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Antifungal Effects of Thyme, Agastache and Satureja Essential Oils on *Aspergillus fumigatus, Aspergillus flavus* and *Fusarium solani*

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Abstract

Growth inhibition of *Aspergillus fumigatus,Aspergillus flavus* and *Fusarum solani* exposed to the essential oils including Thyme, Agastache and Satureja were studied. Disc Diffusion Method was used to evaluate the fungal growth inhibitory effects of the essential oils. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the oils were determined and compared with each other. The results showed that all three essential oils examined, had antifungal effects against three fungi species. The MIC data revealed that Thyme oil was the most effective essential oil with the MIC of 62.5 µl ml^{-1}.

Keywords: Essential oils; Antifungal; *A. fumigatus; A. flavus; F. solani*

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Introduction

Essential oils are naturally occurring as terpenic mixtures. Their insecticidal effect against specific pests and fungicidal effect against some important plant pathogens have been recently reviewed.[1]

Chemical control of fungal pathogens remains as one of the main measures of reducing the incidence of postharvest diseases in various fruits and vegetables. However, due to the development of new strains of pathogens, many of these synthetic chemicals are gradually becoming ineffective.[2] In addition, due to their possible carcinogenicity, teratogenicity, high and acute toxicity, long degradation periods, environmental pollution and side effects on human beings the use of synthetic chemicals for controlling postharvest deterioration of food commodities is restricted.[3] The increasing recognition and importance of fungal infections and the difficulties encountered in their treatment have stimulated the search for alternatives for synthetic chemical fungicides.

Spoilage and poisoning of foods by fungi is a major problem, especially in developing countries. *Penicillium*, *Aspergillus* and *Fusarium* are the most important fungi causing spoilage of foodstuffs in Africa.[4] Fungi are also responsible for off-flavour formation and production of allergenic compounds and mycotoxins which lead to qualitative losses.[5] Aflatoxin-B_1 and B_2 and fumitoxins produced by *Aspergillus flavus* and *Aspergillus fumigatus* are some examples of mycotoxins produced by such fungi.[6] A number of important mycotoxins have been isolated from *Fusarium moniliforme* including moniliformin, fuminosins and zearalenone.[7] Adequate measures to prevent spoilage of grains and foodstuffs are essential to avoid contamination and minimize public health hazards.

Fungi such as *A. flavus* and *A. fumigatus* produce aflatoxins as their secondary metabolites. These fungi grow rapidly on a variety of natural substrates and consumption of contaminated food can pose serious health hazards to people and animals. Aflatoxin B1 is a highly toxic and carcinogenic metabolite produced by *Aspergillus* species on agricultural commodities.[8]

Production of essential oils by plants is believed to be predominantly a defense mechanism against pathogens and pests [9] and it has been shown that essential oils possess antimicrobial and antifungicidal properties.[10,11] Essential oils and their components are gaining increasing interest because of their relatively safe status, wide acceptance by consumers, and their exploitation for potential multi-purpose functional use.[12] Essential oils are composed of many different volatile compounds. It is difficult to correlate the fungitoxic activity to a single compound or classes of compounds. It seems that the antifungal and antimicrobial effects of essential oils are the result of many compounds acting synergistically.[13] Thus, there would be negligible chance of development of resistant strains of fungi after application of essential oils on fruit and vegetables. Tanaoui-Elaraki *et al*., [14] have determined the ability of thyme essential oils of Moroccan *Thymus broussonettii*, *T. zygis* and *T. satureioides* for inhibiting microbial growth and destroying microorganisms. *T. broussonettii* exhibited the greatest potency on all the microorganisms studied, both inhibiting their growth and destroying them.[14] The infra specific variability of oils in the *Thymus* genus has been the subject of several studies reviewed by Stahl-Biskup.[15] However, most of the investigations on the antimicrobial activity of essential oils concern inhibition of microbial growth rather than lethal or antitoxic effects. Therefore, essential oils are one of the most promising groups of natural compounds for the development of safer antifungal agents. This study was undertaken to investigate the inhibitory

effects of Thyme, Agastache *and* Satureja essential oils against food spoilage and mycotoxin producing fungi and comparing them with each other.

Materials and Methods

Chemicals, cultures and media. Essential oils including Thyme oil (Thymus kotschyanus), Agastache oil (Agastache foeniculum), and Satureja oil (Satureja hortensis) were obtained from department of horticulture, faculty of agriculture, Urmia University, Urmia, Iran.

The reference fungal strains, *A. fumigates* (PTCC 5009), *A. flavus* (PTCC 5006) and *F. solani* (PTCC 5284) were obtained from the Iranian Organization for Scientific and Industrial Research and were maintained on Potato Dextrose Agar (E. Merck) slants. Spore suspensions were prepared and diluted in sterile Yeast Extract Sucrose (YES) broth making concentration of approximately 10^8 spores/ml. Spore number was counted using haemocytometer. Subsequent dilutions were made from the above suspension, which were then used in the tests. Incubation temperature was 30°C. All other solvents and reagents of analytical grade were obtained from E. Merck, Germany.

Oil dilution solvent. Di Methyl Sulphoxide (DMSO) was used as a diluting agent as it has no antifungal activity [16]. The stock of treatments was 0.02 ml pure oil in 10 ml DMSO with concentration of 2000 µl ml^{-1}. A number of six dilutions of essential oils including 1000, 500, 250, 125, 62.5 and 32.25 µl ml^{-1} were prepared using DMSO as a solvent. The above dilutions were used in antifungal analysis of the essential oils. Stock oils were used as one of the treatments and DMSO solvent alone also used as the control treatment.

Antifungal analysis. The fresh oils were examined for their antifungal activities. Disc diffusion method was used for antifungal screening as described by Rasooli and Mirmostafa.[17] Brifely, sterile Mueller–Hinton agar medium (Merck) was used for antibiogram assays. The disc size used was 6 mm paper (Whatman No. 1). Different dilutions of the oils were prepared with DMSO. The fungal suspension was streaked over the surface of Mueller–Hinton agar using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. Under aseptic conditions, the discs were placed on the agar plates and then 10 µl of each oil dilutions was put on the discs. Ten microlitre dilution solvent (DMSO) was added to the discs in the control plates. The plates were then incubated at 30 °C for 48 – 72 h in order to get reliable microbial growth. Diameters of microbial inhibition zones were measured using vernier calipers. The minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were assessed based on the modified procedure.[17] For determining MFC a broth dilution method in test tubes was used. Fifty µl of each dilution of the oils was added to 5 mL of Yeast Extract Sucrose broth tubes containing 10^7 spores/ml. Then tubes were incubated while shaking for evenly dispersing the oil throughout the broth in tubes. The highest dilution (lowest concentration) showing no visible growth, was regarded as MIC. Cells from the tubes showing no growth were sub-cultured on potato dextrose agar plates to determine if the inhibition was reversible or permanent. MFC was determined as the highest dilution (lowest concentration) at which no growth occurred on the plates.

Results

Thyme oil. All three essential oils showed antifungal effect (Tables 1-3). Thyme oil severely inhibited fungal growth at the first three concentrations of 1000, 500 and 250 µl ml^{-1} (Table 1) and had the highest fungistatical and fungicidal effect against all the fungi strains using 250 µl ml^{-1} concentration. The 125 µl ml^{-1}

of Thyme oil was effective fungistatistically and fungicidally on *F. solani* only. The fungistatical effect of Thyme oil was observed in concentration of 62.5 µl ml^{-1} against all three fungal species. However, the growth inhibition zone was very small. The concentration of 31 µl ml^{-1} of this oil did not have any effect on fungal growth (Table 1).

Agastache oil. The Agastache oil did not show any fungicidal effect on *A. fumigatus*. This oil was effective as a fungistatic agent when concentrations of 2000 µl ml^{-1} and 1000 µl ml^{-1} of Agastache oil were used. It had fungistatical and fungicidal effects on *A. flavus* and *F. solani* when it was used as stock solution. The concentrations of 1000 µl ml^{-1} and 500 µl ml^{-1} of Agastache essential oil had fungistatical effect on these fungi (Table 2).

Satureja oil. Satureja oil showed antifungal effect on three strains similar to Thyme oil. However the concentration ofdose 250 µl ml^{-1} of this oil had weaker effect than Thyme oil. The concentration125 µl ml^{-1} of Satureja oil had fungistatical and fungicidal effect on *F. solani but had only* fungistatical effect on *A. flavus* and *A. fumigates*. The concentration of 62.5 µl ml^{-1} showed fungistatical effect on all three fungi examined while these fungi showed resistance to the concentration of 32 µl ml^{-1} of this oil (Table 3).

Table 1. Diameter of *A. fumigatus ,A. flavus* and *F. solani*, inhibition zones (mm) determined by disc diffusion method and corresponding MIC/MFC at various dilutions of Thyme oil

	Thyme oil						
Dilution	1	½	1/4	1/8	1/16	1/32	1/64
Dosage(µl ml^{-1})	2000	1000	500	250	125	62.5	31.25
***A. fumigatus* inhibition Zones(mm)**	>	>	>	44	25	11	R
MIC/MFC	+/+	+/+	+/+	+/+	+/-	+/-	-/-
***A. flavus* inhibition Zones(mm)**	>	>	>	39	20	9	R
MIC/MFC	+/+	+/+	+/+	+/+	+/-	+/-	-/-
***F. solani* inhibition Zones(mm)**	>	>	>	54	37	18	R
MIC/MFC	+/+	+/+	+/+	+/+	+/+	+/-	-/-

>=Values equal to or greater than the diameter of petri dish, *R*=resistant

Table 2. Diameter of *A.fumigatus* ,*A.flavus* and *F.solani*, inhibition zones (mm) determined by disc diffusion assay and corresponding MIC/MFC at various dilutions of Agastache oil

	Agastache oil						
Dilution	1	½	1/4	1/8	1/16	1/32	1/64
Dosage(µl ml^{-1})	2000	1000	500	250	125	62.5	31.25
***A. fumigatus* inhibition Zones(mm)**	21	16	7	R	R	R	R
MIC/MFC	+/-	+/-	-/-	-/-	-/-	-/-	-/-
***A. flavus* inhibition Zones(mm)**	24	18	12	7	R	R	R
MIC/MFC	+/+	+/-	+/-	-/-	-/-	-/-	-/-
***F. solani* inhibition Zones(mm)**	28	19	11	7	R	R	R
MIC/MFC	+/+	+/-	+/-	-/-	-/-	-/-	-/-

>=Values equal to or greater than the diameter of petri dish, *R* = resistan

Table 3. Diameter of *A.fumigatus* ,*A.flavus* and *F.solani*, inhibition zones (mm) determined by disc diffusion assay and corresponding MIC/MFC at various dilutions of Satuerja oil

	Satureja oil						
Dilution	1	½	1/4	1/8	1/16	1/32	1/64
Dosage(µl ml^{-1})	2000	1000	500	250	125	62.5	31.25
***A. fumigatus* inhibition Zones(mm)**	>	>	>	35	24	10	R
MIC/MFC	+/+	+/+	+/+	+/+	+/-	+/-	-/-
***A. flavus* inhibition Zones(mm)**	>	>	>	44	25	11	R
MIC/MFC	+/+	+/+	+/+	+/+	+/-	+/-	-/-
***F. solani* inhibition Zones(mm)**	>	>	>	40	24	10	R
MIC/MFC	+/+	+/+	+/+	+/+	+/+	+/-	-/-

>=Values equal to or greater than the diameter of petri dish, *R* = resistant

Discussion

On the basis of diameter of antifungal inhibition zones, Thyme oil at the disc diffusion test and MFC dilutions exhibited > 90 and 39 mm (Table1) indicating antifungal properties of Thyme oil. It is evident that 8- and 4-fold dilutions of Thyme oil and Satureja oil were effective with a complete retardation of fungal growth respectively and somewhat lower concentrations were sufficient to significantly inhibit fungal growth (Table 1).

On the basis of diameter of antifungal inhibition zones, Satureja oil at the disc diffusion test and MFC dilutions exhibited > 90 and 24 mm (Table 3). The results of the present, study clearly showed that of Thyme and Satureja oils are more effective than Agastache oil. Examination of various concentrations of Thyme oil and Satureja oil on a potentially active fungal strains (*A. fumigatu*s PTCC 5009), (*A. flavus* PTCC 5006) and (*F.solani* PTCC 5006) in this study shows promising prospectus on the utilization of essential oils against fungi.

This study shows antifungal effects of three essential oils tested against three food spoilage and mycotoxin producing fungal species. Higher antifungal activity was found for Thyme and Satureja oils. In a study by Amvam Zollo *et al*. they reported a MIC of 2000 µl ml^{-1} against *A. flavus* using Thyme oil and reported antifungal activity of Thyme oil against *A. flavus*.[18] In another study for evaluating of five essential oils from aromatic plants for controlling food spoilage and mycotoxin producing fungi, the examined essential oils expressed different levels of antifungal activities.[19] This different anifungal activity of essential oils may be due to the differences in the content of known antimicrobial compounds of essential oils as previously determined by Amvam Zollo *et al*.[18] and Farag *et al*.[20]. Studying the antifungal effect of different essential oils including Thyme oil against *A. parasiticus* by Farag *et al*. they concluded that Thyme oil was the most antifungal oil among other oils examined and inhibitory effect of the oils was mainly due to the most abundant components such as thymol, cumin aldehyde, eugenol, carvonc, borneol and thujonc respectivly.[20] The study by Nguefack *et al*. demonstrated the potential food preservative ability of the essential oils such as Thyme oil against, *A. flavus*, *A. fumigatus* and *F. moniliforme*.[19]

It was concluded that essential oils examined in the present study could be used as antifungal agents against food spoilage and mycotoxin producing fungi. Further studies on their effect on other important fungal species and also their anti-parasitic activity are required.

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