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In vitro Efficacy of Four Plant Essential Oils against *Botrytis cinerea* Pers.:Fr. and *Mucor piriformis* A. Fischer

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Abstract: The antifungal activities of sweet basil (*Ocimum basilicum* L.), fennel *(Foeniculum vulgare* Mill.), summer savory (*Satureja hortensis* L.) and thyme (*Thymus vulgaris* L.) essential oils were investigated either in a poison food medium (200-1000 µl l^{-1}) or as vapour phase (5-40 µl) against *Botrytis cinerea* Pers.:Fr. and *Mucor piriformis* A. Fischer. All the four essential oils tested had significant inhibitory effects on the growth of both fungi. Thyme and summer savory essential oils exhibited strong antifungal activity against tested fungi in both methods. In the vapour phase fungicidal effects was observed even at low concentration (5 µl). The activity of the vapour phase of all essential oils was significantly higher compared to their activity when incorporated in the medium. Therefore, essential oils could be exploited in treatment of fruits and vegetables against postharvest fungal infections.

Key word: Essential oils, Antifungal activity, *Botrytis cinerea*, *Mucor piriformis.*

Introduction: Considerable postharvest losses of fruits and vegetables can result from different fungal attacks are brought about by decay caused by plant fungal pathogens [1]. It has been estimated that postharvest losses of fruits and vegetables can vary from an estimated 5 % to more than 20 % in the United States and can be as high as to 50 % in developing countries [2]. *Botrytis cinerea* Pers.:Fr. and *Mucor piriformis* A. Fischer are

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two important postharvest pathogens that cause grey mold and mucor rot diseases, respectively. These two species have wide host range and can particularly cause heavy economic losses on fruits, vegetables and flowers [3, 4]. Currently, synthetic fungicides are the primary means of controlling postharvest fungal decays. However, because of consumer awareness of natural food products and concerns about possible risks associated with the use of synthetic fungicides including chemical residues in food chains, pollution of the environment and development of new resistant strains of microorganisms against fungicides, have resulted in enhance interest to find effective and safer alternatives to synthetic fungicides [5, 6, 7]. Several biological methods, including strengthening natural defense mechanisms, microbial antagonists and natural antimicrobial compounds have been proposed as potential alternatives to synthetic fungicides [1]. For example, several aromatic plants used as flavoring agents in foods and their extracts and essential oils have been reported for their antimicrobial properties since antiquity and were used in medicine over time. In the recent past years have seen a growing interest in testing plant extracts, essential oils and their components against several food and plant pathogens [8, 9]. Essential oils are aromatic oily liquids obtained from different plant materials. They are highly complex mixtures of mono (C_{10}) and sesquiterpenes (C_{15}), including biochemically related phenols. These compounds are usually responsible for the characteristic odours and flavours of the plants from which they are obtained [8, 10]. Antifungal activity of several essential oils from aromatic plants against *B. cinerea* has been documented [11, 12, 13, 14, 15], but few of them were tested against *M. piriformis* [16, 17].

The advantage of essential oils is that they are bio-degradable in nature, non-pollutants, posses no residual and they are active in vapour phase [18, 19]. Technological application of essential oils as natural antifungal agents to reduce postharvest plant pathogens requires the establishment of the optimal conditions such as determining the pathogen sensitivity, the optimal essential oil concentrations and adequate period of application.

The objective of this work was to evaluate the effect of essential oils of sweet basil, fennel, summer savory, and thyme using poison food medium and vapour phase methods on growth of *B. cinerea* and *M. piriformis*.

Experimental

Plant materials: The aerial parts of sweet basil (*Ocimum basilicum* L.), summer savory (*Satureja hortensis* L.), and thyme (*Thymus vulgaris* L.) at flowering stage and fennel *(Foeniculum vulgare* Mill.) fruits at ripening stage were harvested, air dried, and stored at room temperature in dark until distillation. Samples were then subjected to hydrodistillation for 3 h using a Clevenger-type apparatus, according to method outlined by European Pharmacopoeia [20]. The obtained essential oils were collected, dried over anhydrous sodium sulfate and stored at 4°C until further use.

Essential oils analysis: The GC analyses were carried out on a Shimutzu 17A gas chromatograph and a DB-5 (non-polar and 95 % dimethyl polysiloxane) capillary column (30 m × 0.25 mm; 0.25 µm film thickness). The oven temperature was held at 30°C for 3

min. and then programmed at 2.1 ml/min to 280°C. Other operating conditions were as follows: carrier gas He, with a flow rate of 2.1 ml/min; injector temperature 230°C; detector temperature 250°C; split ratio, 50 GC-MS analyses were performed on a Shimutzu 17A GC coupled with Shimutzu QGD5050 Mass system. The operating conditions were the same conditions as described above but the carrier gas was He. Mass spectra were taken at 70 e V. Mass range was from m/z 50-450 amu.

The constituents of the oils were identified by calculation of their retention indices under temperature-programmed conditions for identification of individual n-alkanes (C_6-C_{24}) and the oil on DB-5 capillary column. Compounds were made by comparison of their mass spectra with those of the internal reference mass spectra library (NIST 98 and Wiley 5.0) or with those of reported in the literature. Quantitative data was obtained from flame ionization detector (FID) area percentages without the use of correction factors [21, 22].

Pathogens: The tested fungi, *B. cinerea* and *M. piriformis* were isolated from infected table grape fruits and provided by the Plant Protection Department, Urmia University. Stock cultures were maintained on Potato Dextrose Agar (PDA) slants and kept at 4°C for further studies. Fungi were grown on PDA medium and incubated at 25°C in the dark. A 4-7 days old culture of each fungus was used for bioactivity tests.

Antifungal activity assays: The antifungal activities of essential oils were tested against *B. cinerea* and *M. piriformis* following poison food medium and vapour phase methods. In the poison food medium method, requisite amounts (0, 200, 400, 600, 800 and 1000 µl l^{-1}) of essential oils were prepared by dissolving them in Tween 80 (0.5 %, v/v) and added to flasks containing sterile molten medium. The PDA with essential oil, 20 ml/plate, was poured into sterile, 9 cm glass Petri plates. A fungal disc (5 mm in diameter) were cut from the edge of a 4-7 days old culture, and was placed at the center of each Petri plate. Petri plates were incubated at 25°C, and growth inhibition was determined after 7 days by measurement of colony diameter.

In vapour phase assay, different concentrations (0, 5, 10, 15, 20, 25 and 40 µl) of pure essential oils were added to filter paper (70 mm in diameter, Whatmann No.1), and placed on the inner side of the inverted Petri plates. Plates were inoculated with fungi as described above and sealed with parafilm in order to avoid loss of essential oils. Petri plates were incubated at 25°C and vapour phase effects of essential oils were determined after 7 days. Each treatment was replicated four times and antifungal activity was measured in terms of percent mycelial growth inhibition (MGI %) calculated by the followed formula:

$$MGI\ (\%) = ((dc-dt)/dc) \times 100$$

where dc and dt represent mycelial growth diameter in control and treatment Petri plates, respectively.

At the end of the incubation period, fungistatic and/or fungicidal nature of the essential oils was determined by Thompson method [23]. The agar discs of the tested fungi which did not showed any visible growth were transferred on to essential oils-free PDA plates and

incubated for further 7 days to observe the revival growth. The effects of essential oils was considered fungicidal if the pathogen did not grow or fungistatic if the pathogen growth occurred.

Statistical analyses: The data obtained as MGI (%) of essential oils were subjected to analysis of variance for a completely randomized design using SAS software. Mean comparison were performed by Duncan's multiple range test and differences were considered significant at the $P< 0.05$ level.

Results and discussion: The results obtained by GC and GC-MS analyses of the essential oils used in this study are presented in table 1. The main compounds found in essential oils extracted from sweet basil, fennel, summer savory and thyme were linalool (65.3 %), *trans*-anethole (64.4 %), carvacrol (54.1 %) and thymol (10.6 %), respectively.

The MGI % (Mycelial growth inhibition percentage) of the essential oils against *B. cinerea* and *M. piriformis* in both methods are shown in tables 2 and 3. Different essential oils exhibited different antifungal activities against both tested fungi. In poison food medium method (table 2), the oils of fennel, thyme and summer savory completely inhibited the growth of *B. cinerea* when added at concentrations $\geq$ 600 µl l^{-1} for fennel oil and at $\geq$ 800 µl l^{-1} for thyme and summer savory oils, but their inhibitory effects were only fungistatic. Sweet basil oil showed the lowest antifungal activity against *B. cinerea* in poison food medium method. All essential oils showed good inhibitory effects against *M. piriformis* when used in poison food medium method. The highest inhibitory effect against growth of *M. piriformis* was observed with concentrations of $\geq$ 800 µl l^{-1} for sweet basil and fennel oils and at $\geq$ 600 µl l^{-1} for summer savory and thyme oils, which completely suppressed fungal growth. The antifungal properties of essential oils against *M. piriformis* were found to be fungistatic.

All the essential oils tested had good inhibitory effects against both fungi, and the growth of both fungi were completely suppressed at concentrations $\geq$ 10 µl for sweet basil and fennel oils and at $\geq$ 5 µl for summer savory and thyme oils. Antifungal properties (fungistatic/fungicidal) of essential oils in vapour phase method shown in table 4.

Development of new alternative methods for synthetic fungicides to prolong the shelf life of fruits and vegetables are one of the main challenges of agricultural researchers. Many spices and herbs were studied for their antimicrobial properties, which are attributed to their essential oil fractions. In this study, essential oils of some plant species were tested for their antifungal activity against two important plant pathogens.

High inhibition of mycelial growth of *B. cinerea* and *M. piriformis* was provided by essential oils tested. Among them, thyme essential oil had the highest inhibitory effects followed by summer savory, fennel and sweet basil. The antimicrobial activities of essential oils could vary and depend on the composition of essential oils, their concentrations, sensitivity of tested organism and method of application. Soylu *et al*. [24] showed that the essential oils of oregano and thyme were active against *Phytophtora infestans*, while rosemary, lavander, fennel, and laurel essential oils showed reduced bioactivity. Reddy *et al.* [14] evaluated antifungal activity of *Thymus vulgaris* essential oil on *B. cinerea* and *R.*

stolonifer, and concluded that essential oils had fungistatic not fungicidal effects against both fungi, although *B. cinerea* were more sensitive than *R. stolonifer*. Bouchra *et al.* [12] reported that the essential oils of *Origanum compactum* and *Thymus glandulosus* showed strong inhibitory effect against *B. cinerea*. Edris and farrag [16] found that the vapours of peppermint and sweet basil essential oils at different ratios inhibited the growth of *Mucor* sp. and *R. stolonifer*.

Results of this assay showed that the MGI values required to inhibit the growth of fungi in poison food medium were higher than those required on vapour phase. These results are in accordance with Segvic Klaric *et al*. [17], who showed that vapour phase of thyme oil had high antifungal activity, but not agree Yahyazadeh *et al*. [25], who described that sage and fennel essential oils in vapour phase had no inhibitory effect on the growth and even increased the growth of *Penicillium digitatum.*

Biological activities of essential oils depend on the qualitative and quantitative of their components, which it is affected by the plant genotype, plant chemotype, geographical origin, season, environmental and agronomic conditions [26, 27]. Although the exact mechanism(s) of action of the essential oils against fungi was not completely clarified, several authors attributed the antimicrobial activity of essential oils to their phenolic compounds such as thymol, carvacrol and eugenol [28, 29, 30]. Farrag *et al*.[31] reported that antimicrobial activity of essential oils can be attributed to phenolic compounds, followed by alcohols, aldehydes, ketones, ethers and hydrocarbons. Several researchers reported that antimicrobial activity of phenolic compounds may be related to their general ability as a lipophilic compound to dissolve or otherwise disrupt the integrity of cell walls and membranes [32, 33]. The highest antifungal activity of thyme and summer savory oils could be attributed to high levels of phenolic compounds (thymol and carvacrol) in their essential oils. In addition of altering the structure of cell membrane, essential oils break the molecules of intracellular nucleic acids. The antimicrobial action of essential oils could also be related to the interaction of some enzymes, including those involved in the production of energy and synthesis of structural components [34]. Arras and Usai [35] reported that after treatment with thyme oil and its main components, morphological alterations of hyphae and conidia of *P. digitatum* were observed. Kummar *et al*. [36] described that the efficacy of thyme oil on *Aspergillus flavus* is associated with its inhibitory effect on some metabolic pathways of fungi. Rasooli and Owlia [37] found that *Thymus ericalyx* and *Thymus X-porlock* oils dissociated the plasma membrane from the cell wall and caused the formation of lomasoms, similar to that formed by treatment with imidazole fungicides. Sinha and Gulati [38] reported that the antimicrobial activity of essential oils of different species of *Ocimium* was related to linalool and methyl chavicol.

Although, the main components of essential oils are mostly considered to be mainly responsible for their antimicrobial activity, the minor components may also play an additional role. Several authors reported that the antimicrobial activity of essential oils can be related to synergistic correlation between the total components available in essential oil [16, 39, 40, 41].

Our results have demonstrated that thyme and summer savory oils that are rich in phenolic compounds, showed promising antifungal properties. However, their inhibitory

effects were greater in the vapour phase method. Further studies are necessary to confirm antifungal activities of thyme and summer savory oils under *in vivo* conditions, as these oils might be used for preservation and/or extension the shelf-life of agricultural crops, since these essential oils generally recognized as safe products.

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Table 1. The chemical compositions (%) of essential oils

No	Compound [a]	RI [b]	Sweet basil	Fennel	Summer savory	Thyme
1	α-pinene	936	-	-	-	5.2
2	Limonene	964	-	3.4	-	-
3	β-Pinene	971	-	1.5	-	3.8
4	Myrcene	977	2.2	-	-	-
5	Champhene	988	-	-	-	2.6
6	γ-Terpinene	996	-	1.4	-	-
7	α-Phellandrene	997	-	-	5.3	8.5
8	β-Ocimene	1030	-	-	-	12.6
9	(E)-β-Ocimene	1032	2.0	-	-	-
10	p-Cymene	1040	-	-	3.6	-
11	1,8-Cineol	1049	3.6	-	-	-
12	Fenchone	1051	-	14.6	-	-
13	Terpinolene	1075	-	-	20.6	-
14	α-terpinolene	1080	-	-	-	1.2
15	Camphor	1083	-	1.5	-	-
16	Linalool	1090	65.3	-	-	4.2
17	Nerol oxide	1103	-	-	-	3.2
18	Borneol	1107	-	-	-	2.3
19	*trans*-Anethole	1240	-	64.4	-	-
20	Linalyl acetate	1253	-	-	-	1.3
21	Carvacrol	1285	-	-	54.1	6.9
22	Thymol	1289	-	-	-	10.6
23	Geranyl acetate	1352	-	-	-	2.0
24	α-copaene	1358	-	-	-	1.4
25	Methyl eugenol	1368	1.4	-	-	-
26	β-elemene	1381	1.8	-	-	-
27	Eugenol	1389	2.9	-	-	-
28	β-caryophyllene	1420	-	-	-	6.1
29	*trans*-α-bergamotene	1427	3.89	-	-	-
30	Germacrene D	1462	1.9	-	-	1.9
31	Germacrene B	1489	1.2	-	-	-
32	δ-Cadinene	1517	-	-	-	2.1
33	Spathulenol	1550	-	-	-	1.7
34	Widrol	1569	-	-	-	2.1
35	Caryophyllene oxide	1586	-	-	-	5.7
36	β-Eudesmol	1625	4.1	-	-	-
37	Docosane	2300	-	-	-	2.8

[a] The compounds that present lower than 1 % were not showed

[b] Retention indices

Table 2. Effect of essential oils on mycelial growth inhibition percent (MGI%) of *B. cinerea* and *M. piriformis* by poison food medium method

Essential oil concentration(µl l^{-1})	Mycelial growth inhibition percent (MGI%)							
	Sweet basil		Fennel		Summer savory		Thyme	
	B. cinerea	*M. piriformis*	*B. cinerea*	*M. piriformis*	*B. cinerea*	*M. piriformis*	*B. cinerea*	*M. piriformis*
0 (Control)	0 ^{l}	0^{i}	0 ^{l}	0^{i}	0 ^{l}	0^{i}	0 ^{l}	0 ^{i}
200	17.8 ^{k}	0 ^{l}	67.22 ^{b}	3.42 ^{h}	33.05 ^{i}	2.22 ^{h}	21.94 ^{j}	12.62 ^{g}
400	37.78 ^{h}	16.94 ^{f}	66.65 ^{bc}	61.11 ^{e}	42.78 ^{f}	70.28 ^{d}	53.05 ^{e}	95.55 ^{b}
600	40.28 ^{g}	93.61 ^{c}	100 ^{a}	95.83 ^{b}	64.16 ^{cd}	100 ^{a}	66.38 ^{bc}	100 ^{a}
800	62.48 ^{d}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}
1000	68.6 ^{b}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}

Mean values followed by different letters within the column are significantly different according to Duncan Multiple Range Test ($P< 0.05$)

Table 3. Effect of essential oils on mycelial growth inhibition percent (MGI%) of *B. cinerea* and *M. piriformis* by vapour phase method

Essential oil concentration(µl l^{-1})	Mycelial growth inhibition percent (MGI%)							
	Sweet basil		Fennel		Summer savory		Thyme	
	B. cinerea	*M. piriformis*	*B. cinerea*	*M. piriformis*	*B. cinerea*	*M. piriformis*	*B. cinerea*	*M. piriformis*
0 (Control)	0 ^{d}	0 ^{d}	0 ^{d}	0 ^{d}	0 ^{d}	0 ^{d}	0 ^{d}	0 ^{d}
5	36.38 ^{c}	58.61 ^{c}	61.14 ^{b}	61.11 ^{b}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}
10	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}
15	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}
20	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}
25	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}
40	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}

Mean values followed by different letters within the column are significantly different according to Duncan Multiple Range Test ($P< 0.05$)

Table 4. Nature of antifungal activities of plant essential oils on growth of *B. cinerea* and *M. piriformis* in vapour phase assay

Plant name	Essential oil concentration (µl)	*B. cinerea*	*M. piriformis*
Sweet basil	5	g	g
	10	fs	fs
	15	fs	fs
	20	fc	fs
	25	fc	fs
	40	fc	fc
Fennel	5	g	fs
	10	fc	fs
	15	fc	fs
	20	fc	fc
	25	fc	fc
	40	fc	fc
Summer savory	5	fc	fc
	10	fc	fc
	15	fc	fc
	20	fc	fc
	25	fc	fc
	40	fc	fc
Thyme	5	fc	fc
	10	fc	fc
	15	fc	fc
	20	fc	fc
	25	fc	fc
	40	fc	fc

g : Indicates growth of test fungus
fc : Fungicidal activity
fs : Fungistatic activity