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The Potential of Thyme, Clove, Cinnamon and Ajowan Essential Oils in Inhibiting the Growth of *Botrytis cinerea* and *Monilinia fructicola*

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Abstract: In the present study, the antifungal activity of essential oils of *Thymus vulgaris*, *Eugenia caryophyllata, Cinnamomum zeylanicum* and *Carum copticum* against two well-known postharvest fungi, *Monilinia fructicola* and *Botrytis cinerea*, by poison food medium method was assayed. The inhibition of spore germination by essential oils was also determined. The results showed that all of essential oils inhibited completely of *B. cinerea* growth at concentrations ≥ 400 µl/l. Also all of essential oils except *E. caryophyllata* at concentrations ≥ 400 µl/l completely suppressed fungal growth of *M. fructicola*. In addition, results showed that *C. copticum* oil possessed the highest antifungal activity on both fungi. GC-MS analysis showed that main compounds identified in *T. vulgaris*, *E. caryophyllata, C. zeylanicum* and *C. copticum* oils were thymol (37.5 %), eugenol (48.8 %), cinnamaldehyde (90.3 %) and thymol (50.9 %), respectively.

Key words: Essential oil, Thyme, Clove, Cinnamon, Ajowan, *Botrytis cinerea*, *Monilinia fructicola.*

Introduction

The postharvest losses of agricultural products are a chronic problem through the world. It is estimated that postharvest spoilage caused by fungi and bacteria during storage of agricultural crops, caused losses from 5 % to 50 % [1]. *Monilinia fructicola* (G. Wint.) Honey (brown rot) and *Botrytis cinerea* Pers.: Fr. (gray mold) are two most common causes of postharvest fruit decay of stone fruits in most countries [2, 3].

Limitation of postharvest losses can be achieved mainly by pre- and postharvest applications of synthetic fungicides. However, increasing reports of fungi resistance against fungicides, environmental pollution and harmful effects on human health has resulted in negative public perception of chemicals. These restrictions have led to increase interest in alternative methods to

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fungicidal treatment in controlling postharvest fungal disease [4].

Essential oils are interesting biodegradable and relatively safe botanical products that accumulated in the various plant organs and extracted from these organs by hydro-distillation, steam-distillation and etc., methods. Essential oils are thought to play a role in plant defense mechanisms against phytopathogenic microorganisms. Moreover, they possess a great potential as non-toxic antimicrobial material [5, 6]. Bakkali *et al*. [7] and Burt, [8] have summarized a number of works that reveal the essential oils application with diverse methods for food preservation, medical and cosmetic uses.

Antimicrobial properties of essential oils against phytopathogens were observed by several authors in *in vitro*. The essential oil from flowers of *Chrysanthemum coronarium* significantly reduced the growth of *B. cinerea* and *Fusarium moniliforme* under *in vitro* condition [9]. Wilson *et al*. [10, 11] screened the antifungal property of several essential oils against *B. cinerea* and *M. fructicola*. Abdolahi *et al*. [12] reported that *Thymus vulgaris* and *Satureja hortensis* essential oils exhibit strong antifungal activity against *B. cinerea*. Lazar-Baker *et al*. [13] demonstrated that essential oils have potential as antifungal agents to control *M. fructicola*. Also *Cinnamomum zeylanicum* (bark), *C. zeylanicum* (leaf), *C. cassia*, *Syzygium aromaticum* and *Cymbopogon citrates* essential oils showed high antifungal activity on growth and spore germination of *Aspergillus niger* under *in vitro* condition [14].

This study was undertaken to investigate the antifungal activity of essential oils of *T. vulgaris*, *Eugenia caryophyllata*, *C. zeylanicum* and *Carum copticum* against *M. fructicola* and *B. cinerea* by poison food medium method.

Experimental

Plant materials and essential oils extraction

The aerial parts of thyme (*Thymus vulgaris*) in flowering stage, clove (*Eugenia caryophyllata*) buds, cinnamon (*Cinnamomum zeylanicum*) bark and ajowan (*Carum copticum*) seeds 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. The obtained essential oils were collected, dried over anhydrous sodium sulfate and stored at 4°C until further use.

Essential oils analysis

The GC analysis was 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 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 analysis was performed on a Shimutzu 17A GC coupled with Shimutzu QGD5050 Mass system. The operating conditions were the same conditions as described above. Mass spectra were taken at 70 e V. Mass range was from *m/s* 50-450 amu.

The constituents of the oil 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. Identification of compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library (NIST 98 and Wiley 5.0) or with authentic compounds or with those of reported in the literature [15, 16]. Quantitative data was obtained from FID area percentages without the use of correction factors.

Fungi

The test fungi *B. cinerea* and *M. fructicola* were isolated from infected apricot and sweet cherry fruits and identified in the Agriculture Faculty, Plant Pathology Department, Urmia University. The isolated fungi maintained on Potato Dextrose Agar (PDA) medium at 25°C. A 7-day culture of each fungus was used for bioactivity tests.

Determination of antifungal activity

For assay of the antifungal activity of essential oils against *B. cinerea* and *M. fructicola*, the

method of poison food medium was used. PDA medium was autoclaved (20 min at 1.03 kg/cm at 121°C) then had been cooled to about 45°C. The essential oils were mixed with sterile molten PDA to obtain final concentrations: 0, 200, 400, 600, 800 and 1000 µl/l. Tween 80 at 0.01 % were used as a surfactant to disperse the oil. Twenty milliliters of media were poured into each Petri plates. Mycelial disks of 5 mm diameter, cut out from the periphery of 7-day-old cultures of test pathogens, and aseptically inoculated upside down on the PDA. Four replicates were used per treatment. Inoculated Petri plates were incubated at 25 ± 1°C and observations were recorded on day 7. The mean growth value was obtained and was converted into the percentage of mycelial growth inhibition (MGI) in relation to the control treatment using followed formula:

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

Where dc and dt represent mycelial growth diameter in control and treated Petri plates, respectively.

At the end of incubation period, the plates were evaluated for the presence or absence of growth, to find out the nature of antifungal activity (fungistatic/ fungicidal) of the oils. For this purpose, fungal discs which did not showed any visible growth, were transferred on to fresh PDA plate and incubated for 7 days to observe the revival growth. The effect of essential oils was considered fungicidal if the pathogen did not grow or fungistatic if the pathogen growth occurred [17].

Essential oils effects on spore germination of *B. cinerea*

To determine the effect of *T. vulgaris*, *E. caryophyllata, C. zeylanicum and C. copticum* essential oils on spore germination of *B. cinerea*, a spore suspension of *B. cinerea* at 10^6 spore/ml was prepared and 10 µl of spore suspension was spread in the Petri plates containing different concentrations (0, 200, 400, 600, 800 and 1000 µl/l) of essential oils that mixed with molten PDA media. Control plates containing Tween 80 mixed with PDA were prepared in the same way. After 24 h of incubation at 25 ± 1°C, plates were evaluated using light microscopy (Olympus. BX41, Japan) and number of germinated spores assessed.

Statistical analysis

Statistical analysis of the data was performed using MSTATC statistical software, Version 4 [18], and the means were separated by Duncan's multiple range test. Statistical differences at $P < 0.05$ were considered significant.

Results and Discussion

Hydrodistilled essential oils from *T. vulgaris*, *E. caryophyllata*, *C. zeylanicum* and *C. copticum* were analyzed using GC-MS system. The identified components are given in table 1 with their relative percentages. Twenty six compounds were identified in the *T. vulgaris* oil, representing 97.7% of the total components detected. Eight components were characterized in the *E. caryophyllata* oil, representing 98.3 % of the total. At the same time, twenty one compounds determined in the *C. zeylanicum* sample, representing of 98.3 % of total compounds. Finally, in the *C. copticum* sample, twenty components representing 99.5 % of the oil were identified.

The major compounds found in *T. vulgaris* oil were thymol (37.5 %), γ-terpinene (15.1 %), carvacrol (10.8 %) and ρ-cymene (10.6 %). This result was in accordance with the investigation conducted by Hudaib *et al.* [19], which *T. vulgaris* oil was rich in the active monoterpene phenols (thymol and carvacrol) and their corresponding monoterpene hydrocarbon precursors ρ-cymene and γ-terpine. Also it is identified that thymol (44.4-58.1 %), p-cymene (9.1-18.5 %), γ-terpinene (6.9-18.9 %) and carvacrol (2.4-4.2 %) were the dominant constituents of *T. vulgaris* oil [20]. The oil of *E. caryophyllata* was particularly rich in eugenol (48.8 %), eugenyl acetate (27.8 %) and *trans*-caryophyllene (16.8 %). These are in accordance with previous study that showed eugenol (78.5 %) was the predominant compound in the clove oil [21].

Cinnamaldehyde (90.3 %) was the main compound of *C. zeylanicum* oil. our results confirm the published data by Singh *et al.* [22],

Pawar and Thaker [14] and Xing *et al*. [23] that reported cinnamaldehyde was the major compound in the *C. zeylanicum* oil. At the same time, *C. copticum* oil contained thymol (50.9 %), γ-terpinene (16.0 %), ρ-cymene (10.6 %) and carvacrol (8.0 %), as the main components. The present results were agree with our previous study that showed thymol (63.2 %), ρ-cymene (21.4 %) and γ-terpinene (13.8 %) were the main constituents of *C. copticum* oil [1].

Table 1. Chemical composition of essential oils

Component	RI [a]	*Thymus vulgaris*	*Eugenia caryophyllata*	*Cinnamomum zeylanicum*	*Carum copticum*
α-Thujene	930	1.8	-	-	0.3
α-Pinene	939	1.1	-	0.1	0.3
Comphene	954	0.7	-	0.1	-
Benzaldehyde	960	-	-	0.2	-
β-Pinene	979	-	-	0.1	1.9
Myrcene	991	3.0	-	-	0.7
α-Phellandrene	1003	0.4	-	-	-
α-Terpinene	1017	2.3	-	-	0.3
ρ-Cymene	1025	10.6	-	0.1	15.4
β-Phellandrene	1030	-	-	-	0.7
1,8-Cineole	1031	0.9	-	-	-
γ-Terpinene	1050	15.1	-	-	16.0
cis-Sabinene hydrate	1070	2.7	-	-	0.3
Linalool	1097	2.7	-	-	-
Trans-Sabinene hydrate	1098	-	-	-	0.3
Camphor	1146	0.3	-	-	1.6
α-Terpineol	1189	0.3	-	-	0.5
Carvacrol methyl ether	1245	0.9	-	-	-
Chavicol	1250	-	0.3	-	-
Cinnamaldehyde	1270	0.2	-	90.3	0.2
Thymol	1290	37.5	0.3	-	50.9
Carvacrol	1299	10.8	-	-	8.0
Thymyl acetate	1352	0.6	-	-	-
Eugenol	1359	0.3	48.8	0.4	1.0
α-Copaene	1377	-	-	1.2	-
trans-Caryophyllene	1419	3.1	16.8	0.1	0.2
β-Bergamotene	1435	-	-	0.1	-
Cinnamyl acetate	1446	-	-	0.3	-
α-Humulene	1455	-	2.4	-	-
Geranyl propionate	1478	0.5	-	-	-
Germacrene D	1485	0.2	-	0.3	-
α-Muurolene	1500	-	-	0.4	-
β-Bisabolene	1506	-	-	0.2	-
(Z,E)-α-Farnesene	1506	-	0.2	-	-
Eugenyl acetate	1523	-	27.8	-	0.3
δ-Cadinene	1523	0.4	-	0.6	-
cis-Calamenene	1543	-	-	0.4	-

table 1. (continued).

Component	RI [a]	*Thymus vulgaris*	*Eugenia caryophyllata*	*Cinnamomum zeylanicum*	*Carum copticum*
ρ-Methoxy cinnamic alidahyde	1563	-	-	2.3	-
Caryophyllene oxide	1583	0.8	1.7	0.2	0.2
Dillapiole	1621	0.3	-	0.4	0.4
δ-Cadinol	1649	0.2	-	-	-
Torreyol	1667	-	-	0.3	-
Farnesol	1725	-	-	0.2	-

[a] Retention indices

A corresponding decrease in fungal mycelia growth and spore germination with increasing concentration of oils was observed in the present study (Table 2, 4). It is evident from table 2 that all of essential oils showed complete inhibition of growth of *B. cinerea* at concentrations ≥ 400 µl/l. Bouchra *et al*. [23] showed that *Origanum compactum* and *T. glandulosus* completely inhibited the mycelia growth of *B. cinerea* at 100 µl/l concentrations. Also all of essential oils except *E. caryophyllata* at concentrations ≥ 400 µl/l completely suppressed fungal growth of *M. fructicola*. In addition, results showed that *C. copticum* had the highest antifungal activity on both fungi (Table 2).

As shown in table 3, although all of tested essential oils demonstrated good inhibitory effects on mycelia growth of fungi, but assessment the nature of antifungal activity of these oils showed that *C. zeylanicum* at concentrations ≥ 400 µl/l against *B. cinerea* and at concentrations ≥ 600 µl/l against *M. fructicola* showed fungicidal character. Also results showed that *E. caryophyllata* oil on *B. cinerea* and *C. copticum* oil against *M. fructicola* had not fungicidal activity (Table 3).

Evaluation the efficacy of essential oils on spore germination of *B. cinerea* showed that *T. vulgaris*, *C. zeylanicum* and *C. copticum* at concentrations ≥ 600 µl/l oils completely inhibited spore germination. Also, *E. caryophyllata* oil had the highest inhibitory effect on spore germination and at all concentrations suppressed spore germination of *B. cinerea* (Table 4). In accordance with these results Tzortzakis [25] identified that fungal spore production inhibited up to 63 % at 25 µl/l of cinnamon oil concentration and fungal sporulation was completely retarded at the 500 µl/l concentrations. Pawar and Thaker [14] showed that *C. zeylanicum* (bark), *C. zeylanicum* (leaf), *C. cassia*, *Syzygium aromaticum* and *Cymbopogon citrates* inhibited hyphal growth and spore formation of *Aspergillus niger*.

Although, antifungal property of essential oils have been evaluated in many studies. However, insignificant attention has been given to the mechanism efficacy of these substances as antifungal agents and the exact mechanism (s) action of the antifungal products not distinguished. In general, the differences in antifungal activities of essential oils could be related to their chemical composition [26]. Which chemical composition of essential oils could be changed by genetic and agronomic conditions [27].

Generally, the antifungal action of essential oils on fungal cells involves cytoplasm granulation, cytoplasm membrane rupture and inactivation and/or inhibition of synthesis of intracellular and extracellular enzymes [28]. These actions can occur in an isolated or in a concomitant manner and culminate with mycelium germination inhibition [29]. In according to Pawar and Thaker [14] the physical nature of essential oils, i.e. low molecular weight combined with pronounced lipophilic tendencies allow them to penetrate cell membrane more quickly than other substances.

Several authors attributed the antimicrobial property of essential oils to their dominant compound especially phenolic compounds such as thymol, carvacrol and eugenol [30, 31,32]. Farag *et al*. [30] reported that antimicrobial activity of essential oils can be attributed to phenolic compounds, followed by alcohols, aldehydes, ketones, ethers and hydrocarbons. Nychas [31] indicated that phenolic compounds could denature the enzymes responsible for spore germination or interfere with the amino acid involved in germination. Cinnamaldehyde may be potential lead compound for the development of antifungal compounds through the control of B-(1-3)-glucan and chitin synthesis in yeast and molds [33].

Thus, the presence of thymol, carvacrol, eugenol and cinnamaldehyde as major components of tested oils might have played role in inhibiting mycelia growth and spore germination in the present study. Also, it is reported that the antifungal activity of essential oils could be related to all components available in the oils and existence of synergistic correlation between the total constituents available in essential oil [34].

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

Essential oil (µl/l)		Mycelial growth inhibition (%)	
		Botrytis cinerea	***Monilinia fructicola***
T. vulgaris	0 (Control)	0 ^{e}	0 ^{e}
	200	9.55 ^{d}	76.25 ^{c}
	400	100 ^{a}	100 ^{a}
	600	100 ^{a}	100 ^{a}
	800	100 ^{a}	100 ^{a}
	1000	100 ^{a}	100 ^{a}
E. caryophyllata	0 (Control)	0 ^{e}	0 ^{e}
	200	13.5 ^{d}	20.5 ^{d}
	400	100 ^{a}	61.75 ^{c}
	600	100 ^{a}	83.75 ^{b}
	800	100 ^{a}	100 ^{a}
	1000	100 ^{a}	100 ^{a}
C. zeylanicum	0 (Control)	0 ^{e}	0 ^{e}
	200	55.5 ^{c}	0 ^{e}
	400	100 ^{a}	100 ^{a}
	600	100 ^{a}	100 ^{a}
	800	100 ^{a}	100 ^{a}
	1000	100 ^{a}	100 ^{a}
C. copticum	0 (Control)	0 ^{e}	0 ^{e}
	200	80 ^{ab}	69.75 ^{c}
	400	100 ^{a}	100 ^{a}
	600	100 ^{a}	100 ^{a}
	800	100 ^{a}	100 ^{a}
	1000	100 ^{a}	100 ^{a}

Mean values followed by different letters within the column are significantly different according to Duncan Multiple Range Test ($P < 0.01$).

Table 3. Nature of antifungal activities of essential oils on growth of *Botrytis cinerea* and *Monilinia fructicola* by poison food medium method

Essential oil concentration (µl/l)		*Botrytis cinerea*	*Monilinia fructicola*
T. vulgaris	0 (Control)	g	g
	200	g	g
	400	fs	fs
	600	fs	fc
	800	fs	fc
	1000	fc	fc
E. caryophyllata	0 (Control)	g	g
	200	g	g
	400	fs	g
	600	fs	g
	800	fs	fs
	1000	fs	fc
C. zeylanicum	0 (Control)	g	g
	200	g	g
	400	fc	fs
	600	fc	fc
	800	fc	fc
	1000	fc	fc
C. copticum	0 (Control)	g	g
	200	g	g
	400	g	g
	600	fc	fs
	800	fc	fs
	1000	fc	fs

g- Indicates growth of test fungus; fs- Fungistatic activity; fc- Fungicidal activity

Table 4. Effect of essential oils on spore germination of *Botrytis cinerea* under *in vitro* condition

Essential oil concentration (µl/l)		Spore germination inhibition (%)
T. vulgaris	0 (Control)	9.37 [h]
	200	23.5 [g]
	400	92.5 [b]
	600	100 [a]
	800	100 [a]
	1000	100 [a]
E. caryophyllata	0 (Control)	9.75 [h]
	200	100 [a]
	400	100 [a]
	600	100 [a]
	800	100 [a]
	1000	100 [a]

table 4. (continued).

Essential oil concentration (µl/l)		Spore germination inhibition (%)
C. zeylanicum	0 (Control)	9.5 [h]
	200	82.5 [d]
	400	87.5 [c]
	600	100 [a]
	800	100 [a]
	1000	100 [a]
C. copticum	0 (Control)	9.5 [h]
	200	34 [f]
	400	63 [e]
	600	100 [a]
	800	100 [a]
	1000	100 [a]

Mean values followed by different letters within the column are significantly different according to Duncan Multiple Range Test ($P < 0.01$).

Conclusions

In contrast to synthetic fungicides based on single products, essential oils are a complex mixture of compounds, such as terpenes, phenolics and their derivatives which act synergistically within plant as a defense strategy [35]. Hence it is likely that such bioactive essential oils are more durable even against those strains of fungi, which are resistant towards synthetic fungicides. Thus the use of plant essential oils as botanical fungicides would of immense significance in view of the problem of increasing fungi resistant due to indiscriminate use of chemicals. The finding of our study may draw the attention to conduct further experiments regarding large scale exploitation of essential oils as plant based preservatives of horticultural crops in postharvest stage.

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