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Antifungal Effects of *Zataria multiflora* Essential Oil on the Inhibitory Growth of some Postharvest Pathogenic Fungi

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Abstract

The present study aimed to determine minimum inhibitory concentration and minimum fungicidal concentration of the essential oil of *Zataria multiflora* to control *Alternaria solani, Rhizoctonia solani, Rhizopus stolonifer, Aspergillus flavus, Aspergillus ochraceus* and *Aspergillus niger*. The essential oil of *Zataria multiflora* was tested *in vitro* on PDA (malt extract agar medium) with eight concentrations: 0, 10, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ppm. This investigation followed the completely randomized design (CRD) with three replications. GC-MS evaluations of the essential oil revealed that thymol (35%), carvacrol (34%), cymene-p (9.89%), gamma-terpinene (5.88%) and alpha-pinene (4.22%) were the main compounds of *Zataria multiflora* oil. The results showed that the essential oil of *Zataria multiflora* has antifungal activity; the lowest inhibition (75%) was observed in the *A. niger*, while the highest inhibition (95.3%) was observed in *A. solani*. Minimum inhibitory concentration for *A. solani, R. solani, R. stolonifer, A. flavus, A. ochraceus* and *A. niger* was 200, 200, 200, 300, 300 and 200 ppm respectively. In addition, the present results showed that minimum fungicidal concentration (MFC) for *A. solani, R. solani, R .stolonifer, A. niger* and *A.ochraceus* was 600, 400, 300, 900 and 700 ppm respectively and none of the tested concentrations were fatal for *A. flavus*. *A. solani* and *R. solani* showed a strong sensitivity to *Zataria multiflora* essential oil at all concentrations. Findings of the current study suggest that essential oils of *Zataria multiflora* could be used for control of postharvest phytopathogenic fungi on fruits or vegetables.

Keywords: carvacrol, thymol, fungicidal, fungistatic, Shiraz thyme

Introduction

Fruits and vegetables are often subject to varying levels of microbial decay during storage. Pathogenic fungi usually infect the host through wounds and cause significant economic losses in the commercialization stage (Gatto *et al*., 2011).

The use of manufactured chemicals as fungicides is a main method to prevent or delay diseases and the post harvest rot is well known. Outspread use of fungicides has significant disadvantage including increased cost, worry about fungicides residues on crop, as well as risk for human health and environment (Nikos and Costas, 2007). As alternatives to synthetic fungicides, natural crop protective products are currently in the highlight (Combrinck *et al*., 2011). These include plant essential oils, a number of which have been reported to show antimicrobial activity against a wide array of plant pathogenic agents.

Essential oils are represent a defence mechanism against pathogens and pests, produced in different plant section and they also have been shown to own antimicrobial and antifungicidal properties (Znini *et al*., 2011). Several studies have investigated the antifungal properties of essential oils against postharvest pathogens (Giamperi *et al*., 2002; Bouchra *et al*., 2003; Bagamboula *et al*., 2004).

Zataria multiflora belongs to *Laminaceae* family and is mainly distributed in Iran, Pakistan and Afghanistan (Ali *et al*., 2000; Hosseinzadeh *et al*., 2000). It is greatly used for medicinal and spice uses in these countries. Commonly named Avishan Shirazi in Iran, *Z. multiflora* has various traditional uses such as antiseptic, anesthetic and antispasmodic (Zargari, 1990).

The antimicrobial properties of essential oils are principally related to their phenolic compounds (Bagamboula *et al*., 2004). Carvacrol and thymol are the main components of *Zataria multiflora* essential oil that are phenolic compounds (Alizadeh-Alteh *et al*., 2010).

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Table 1. Chemical composition of *Z. multiflora* essential oil

No	Components	Amount (%)	Retention index (RI)
1	Alpha-Thujene	0.36	930
2	Alpha-Pinene	4.22	939
3	Camphene	0.1	954
4	Beta-Pinene	0.34	979
5	Beta-Myrcene	1.01	990
6	Alpha-Phellandrene	0.19	1002
7	Alpha.-Terpinene	1.26	1017
8	Cymene<p->	9.89	1024
9	1,8-Cineole	0.37	1031
10	Gamma-Terpinene	5.88	1059
11	Linalooll	0.37	1096
12	Terpinene-4-ol	0.36	1177
13	Terpineneol<gama>	0.29	1199
14	Thymyl Methyl Ether	0.41	1235
15	Carvacrol Methyl Ether	0.73	1244
16	Thymoquinone	0.63	1248
17	Thymol	35.3	1289
18	Carvacrol	33.9	1299
19	Trans-Caryophyllene	1.12	1417
20	Aromadendrene	0.36	1439
21	Ledene	0.22	1475

Based on the distribution and abundance of *Zataria multiflora* in Iran, the studies on these plants in the term of their antifungal properties provide a solid ground that their results might be used to replace the fungicides of natural origin to control the postharvest diseases of crops, and this can lead to significant decreasing of the fungicides application and thus minimising their effects. Therefore, the objective of the present study was to evaluate the *in vitro* activity of plant essential oils from *Zataria multiflora* against postharvest fungal pathogens (*Alternaria solani, Rhizoctonia solani, Rhizopus stolonifer, Aspergillus flavus, Aspergillus ochraceus* and *Aspergillus niger*) and to determine the minimum inhibitory concentration and the minimum fungicidal concentration of *Zataria multiflora*.

Materials and Methods

Essential oils and fungi species

The essences of *Z. multiflora* were obtained from Barij Essence Pharmaceutical Co (Kashan, Iran). The strain of the studied fungus was obtained from the Agriculture and Natural Resources Research Center of Khorasan Razavi (Mashhad, Iran).

Gas chromatography/mass spectrometry and component identification

Gas chromatography/mass spectrometry (GC/MS) analysis was carried out in a Varian 3400 GC/MS (California, USA) system equipped with a DB-5 fused silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J and W Scientific). The oven temperature was raised from 50 to 240 °C at a rate of 4 °C min^{-1}, the transfer line temperature was 260 °C, the carrier gas was helium at a linear velocity of 31.5 cm s^{-1}, the split ratio was 1 : 60, the ionization energy was 70 eV. The components of the oils were identified by comparison of their mass spectra with those of a computer library or with those of authentic compounds and confirmed by comparison of their retention indices with those of authentic compounds or with data published in the literature. The retention indices were calculated for all volatile constituents using a homologous series of alkanes.

Antifungal effects of Zataria multiflora essential oil on mycelia radial growth by in vitro method

Antifungal activity was studied using a contact assay (*in vitro*) that produced hyphal growth inhibition. The assay was previously used for essential oil treatment on potato dextrose agar (PDA) medium by the 'solution method' (SM) (Özden and Bayindirli, 2002). Briefly, the essential oil was dissolved in 50 ml L^{-1} Tween 80/water solution and the required amounts of these solutions were added to individual Petri dishes containing 20 mL of PDA medium at 45 °C. Then a 0.5 mm disc of mycelium was placed on the PDA medium in each dish. The treated media were incubated at 27 °C and mycelia growth was measured daily. The inhibitory percentage (IP) was determined from the formula $IP=[(dc-dt)/dc]\times100$, where dc is the mycelium diameter in the control Petri dish and dt is the mycelium diameter in the essential oil treated Petri dish.

Nature of toxicity of the essential oil

The fungi-toxicity (fungistatic/fungicidal) of the essential oil was evaluated using the technique described by Thompson (1989). Different experiments were performed to demonstrate the toxicity nature of the oil at its minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) by the poisoned food technique assay. On day 7, the inhibited fungal discs of the treatment groups were acquired, washed with sterilized water and separately reinoculated into Petri plates containing fresh medium which were similarly incubated. On day 7, the assessment for the revival of growth of the reinoculated fungal discs was performed based on the observation (presence or absence) of mycelia growth.

Statistical analysis

Data were analysed statistically using analysis of variance (ANOVA) and differences among the means were determined for significance at $P<0.05$ using Duncan's test (by MSTATC software). The experiment was arranged as a completely randomized design (CRD) with three replications for each treatment.

Results

Chemical compositions of essential oil

The chemical composition of the essential oils was determined by GC/MS analysis. The identified components are given in Table 1 with their relative percentages. The twenty one compounds were characterized in the oil of *Z. multiflora*, making up of 98.71% of the oil. Major components of Shiraz thyme oil were: thymol (35%), carvacrol (34%), cymene.p (9.89%), gamma-terpinene (5.88%) and alpha-pinene (4.22%) (Fig. 1). The major components of Shiraz thyme (carvacrol, thymol, p-cymene and linalool) have key roles in regard to the antifungal activity (Burt, 2004; Omidbeygi, *et al.*, 2007; Solaimani, 2009).

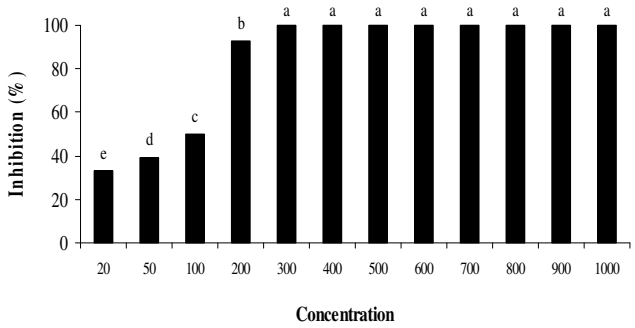

Fig. 1. Effect of different concentrations of *Zataria multiflora* essential oil on the inhibition percentage of fungus *Alternaria solani*, *Rhizoctonia solani*, *Rhizopus stolonifer*, *Aspergillus flavus*, *Aspergillus ochraceus* and *Aspergillus niger*. Different letters above columns indicate significant differences according to Duncan's multiple range tests at P≤0.01

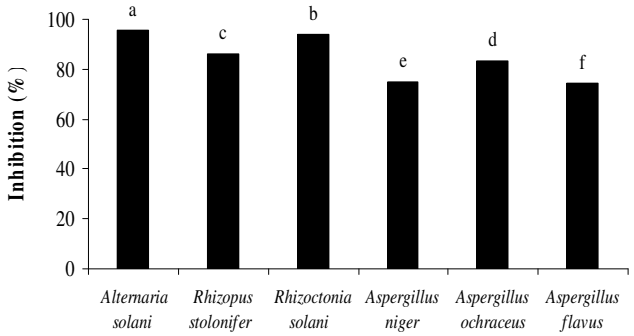

Fig. 2. Effect of *Zataria multiflora* essential oil on the inhibition percentage of fungus *Alternaria solani*, *Rhizoctonia solani, Rhizopus stolonifer*, *Aspergillus flavus*, *Aspergillus ochraceus* and *Aspergillus niger*. Different letters above columns indicate significant differences according to Duncan's multiple range tests at P≤0.01

Table 2. Effect of different concentrations of *Zataria multiflora* essential oil on the inhibition percentage of fungus *Alternaria solani*, *Rhizoctonia solani*, *Rhizopus stolonifer*, *Aspergillus flavus*, *Aspergillus ochraceus* and *Aspergillus niger*

Concentration (ppm)	*R. stolonifer*	*R. solani*	*A. solani*	*A. flavus*	*A. ochraceus*	*A. niger*
Control (0)	–	–	–	–	–	–
20	(s) 69 e	(s) 22 k	(s) 77 d	(s) 0 m	(s) 30 j	(s) 0 m
50	(s) 67 f	(s) 44 h	(s) 83 c	(s) 0 m	(s) 42 i	(s) 0 m
100	(s) 89 b	(s) 66 f	(s) 83 c	(s) 11 l	(s) 50 g	(s) 0 m
200	(s) 100 a	(s) 100 a	(s) 100 a	(s) 78 d	(s) 78 d	(s) 100 a
300	(c) 100 a	(s) 100 a	(s) 100 a	(s) 100 a	(s) 100 a	(s) 100 a
400	(c) 100 a	(c) 100 a	(s) 100 a	(s) 100 a	(s) 100 a	(s) 100 a
500	(c) 100 a	(c) 100 a	(s) 100 a	(s) 100 a	(s) 100 a	(s) 100 a
600	(c) 100 a	(c) 100 a	(c) 100 a	(s) 100 a	(s) 100 a	(s) 100 a
700	(c) 100 a	(c) 100 a	(c) 100 a	(s) 100 a	(c) 100 a	(s) 100 a
800	(c) 100 a	(c) 100 a	(c) 100 a	(s) 100 a	(c) 100 a	(s) 100 a
900	(c) 100 a	(c) 100 a	(c) 100 a	(s) 100 a	(c) 100 a	(c) 100 a
1000	(c) 100 a	(c) 100 a	(c) 100 a	(s) 100 a	(c) 100 a	(c) 100 a

(s): fungistatic , (c): fungicidal
Different letters indicate significant differences according to Duncan's multiple range tests at P≤0.01.

These results are in agreement with those published by Omidbeygi *et al.* (2007), Suhr and Nielsen (2003) and Solaimani *et al.* (2009) who reported that the major components of thyme essential oil are thymol, carvacrol, and á-pinene.

Inhibitory effect of the Z. multiflora essential oil on the studied fungus

The effects of different concentrations of the essential oils on the inhibition percentage of fungus are shown in Fig. 1. The highest inhibition (100%) was observed in the 300 ppm and more than it, while the lowest (33%) inhibition was obtained with 20 ppm essential oil of *Zataria multiflora* to control of the tested fungus. These results indicate that the percentage inhibition of mycelia growth increased with increasing concentrations of *Z. multiflora* essential oil for all fungus tested. The present results suggested that the essential oil of *Z. multiflora* has a significant activity (P<0.05) and inhibited the mycelia growth of all fungus.

The effects of essential oils on the inhibition percentage of fungus of *A. solani, R. solani, R. stolonifer, A. flavus, A. ochraceus* and *A. niger* are shown in Fig. 2. The lowest inhibition (75%) was observed in the *A. niger* and the highest inhibition (95.3%) was observed in *A. solani*. It was clear that *A. solani* and *R. solani* showed a high sensitivity to *Zataria multiflora* essential oil at all concentrations.

The lowest inhibition (0%) was observed in the concentrations of 20, 50 and 100 ppm in *A. niger* and concentrations of 20 and 50 ppm in *A. flavus.* Also the highest inhibition (100%) was observed in the 200 ppm and more than it in *A. solani, R. solani, R. stolonifer, A. niger* and 300 ppm and more than it in *A. flavus* and *A. ochraceus* (Table 2). Also, the results showed that minimum inhibitory concentration (MIC) for *A. solani, R. solani, R. stolonifer, A. flavus, A. ochraceus and A.niger* was 200, 200, 200, 300, 300 and 200 ppm respectively (Table 2).

Minimum fungicidal concentration (MFC) for *A.solani, R. solani, R.stolonifer, A. niger* and *A. ochraceus* was 600, 400, 300, 900 and 700 ppm respectively and of the concentrations were fatal for *A. flavus* (Table 2). This indicated that this oil has fungistatic effect on *A. flavus*.

Discussion

Zataria multiflora is a thyme-like plant that wildly grows in central and southern parts of Iran (Ali *et al*., 2000). It is a member of the *Labiate* family to which mint, rosemary and several other medically useful plants also belong. The Shiraz thyme essential oil used in the present study consisted of 21 components of which thymol (35%) and carvacrol (34%) were the major components. These results are in agreement with those of Soleimani *et al.* (2009) who reported that the major components of thyme essential oil are thymol, carvacrol and linalool. Mirzabagheri *et al.* (2014) reported that *Zataria multiflora* consisted of 25 components which thymol (13%) and carvacrol (50%) were the most abundant ones. Shaffiee and Javidnia (1997) found that the major components of *Zataria multiflora* oil prepared from Yazd Province of Iran were carvacrol (61.3%) and thymol (25.18%). Sharififar *et al.* (2007) observed that thymol (37.6%), carvacrol (33.6%) were the main components of *Zataria multiflora* oil. The composition of the essential oil of plants can change extensively depending upon the geographical conditions, variety, age of the plant and due to the method of drying and extraction of the oil (Valero and Salmeron, 2003; Bagamboula *et al*., 2004).

Essential oils complex mixtures of volatile compounds are produced by plants as secondary metabolites. The antibacterial and antifungal properties of essential oils have been known and used for duration (Danuta Kalemba *et al.*, 2012). The power of any biological activity of essential oils is severely connected with the oil composition and especially with the content of some very active constituents. It is reported that phenols and monoterpenes (thymol and carvacrol, the most phenols found in essential oils) have the highest activity (Bagamboula *et al*., 2004). Antifungal activities of these compounds have been reported by others (Kalemba and Kunicka, 2003; Chmai *et al*., 2004; Bagamboula *et al.*, 2004). However, the mechanisms of action of these compounds have not been completely explained. Lis-Balchin and Deans (1997) reported that strong antimicrobial activity could be correlated with essential oils containing high percentage of monoterpenes, thymol and carvacrol.

In the present study, *Zataria* essential oil showed antifungal activity against the mycelia growth of all the phytogenic fungi studied (Fig. 2). This activity is related to its high levels of oxygenated monoterpenes. Zambonelli *et al.* (1996) reported that the high activity of monoterpenes against pathogens result from their interference with enzymatic reaction during cell-wall synthesis. The effect of essential oils on microbial growth has been reported by Fung *et al.* (1977) who thought it may be the result of phenolic compounds in essential oils that cause an alteration in microbial cell permeability by interacting with membrane proteins.

The inhibition rate reached 78% for *A. flavus, A. ochraceus* and 100% for *A. solani, R. solani, R. stolonifer* and *A. niger* at 200 ppm. This indicated that 200 ppm was the minimal inhibitory concentration (MIC) of Shiraz thyme essential oil against *A. solani, R. solani, R. stolonifer, A. niger* and 300 ppm for *A. flavus, A. ochraceus* (Table 2). This might be attributable to the mechanism of resistance of the fungi against various substances present in the essential oil. The plant pathogens studied can be classified according to their sensitivity to the oil in the following order: *A. solani* > *R. stolonifer* > *R. stolonifer* > *A. ochraceus* > *A. niger* > *A. flavus* (Fig. 2).

After the transfer of the mycelia disk on plates containing a PDA medium and essence on fresh PDA (without oil) was made, the mycelia of *A. flavus* grew after incubation for seven days. It indicated that this oil has fungistatic effect (no fungicidal activity) on *A. flavus* (Table 2). Gandomi Nasrabadi *et al.* (2008) reported that MIC of Shiraz thyme essential oil against *A. flavus* was 400 ppm and MFC was 1000 ppm. Mahmoudi *et al.* (2012) determined the MIC of Shiraz thyme essential oil against *Alternaria alternate* by the poisoned food technique was 500 ppm. The fungicidal nature of the oil indicates its potential economic exploitation as fungi toxicant which has not been reported before (Basti *et al*., 2007).

Conclusions

The current results demonstrated that *Zataria essential* oil had an antifungal activity and may be considered a potential alternative to artificial fungicides for the defend of fruit and vegetable against phytopathogenic fungi. Nevertheless, before it can be marketed the evaluation of the antifungal activity of *Zataria multiflora* essential oil on *in vivo* condition is required.

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