceptible crops in the field (Razzaghi-Abyaneh et al., 2008).

It is well known that the chemical composition and yield of essential oils are affected by exogenous factors, such as geographical position, altitude, and climate and soil composition (Mihajilov-Krstev et al., 2010). For this reason, in the present study, the growth inhibitory and fungicidal effect of the alcoholic extract of *S. hortensis* on some hospital isolates of *Aspergillus niger*, *A. flavus*, *Aspergillus fumigatus* and *Candida albicans* obtained from clinical sources in Iran were studied as a model system for fungi.

MATERIALS AND METHODS

Collection and identification of plants

About 2000 g of the *S. hortensis* seeds (called Marzea in Persian word) were obtained from a private traditional plant farm in the North-west of Iran, and confirmed by the Plants Systematic Laboratory, Pharmacology Dep. UMSU, Iran.

Preparation of alcoholic extract

Air-drying of *S. hortensis* seeds was performed in a dark place at room temperature for 48 h. The dried seeds (1000 g) were ground and subjected to alcoholic (ethanol) extraction using a dehydrator apparatus. In brief, the Marzea powder was added to 3200 ml of ethanol 96%, shaken for 2 h and incubated over night at room temperature to remove alcoholic solvent from the crude extract. Again, the same amount of solvent was added to the crude extract and the process was repeated for five times. The alcoholic solvent was separated completely by a vacuum filtering system and the final product was collected in a 250 ml container and stored at 4°C until use.

Microorganisms

The main subjects were *Candida* and *Aspergillus* species (pathogenic opportunistic fungi) isolated from patients with symptomatic infections. The species used in this study included; *C. albicans*, *A. fumigatus*, *A. flavus* and *A. niger*, and some other *Candida* species with low frequency of isolations. All organisms were identified in the Department of Medical Mycology, Urmia University of Medical sciences, Urmia, Iran. *Candida* isolates were identified in the level of species using differential media CHROMagar *Candida* and the restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) method included *C. albicans*, *Candida glabrata*, *Candida kefyre* and *Candida parapsilosis*. The identification of *Aspergillus species* was performed using morphological characteristics, and also confirmed by the molecular method as earlier mentioned so that three species of *A. fumigatus*, *A. flavus* and *A. niger* were identified and used in the study.

We also used standard control strains, including *Candida tropicalis* (PTCC 5028), *C. albicans* (ATCC 10231) and *C. glabrata* (UMSU C152), for the evaluation of the findings of clinical fungal isolates. The fungal suspensions were prepared from 48 - 72 h Sabouraud broth cultures at room temperature and then adjusted to the McFarland 0.5 standard dilution (corresponding to 10^3-10^4 spore/ml for fungal strains consensus standard by the NCCLS M38 [ISBN 1-56238-480-8).

S. hortensis alcoholic extract serial dilutions

Serial dilutions in tubes

A small volume (1 ml) of Sabouraud broth in capped tubes was used as substrate. Briefly, 1 ml of 10% solution of the crude extract in sterile water was added into the substrate and mixed thoroughly. Then, 1 ml of the solution was transferred into the next tube containing 1 ml of substrate, mixed and transferred into the next tube in same way up to 6th tube, so that six decreasing titers of *S. hortensis* alcoholic extract including: 1/20, 1/40, 1/80, 1/160, 1/320, 1/640 were made.

Serial dilutions in agar media

The Sabouraud glucose agar media (SGA) was boiled, autoclaved and aliquated 10 ml in plates (8 cm in diameter). Then 1, 0.5, 0.25, 0.125 and 0.0625 ml of a prepared 1/10 dilution were added to the plates 1 to 5, respectively.

Treatment of fungal suspension with diluted extracts

In an aseptic condition, 1 ml of *Candida* suspension was added to serial diluted tubes. Negative and positive controls were prepared by adding 1 ml to ½ diluted extract and *Candida* suspension, respectively. Agar dilution method was used only for *Aspergillus* isolates, using Sabouraud glucose agar media that was mixed in advance with serial diluted *Satureja* alcoholic extract. The spore suspensions (0.02% agar and 0.05% Tween 20) inoculated on the mixed media except the control negative plate and control positive included agar media free of extract. All treatments were incubated at room temperature (RT), respectively.

Minimum inhibitory concentration (MIC)

After 24 - 48 h of incubation at RT, the tubes of reactions were observed for *Candida* growth turbidity. This was performed by comparing the growth turbidity of test tubes with that of McFarland standard 0.5. The tubes without any turbidity were considered as positive and the other as negative. MIC was determined at the point of negative turbidity. Also, the first SGA plate in the serial agar dilution, without growth of Aspergilli showed MIC of *Satureja* on

Table 1. The results on the effects of *Satureja hortensis* alcoholic assay on the tested *Candida* and *Aspergillus* isolates in broth micro dilution assay.

Fungus MIC (mg/ml)	*C. albicans*		*Aspergillus spp.*		*A. fumigatus*		*A. flavus*		*A. niger*	
	No.	%	No	%	No.	%	No.	%	No.	%
5.0	3	9.7	4	20	1	20	0	0	0	0
2.5	5	16.1	8	40	2	40	1	33.3	1	8.3
1.25	9	29	7	35	0	0	2	66.7	6	50
0.625	12	38.7	0	0	2	40	0	0	5	41.7
0.312	2	6.5	0	0	0	0	0	0	0	0
Total	31	100	20	100	5	100	3	100	12	100

Aspergillus isolates.

Minimum fungicidal concentration (MFC)

All tubes without turbidity (negative tubes) were tested for fungicidal effect of *Satureja* alcoholic extract. MFC resulted from negative growth of the isolate on Sabouraud glucose agar media after 24 - 72 h of incubation at RT. After the incubation time, all SGA plates were tested for the growth of *Candida* colonies. The plates with fungal growth were excluded, while the first plate without fungal growth was the cutoff point, and this showed the fungicidal activity of *S. hortensis* alcoholic extract.

RESULTS

In total, 100 fungal isolates including *C. albicans* (mostly), *C. parapsilosis*, *C. kefyre*, *C. glabrata* (rarely) and 3 *Aspergillus* species were tested for MIC and MFC. The results of broth micro dilution assay (Table 1) showed that the alcoholic extract was active against all the yeasts tested in the following range of concentrations: MIC = 0.312 to 1.25 mg/ml; MIC: 0.312 mg/ml for *C. glabrata* as the highest and MIC: 1.25 mg/ml for *C. albicans* as the lowest inhibitory effect of *S. hortensis* the alcoholic extract. Minimum fungicidal concentration of the alcoholic extract on *Candida* isolates showed a range of 0.312 to 2.5 mg/ml. From our results, the highest fungicidal activity of the extract included MFC: 0.625 mg/ml for *C. glabrata*, and 2.5 mg/ml was the lowest MFC for *C. albicans*.

Furthermore, the *S. hortensis* alcoholic extract was active against the tested *Aspergillus* species in the range of MIC/MBC = 0.05–0.39/0.05-0.78 µL/ml, which exhibited higher activity for *A. niger* and *A. fumigatus* compared to *A. flavus*. In fact, *A. flavus* cultures treated by *S. hortensis* extract resulted only in morphologic alterations of macroscopic features, including sporulation rate, color and size of colonies. There was no microscopic variation considerable for *Aspergillus* species.

It is clear that 24 h treatment at fungal suspension with *S. hortensis* is much more effective than 2 and 6 h treatment.

DISCUSSION

S. hortensis is considered from ancient time to date as a plant with drug properties. Some compounds of this plant have anti-parasite and anti-microbial effects. The essential oil of *S. montana* have a broad-spectrum of antimicrobial activity against 46 species of yeasts. This high and diffused activity could be used to control potential pathogenic and spoilage yeasts (Ciani et al., 2000).

In the present study, the anti-fungal activity of *S hortensis* on some species of *Candida* and *Aspergillus* was tested *in vitro*, respectively. The inhibitory effects of alcoholic extract of Peganum *harmala* have been measured and indicated by MIC and MFC parameters (Diba et al., 2011). The activity of this extract against *Candida spp.* was in the range of: MIC=0.312 to 1.25 mg/ml.

The least MIC has indicated in *C. albicans* and the most MIC in *C. glabrata*. The least measured MIC in *C. albicans* can be explained by the resistance of most *C. albicans* strains to antifungal drug. Its MIC/MBC values are very low (lower than the referent antibiotics) and in most cases they are at the same concentration. The results of bacterial studies indicate that *S. hortensis* alcoholic extract, showed various activities in relation studies.

The activity of this extract against *Candida spp.* was in the range of MIC/MBC=0.39/0.78 µL/ml, while the oil collected from Yusufli in Turkey did not show any effect against this strain. Also, it exhibited higher activity against two strains of *S. aureus* (MIC=0.20 and 0.39 µL/ml, respectively) in relation to the oil from Turkey (MIC=15.62-62.50 µL/ml, respectively) (Adiguzel et al., 2007). The result of *Pseudomonas aeruginosa* susceptibility is also in accordance with the effect of the Turkish *S. hortensis* essential oil against this strain (MIC=31.25, 62.50 and 125 µL/ml, respectively) (Adiguzel et al., 2007).

In previous studies, the alcoholic extract of *S. hortensis* showed antifungal activity against phytopathogenic fungi (Boyraz and Ozcan, 2006) and against food spoilage

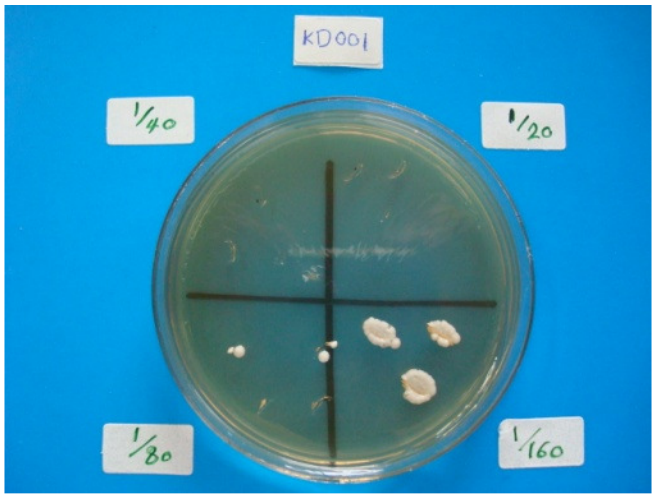

Figure 1. Fungicidal effect of *S. hortensis* on *Candida albicans* in the titers of 1/20 to 1/160.

fungi (Adiguzel et al., 2007). The dominant component of this oil, carvacrol, is capable of inhibiting aflatoxin production by *A. parasiticus* (Razzaghi-Abyaneh et al., 2008) and *A. flavus* in a liquid medium and tomato paste (Dikbas et al., 2008; Omidbaigi et al., 2005). The same authors suggested that carvacrol could be useful in controlling aflatoxin contamination of susceptible crops. In the present study, the extract exhibited a partial antifungal activity against *A. niger* (MIC/MBC= 0.78 µL/ml), *Saccharomyces cerevisiae* (MIC/MBC = 0.39/0.20 µL/ml) and *C. albicans* (MIC/MBC = 0.20 µL/ml). Against *C. albicans*, however, the oil from Turkey did not show any effect (Adiguzel et al., 2007; Mihajilov-Krstev et al., 2010). Moreover, incubation time at 2 h did not show fungicidal effects on *Candida* and *Aspergillus* isolates, although there was a fungicidal result for 2 of 14 *Candida* tested isolates (Figure 1). Nevertheless, we got results on 24 h incubation for all the tested *Aspergillus* isolates.

The essential oil of *S. hortensis* L. collected in Serbia yielded 2.05%, which was higher than previously reported (4). The oil exhibited very high antibacterial and anti-fungal activity, owing to high content of monoterpene carvacrol, which is a well known antimicrobial compound. Active concentrations were much lower than those of the referent antibiotics. These values, together with high yield and lack of toxicity economically justify the use of essential oil derived from *S. hortensis* L. for many purposes: food conservation, treatment of different human diseases, and also for the treatment of phytopathogens that infect economically important plants (Adiguzel et al., 2007; Stammati et al., 1999).

To our knowledge, the *Satureja* alcoholic extract is active against opportunistic and food borne fungi (Candida spp). The present study clearly demonstrates that the alcoholic extract of *S. hortensis* contains com-pounds possessing antifungal properties. Therefore, on the basis of our results in present and previous studies, *S. hortensis* can be added as a protective agent to various food products (Razzaghi-Abyaneh et al., 2008).

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Peyman Mikaili from the Department of Pharmacology, UMSU for his help in the plant identification. This study was financially supported by the Research Deputy of Urmia University of Medical Sciences.

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