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Control of *Aspergillus flavus* with essential oil and methanol extract of *Satureja hortensis*

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ABSTRACT

The essential oil and methanol extract of *Satureja hortensis* were tested 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. The very low concentrations of them also reduced wet and dry mycelium weight of pathogen fungus in liquid culture. When the oils at 25, 12.5 and 6.25 µl/mL concentrations were applied to lemon fruits before seven days of pathogen inoculation on storage conditions, the decay on fruits caused by the pathogen could be prevent completely. The results in this study showed that the essential oil of *S. hortensis* had strong antifungal activity against pathogen fungi tested. So, the essential oil of *S. hortensis* could be used for management of this pathogen as a potential source of sustainable eco-friendly botanical fungicides.

1. Introduction

Aspergillus flavus is responsible for spoilage of many foods and feeds, and causes decay on stored fruits damaged by insects, animals, early splits, and mechanical harvesting. Furthermore, *A. flavus* is able to produce aflatoxins in foods and feedstuffs (Rojas et al., 2005). Post harvest pathogens are controlled by a combination of storage technologies, physical methods and synthetic chemical fungicides (Eckert and Ogawa, 1988). But, alternative control methods are needed because of negative public perceptions about the use of pesticides, development of resistance to fungicides, and high cost for development of new chemicals preservatives.

During the last years, numerous studies have documented the antifungal effects of plant essential oils against post harvest fungal diseases (Shahi et al., 2003; Guynot et al., 2005; Mercier and Smilanick, 2005; Neri et al., 2006; Irkin and Korukluoglu, 2007; Kumar et al., 2007; Omidbeygi et al., 2007). According to our knowledge, antifungal property of the extracts or essential oils obtained from some plants against *A. flavus* has been studied before (Bankole, 1997; Montes-Belmont and Carvajal, 1998; Mahmoud, 1999; Yin and Tsao, 1999; Paranagama et al., 2003; Kumar et al., 2007). These authors reported that some plant oils and/or extracts could effectively inhibit the growth of this pathogen fungus.

Satureja hortensis L. (summer savory) is well known aromatic and medicinal plant, which is widely distributed in the Eastern Anatolia region of Turkey, and locally named as 'Koc Otu' (Sahin et al., 2003). There are also many studies related to the essential oil and extract of *S. hortensis* which has antifungal activity against *A. flavus* under in vitro conditions (Güllüce et al., 2003; Sahin et al., 2003; Boyraz and Ozcan, 2006). But, there is only one study reported that *S. hortensis* was tested against *A. flavus* under storage conditions (Omidbeygi et al., 2007).

The first objective this study was to examine the antifungal activity of the essential oil and methanol extract obtained from *S. hortensis* against *A. flavus* on Petri plates and in liquid medium. Second, the minimal inhibition concentration of them against the growth of *A. flavus* was determined. Third, to suppress or protect the growing of *A. flavus* and under storage conditions on lemon fruits was tested.

2. Materials and methods

2.1. Plant material

The aerial parts of *S. hortensis* L. plants were collected from Gaziler valley of Senkaya in the Eastern Anatolia region of Turkey in July 2006 at full flowering stage. The taxonomic identification of plant materials was confirmed by a senior plant taxonomist, Saban Kordali, in the Department of Plant Protection, Atatürk University, Erzurum (Turkey). They have been deposited in the Biotechnology Research and Application Centre at Atatürk University, Erzurum (Turkey).

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2.2. Preparation of the methanol extract

Collected plant material was dried in the shade and ground in a grinder with 2 mm in diameter mesh. The dried and powdered plant materials (500 g) were extracted successively with 1 L of methanol (MeOH) by using Soxhlet extractor for 72 h at a temperature (65 °C) not exceeding the boiling point of the solvent (Sahin et al., 2003). The extracts were filtered using Whatman filter paper (no. 1) and concentrated in vacuo at 40 °C using a Rotary evaporator. The residues obtained were stored in a freezer at −80 °C until further tests. The extract yield was 5.96% (w/w).

2.3. Isolation of the essential oil

The air-dried and ground aerial parts of plants collected were submitted to water distillation for 3 h using a Clevenger-type apparatus. The obtained essential oil was dried over anhydrous sodium sulfate and, after it was filtered, stored in a sealed vial at 4 °C until tested. The essential oil yield was 2.3% (v(w).

2.4. Pathogen fungus

The pathogen fungus *A. flavus* was isolated from lemon fruit used in this study. The Identity of the fungus was identified by the Microbial Identification System (Sim index: 0.635) (MIDI, Inc., Newark, DE), and confirmed by the standard morphological methods (Klich, 2002). Fungus culture was maintained on potato-dextrose agar (PDA) at 4 °C for using further studies. Pathogenisity test of the fungus was performed by injecting suspensions of fungal conidia (1×10^6 spores/mL) into the rind of lemon fruits cultivars Meyer.

2.5. Source of fruit materials

Lemon fruits (cultivars Meyer) used in the experiments were obtained from market place located in Erzurum (Turkey). Fruits were selected free of wounds and rots and as much as possible homogeneous in maturity and size, and were stored at 5 °C for 3–5 days until use. Fruit were surface-disinfected by immersion for 1 min in a dilute solution of ethanol (70%), washed twice by immersion in distilled and sterile water, and left in a dry place to remove excess water on the surface until using for in vivo assays.

2.6. Screening for antifungal activity

Antifungal activity tests were carried out by disc diffusion method (Murray et al., 1995). The tested fungus was grown on Potato Dextrose Agar (PDA) medium for sporulation on Petri dishes for 5–7 days. The concentration of spores was determined using a hemocytometer, adjusted to 1×10^6 spores/ml by appropriate dilution and used fresh as a stock suspension. Using 100 μL of suspension containing 10^6 spore/mL fungi spread using glass L-rod spreader on PDA. The dried plant extracts were dissolved in the some solvent (methanol) to a final concentration of 30 mg/mL, and prepared stock solutions. The extracts and oils were sterilized by filtration through 0.45 μm Syringe Driven Filter Unit (MILLEX®-HA). The sterile filter papers discs (6 mm in diameter) were impregnated with 10 μL of the essential oil, or the 30 mg/mL extracts (300 μg/disk) were put in the middle of inoculated agar plates. Methanol and DMSO 10% were added on the discs to provide negative control. The inoculated plates were incubated at 37±2 °C and 72 h. At the end of the period, antifungal activity was evaluated by measuring the zone of inhibition (mm) against the test fungus. All treatments consisted of three replicates, and experiments were repeated three times, and determined the averages of the repeated experimental results.

2.7. Determination of the minimum inhibitory concentration (MIC)

The minimal inhibition concentration (MIC) value of the oil was determined by using the modified agar-well diffusion method (Okeke et al., 2001). The essential oils dissolved and prepared stock solutions in 10% dimethyl sulfoxide (DMSO), were first diluted to the highest concentration (500 μL/mL) to be tested, and than serial 2-fold dilutions were made in order to obtain a concentration range from 6.25 to 500 μL/mL in 2 mL sterile Eppendorf test tubes. A 100 μL amount from suspension contained 10^6 spore/mL of fungus spread on PDA plates. The discs were impregnated with 10 μL of the serial essential oils dilutions, and than were put in the middle of inoculated agar plates. The inoculated plates were incubated at 37±2 °C and 72 h. At this period, inhibition zones were determined. The least concentration of each the essential oils showing a clear zone of inhibition were taken as the MIC. All treatments consisted of three replicates, and experiments were repeated three times.

2.8. Determination of wet and dry mycelium weight

A 1000, 500 and 250 μL amount from the stock solutions of the essential oils (500 μL/mL) and extracts (30 mg/mL) was added in separately to each Erlenmeyer flask with 20 mL nutrient broth (NB). Three different final concentrations of the essential oil (25, 12.5 and 6.25 μL/mL) and methanol extract (30, 15 and 7.5 mg/mL) in liquid medium were prepared. A 100 μL amount from the fungal suspension containing 10^6 spore/mL of fungus suspension was inoculated in these flasks. The control contained NB medium plus 100 μL of fungal suspension. It was incubated at 37±2 °C until a strong mycelial growth was obtained at 10 days. The fungal myceliums were harvested by filtrating to separate from liquid culture and pre-weighed filter paper (Whatman no. 1), followed by washing twice with distilled sterile water. The wet weight of myceliums was determined. And then they were dried at 60 °C. The dry weight of mycelium was determined. All treatments consisted of three replicates, and experiments were repeated three times and determined the averages of the repeated experimental results.

2.9. In vivo assays on storage condition

The selected lemon fruits cvs Meyer for the experiments were washed in running, dipped in ethanol (70%) for 2 min, rinsed twice with double distilled sterile water (10 min each) and air-dried. Surface-sterilized lemon fruits were wounded with a flame-sterilized nail to a uniform depth of 3 mm. The fungal inoculums containing 10^6 spore/mL was prepared by scraping spore material from the surfaces of the colonies with a wet cotton swab and resuspending the material in distilled water containing 0.5% Tween

Table 1
Means wet and dry mycelium weight (g) of *Aspergillus flavus* in liquid medium added the essential oil and methanol extract from *Satureja hortensis* at three different concentrations (25, 12.5, 6.25 μl/ml)

Treatments	25 (μl/ml)	12.5 (μl/ml)	6.25 (μl/ml)
*Wet mycelium weight (g)**			
Essential oil	8.00±0.27^c	9.63±0.08^b	10.50±0.23^c
Methanol extract	11.06±0.12^b	12.53±0.08^a	14.56±0.29^b
Control (only pathogen)	12.60±0.23^a	12.60±0.23^a	12.60±0.23^a
*Dry mycelium weight (g)**			
Essential oil	0.30±0.00^b	1.73±0.08^b	1.76±0.03^c
Methanol extract	3.26±0.12^a	3.90±0.10^a	5.10±0.17^b
Control (only pathogen)	3.86±0.16^a	3.86±0.16^a	3.86±0.16^a

*Values are means±standard deviation; data in columns with different letters are statistically different according to Duncan's multiple range tests at $p<0.01$ and all treatments consisted of three replicates, and experiments were repeated three times.

Table 2
Means of decay diameters (mm) which measured after 6, 8, 12 and 20 days on lemon fruits applied with 25, 12.5 and 6.25 µl/ml concentrations of essential oil from *Satureja hortensis* and inoculated *Aspergillus flavus* under standard storage condition

Treatments	Means of decay diameters (mm)*			
	6th days	8th days	12th days	20th days
Controls				
Negative (not pathogen was inoculated)	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
Positive (only pathogen was inoculated)	6.4±1.8^{b-d}	8.6±2.4^{bc}	11.3±3.2^{b}	20.3±3.3^{bc}
Oil was applied before 7 days of pathogen				
25 µl/ml	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
12.5 µl/ml	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.6±0.6^{a}
6.25 µl/ml	0.0±0.0^{a}	0.6±0.6^{a}	2.3±1.6^{a}	3.5±2.4^{a}
Oil was applied before 3 days of pathogen				
25 µl/ml	1.3±0.9^{ab}	3.3±1.9^{ab}	18.3±4.3^{bc}	18.3±4.17^{bc}
12.5 µl/ml	7.5±3.5^{cd}	9.7±3.5^{bc}	24.8±4.3^{cd}	26.2±4.2^{c}
6.25 µl/ml	8.7±3.5^{cd}	11.5±3.8^{cd}	24.8±4.3^{cd}	26.4±4.1^{c}
Oil was applied simultaneously with pathogen				
25 µl/ml	5.3±1.7^{a-c}	9.1±2.4^{bc}	12.8±2.9^{b}	15.0±3.3^{b}
12.5 µl/ml	5.7±1.5^{a-c}	9.7±1.7^{bc}	13.4±2.3^{b}	20.1±2.8^{bc}
6.25 µl/ml	11.7±2.0^{d}	16.3±2.5^{d}	29.4±2.5^{d}	35.3±2.7^{d}

*Values are means±standard deviation; data in columns with different letters are statistically different according to Duncan's multiple range tests at $p<0.05$ and all treatments consisted of three replicates, and experiments were repeated three times.

80. Three different concentrations (25.0, 12.5 and 6.25 µL/mL) of the essential oils were prepared for testing antifungal activity against *A. flavus*. The essential oil of *S. hortensis* at three different concentrations and fungal inoculum were sprayed on wounded lemons fruits. A total of eleven lemon fruits were tested for per concentrations groups. These experiments were arranged as three different applications.

The oils and the pathogen inoculums were applied simultaneously, before 3 and 7 days of pathogen inoculation, and than fruits were stored at 10 °C in 70% relative humidity (rh) under a photoperiod of 12-h light and 12-h dark under storage condition. Fruits inoculated with only pathogen were used as positive control for each experiment. Not inoculated fruits with pathogen were used as negative control. The fruits were sealed in polyethylene-lined plastic boxes to retain 70% humidity and incubated at 10 °C storage condition. The diameters of decay on fruits were measured at 6, 8, 12 and 20th days after inoculation. It was accepted that the diameters of full decayed fruits were as 45 mm (Tamamen çürüyen meyvelerdeki çürüme çapı 45 mm olarak Kabul edildi). All treatments consisted of three replicates, and experiments were repeated three times and determined the averages of the repeated experimental results.

2.10. Statistical analyses

An analysis of variance (ANOVA) and Duncan's multiple range test (at $P<0.05$) were performed to analyze statistical differences and to discriminate between means (StatSoft Inc., 1998).

3. Results and discussion

The results of in vitro assays showed that the essential oil of *S. hortensis* had a strong fungicidal effect against *A. flavus* on Petri plate. The mean inhibition zone and the minimal inhibition concentration value of the oil were recorded as 61 mm and at 6.25 µl/mL, respectively. The methanol extract, DMSO 10% and methanol didn't show any antifungal activity.

The effects on wet and dry mycelium weight of pathogen fungus in liquid culture of the essential oil were showed in Table 1. All concentrations of the essential oil significantly reduced the wet and dry weight of mycelium of the pathogen. But, the extract did not reduce. Furthermore, 6.25 µl/mL concentration of the extract increased wet and dry weight of mycelium. The reason of this increase may be related to high incubation temperature and/or the chemical composition of *S. hortensis* methanol extract. That is, they may contain high proportion of some components such as sugar used as a nourishment for microorganisms.

The antifungal activity results of essential oils on lemon fruits under storage conditions were given in Table 2. According to the results of lesion diameters on the fruits, all concentrations (25, 12.5 and 6.25 µl/mL) of the essential oil applied before 8 days of pathogen inoculation showed strong antifungal activity even at the and of 20th days. Furthermore, there was no significant difference in lesion diameters among those treatments in comparison to the negative control even at the and of 20th days. The results obtained from 6.25 µL/mL concentration of the essential oil when oil applied seven days ago from pathogen inoculation was showed on Fig. 1. But, other applications showed a weak effect on disease.

It is widely accepted that higher concentrations of plant essential oils are required in foods than in laboratory media (Farbood et al., 1976). This may be a result of changes to the site of action of this oil at the lower temperature, or alterations in the fungal membrane, so reducing the penetration of the oil to the interior of the cell. But in this study, even though a 6.25 µL/mL concentration of the oil is applied before 7 days of pathogen inoculation, growing of *A. flavus* on foods can protect.

Although the extracts or essential oils of *S. hortensis* have been screened for their antifungal activity under in vitro conditions (Montes-Belmont and Carvajal, 1998; Güllüce et al., 2003; Sahin et al., 2003; Boyraz and Ozcan, 2006), there are no reports on the control of *A. flavus* under in vivo conditions by using the essential oil of *S. hortensis* except that a study was done by Omidbeygi et al. (2007). In the present study, the essential oil from *S. hortensis*

Fig. 1. Decayed lemon fruit (left) that was inoculated with only pathogen *Aspergillus flavus* (positive control), and not decayed lemon fruit (right) that oil was applied (6.25 µl/ml concentration) before 20 days of pathogen inoculation, and kept under storage condition.

showed a strong antifungal activity against *A. flavus* under in vitro and storage conductions. It is known that phenolic compounds such as γ-Terpinene, carvacrol and thymol a major fungicidal effect (Nychas 1996; Sefidkon et al., 2006). Güllüce et al., (2003) stressed that thymol (29.0%) and carvacrol (26.5%) were the main components of the *S. hortensis*. These compounds can be responsible for the antifungal activity of *S. hortensis* oil.

In conclusion, this study showed that *S. hortensis* oil has a strong antifungal activity against *A. flavus*. So this essential oil can be used as a potential source of sustainable eco-friendly botanical fungicides to protect some stored food products from pathogen and saprophytic fungi.

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