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Antifungal activity and chemical composition of eucalypt (*Eucalyptus leucoxylon* L.) essential oil

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Abstract

The objective of this study was to investigate the antifungal activity of eucalypt essential oil against some microorganisms during fruit storage of Washington Navel Orange. In this experiment, we used of 0, 200 and 400 µl concentration of eucalypt essential oil as spray and floating for 10 and 20 min on polluted fruits against *Penicillium digitutum* and P. *italicum.* Also, the chemical composition of the eucalypt oil was investigated using thin Gas chromatography and Gas chromatography-mass spectrometry methods. This investigation was performed by the completely random design with three replications.

Essential oil of eucalypt showed antifungal activity against P. *digitutum* in both of spray and floating applications. High inhibitory effect on *P. digitutum* in spray and floating were obtained at 400 and 200 µl for 10 min, respectively. No activity was observed against P. *italicum* in spray method, whereas Antifungal activity was observed in floating (400 µl for 10 min).

The GC and GC-MS screening revealed the presence of 1,8-cineol (43.48%), p-cymene (40.997%), α-Pinene (9.4%), α- phellandrene (1.399%), δ-terpinen (1.53%), 4-terpineol (0.547%) and α- terpineol (0.403%) in eucalypt oil. The total flavonoid and phenolic contents were high, which suggests that the strong antifungal activity of eucalypt may be due to high levels of phenolic and flavonoid compounds.

Key words: Antifungal activity, Eucalypt Essential oil, *Penicillium digitutum, Penicillium italicum*.

INTRODUCTION

In recent years, scientists have focused on the increase of food production needed for the fast expansion of world population. Unfortunately, substantial yield losses occur due to insects and plant diseases caused by fungi, bacteria and viruses (Fletcher et al. [16]; Kordali et al. [21]). Fungi and bacteria have also unfavorable effects on quality, safety and preservation of agricultural products. Synthetic chemicals are widely used in the control of plant diseases. However, these chemicals may cause toxic residues in treated products (Barnard et al. [4]; Isman [18]). Synthetic chemicals can also cause environmental pollution owing to their slow biodegradation (Barnard et al. [4]; Misra and Pavlostathis [25]). In addition, the risk of developing the resistance by microorganisms and the high cost-benefit ratio are other disadvantages of synthetic fungicide usage (Brent and Hollomon, [7]).

The antifungal properties of plant volatile oils and their constituents from a wide variety of plants have been assessed (Lis-Balchin and Deans [22]; Batish [5]; Ceferino et al. [9]) and reviewed (Janssen et al. [19]; Rı´os et al. [28). It is clear from these studies that these secondary plant metabolites have potential uses in medical procedures and applications in the cosmetic, pharmaceutical and food industries (Baratta et al. [2,3]; Iocabellis et al. [17]; Lo Cantore et al. [23]; Youdin et al. [36]). Biological activity of essential oils depends on their chemical composition which is determined by the genotype and influenced by environmental and agronomic conditions (Marotti et al. [24]).

The genus *Eucalyptus* (family Myrtaceae) comprises about 800 species widespread in the tropical and subtropical regions. *Eucalypt* plants are widely used as a medicine herb (Coppen [11]). The antifungal, antibacterial and antimicrobial activity exhibited by *Eucalyptus* genus essential oil has been demonstrated by several researchers (Delaquis et al. [13]; Batish et al. [5]; Cimanga et al. [10]; Fiori et al. [15]; Rota et al. [29]; Beuchat [6]), but unfortunately, there aren't quantitative data (inhibitory effect in laboratory or storage condition) related to the antifungal activity of essential oil.

Therefore, this study was undertaken in order to investigate the effectiveness 'in vivo' of *Eucalyptus leucoxylon* L. essential oils on growth of *Penicillium digitutum* and *P. italicum* pathogens. These results will allow deduction of which components are likely to contribute to the antifungal activity according to GC characterization of essential oils and determination of any relationships between the components and their antifungal activity.

MATERIALS AND METHODS

Plant material and oil isolation

Eucalypt (*Eucalyptus leucoxylon* L.) plants were obtained from the college of Agricultural at Shiraz University at Badjgah (29º 36' N, 52º 32' W, 400 mm annually Rain fall and 1810 m above sea level) in the region of Fars province (Iran).

One hundred of dried aerial parts (leaves) of plants were waterdistilled for three hours using a Clevenger-type apparatus, to produce oil according to the method recommended by the European Pharmacopoeia. Essential oils were dried with anhydrous sodium sulfate and kept in dark vials at 4 ºC until chromatographic analysis.

Gas chromatography

Samples of 0.1 μL were subjected to analysis by capillary gas chromatography. A Hewlett-Packard 5890 gas chromatograph (GC) (Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and a 30 m × 0.25 mm HP-5 (cross-linked Phenyl–Methyl Siloxane) column with 0.25 μm film thickness (Hewlett-Packard, Palo Alto, CA, USA), was used for this study. The FID and the injector were maintained at 280 ºC and 250 ºC, respectively.

Helium was used as carrier gas, the flow through the column was 1 mL/min, and the split ratio was set to 100:1. The column was maintained at 60 ºC for 4 min, increased to 64 ºC at a rate of 1 ºC/min, then increased to 155 ºC at a rate of 2.5 ºC/min and finally raised from 155 ºC to 250 ºC at a rate of 5 ºC/min. For the identification of the compounds, retention times and retention index were confirmed with commercially available standard compounds (Acros Organics BVBA/ SPRL, Fisher Scientific S.A. and Sigma Aldrich Quı´mica S.A.).

Mass spectrometry analysis

Gas chromatography–mass spectrometry (GC–MS) was used for the identification of volatile components in thyme essential oil. For this portion of the work, a Hewlett-Packard 5890 Series II Plus gas chromatograph (GC), equipped with a 30 m × 0.25 mm HP-5 column with 0.25 μm film thickness was used. The GC was linked to a Hewlett-Packard Model 5972 mass spectrometry detector. The chromatographic conditions were identical to those used for gas chromatography analysis.

Qualitative and quantitative analysis

The individual peaks were identified by retention times and retention index (relative to C6–C17 n-alkanes), compared with those of known compounds, and by comparison of mass spectra using the NBS75K library (United States, National Bureau of Standards, 1986) and spectra obtained from the standard, which were tentatively identified considering the NBS75K library spectra and their corresponding retention index.

Percentage compositions of samples were calculated according to the area of the chromatographic peaks. Samples were analyzed according to a previously developed methodology (Stenhagen et al. [33]; Adams [1]; Shibamoto [31]).

Suspension preparing

At first, *Penicillium digitutum* and P. *italicum* separately in medium PDA (potato dextrose agar) for four days had been cultivated. By sterile lob, spores

Table 1. Chemical composition of *Eucalyptus leucoxylon* L. essential oil.

No.	Compound	Retention index (RI)	Amount (%)
1	α-Pinene	942.25	9.4
2	β- Pinene	985.66	0.04
3	Myrcene	989.92	0.054
4	α- phellandrene	1010.2	1.399
5	p-cymene	1033.6	40.997
6	1,8-cineol	1044.9	43.48
7	δ-terpinen	1063.3	1.53
8	α-terpinolene	1093.6	0.389
9	Isoamylisovalerate	1101.4	0.086
10	4-terpineol	1184.4	0.547
11	α- terpineol	1195.8	0.403
12	Piperitone	1261.0	0.022
13	Thymol	1287.7	0.15
14	Carvacrol	1299.3	0.42
15	α-Gurjunene	1427.6	0.088
16	Aromadendrene	1459.1	0.266
17	allo Aromadendrene	1481.1	0.087
18	Ledene	1512.8	0.057
19	Epiglobulol	1580.6	0.106
20	Globulol	1607.1	0.33
21	Veridiflorol	1615.9	0.108
22	β-Eudesmol	1644.2	0.023

at petridish were moved in to 100 ml sterile distillated water. For separating spore from each other and spreading them in to water, was added 2-3 drops of mixed NPX. After 5 min, it's located on shaker set. Number of cells in one milliliter of suspension was determined by hemacytometer. From present suspension, 105 (spore/ml) densities were prepared.

Preparing of fruits and antifungal activity assay

Orange fruits (Washington Navel type) were harvested from commercial garden located in Darab (Fars Province, Iran) and immediately move to laboratory. The fruits were selected free of wounds and rots and as much as possible homogeneous in physiological maturity stage and size. Based on Karimi [20] research, fruits washed with distilled water and surface-disinfected by spray of dilute solution of ethanol (70%). Surface of fruits were dried by below in lab. Four points on the fruits hole by sterile nail in 2 mm depth. One milliliter of *Penicillium digitutum* and P. *italicum* suspension injected in hole and then fruits kept during 4 hr for establishing of fungus spore in holes.

In this experiment, we used o, 200 and 400 µl of *Eucalyptus leucoxylon* L. essential oil as two method of application: 1) Spray on the polluted fruits and 2) floating of polluted fruits for 10 and 20 min in essential oil solution. After apply of treatments, the fruits kept in plastic bag and storage in 11 ºC for one month. Data recording performed every two weeks and fruits were removing from bags. For providing of various concentrations of essential oil, we used 2 ml of tween 80 as dissolver. Control fruits were treated with distillated water contains same dissolver.

Statistical analysis

The experiment was arranged as a completely randomized design (CRD) with three replications for each treatment. Each replication included 4 fruits. Each fruit include 4 holes. All analyses were performed with a statistical software package (SPSS version 13) and the means were compared by Duncan's multiple Range test at 5% level of probability.

RESULTS AND DISCUSSION

Chemical compositions of essential oils

The components identified in the essential oil are shown in Table 1 in order of their experimental retention times and retention index. About 22 compounds, representing 99.982% of the oil, were identified by GC and GC/MS analyses. As seen, major volatile components of the aerial parts of E. leucoxylon L. oil were 1,8-cineol (43.48%), p-cymene (40.997%), α-Pinene (9.4%), α-phellandrene (1.399%), δ-terpinen (1.53%), 4-terpineol (0.547%) and α- terpineol (0.403%).

These results are in agreement with those published by Yang et al. [35]; Ceferino et al. [9]; Sartorelli et al. [30], who reported that the major components of eucalypt essential oil are 1,8-cineol, p-cymene and α-Pinene. But, these results are different in comparison with Delaquis et al. [13], who reported that the major components of eucalypt essential oil are piperitone, terpinen-4-ol and a-terpinolene.

Antifungal activity

Penicillium digitatum

The results of antifungal activity of eucalypt essential oil against the *Penicillium digitatum* are presented in Table 2. Essential oil of eucalypt showed antifungal activity against P. *digitutum* in both of spray and floating applications. From these data, it emerges that method of essential oil application is important. So, floating method was significantly better than spray method on growth reduction of green mould. The spray application of various concentration of eucalypt essential oil had low effect on control of orange fruit green mould. Application of 400 µl resulted in decrease of green mould growth in comparison with control.

Floating method had maximum inhibitory effect on P. *digitatum*. High inhibitory effect of eucalypt oil was obtained in 200 µl for 10 min (31.5%) and no significant difference in comparison with 400 µl for 10 min (33.5%). these treatments had significant different with control and others (P≤0.05).

Table 2. Effect of *eucalypt* essential oil on P. *digitatum* growth on the surface of orange fruit

Method	Percentage	
spray		
0		100 a
200		93.33 ab
400		80 b
Floating		
Time	con*.	
0	10	83 b
0	20	100 a
10	200	31.5 c
10	400	33.5 c
20	200	100 a
20	400	95.83 ab

*. Concentration

Table 3. Effect of *eucalypt* essential oil on P. *italicum* growth on the surface of orange fruit

Method	Percentage	
spray		
0		91.67 ab
200		91.67 ab
400		91.67 ab
Floating		
Time	con*.	
0	10	83.33 b
0	20	100 a
10	200	91.67 ab
10	400	83.33 b
20	200	100 a
20	400	100 a

*. Concentration

Penicillium italicum

The results obtained in assays of antifungal activity (spray and floating methods) of the *eucalypt* oil on *Penicillium italicum* are shown in table 3. No activity was observed against P. *italicum* in spray method, whereas Antifungal activity was observed in floating (400 µl for 10 min). Spray of various concentration of eucalypt oil was not significant difference in comparison control at 5% level.

The strong antifungal activity of *eucalypt* could be due to their high content of phenolic (and terpene) compounds have been reported in different reviews (Beuchat [6]; Davidson [12]; Nychas [27]; Burt [8]; Nguefack, et al. [26]; Singh et al. [32]). Our results are in agreement with those reported by Faid et al. [14] and Yahyazadeh et al. [34], in where it has been established that a relationship exists between the high activity of the oils and presence of phenolic components. According to Faid et al. [14] antimicrobial activity of major oil compounds is in the order: phenols (highest activity)> alcohols> aldehydes> Ketones> ethers> hydrocarbons.

In our opinion major components of eucalypt [1,8-cineol (43.48%), p-cymene (40.997%), α-Pinene (9.4%), α-phellandrene (1.399%), δ-terpinen (1.53%), 4-terpineol (0.547%) and α-terpineol (0.403%)] have key roles in their antifungal activities. However, the mechanisms of action of these compounds have not been completely elucidated.

In this experiment, we observed that methods of essential oil application are effective. So, floating method was more effective than spray for control of P. *italicum.* But two mentioned method were effective in P. *digitatum* control.

Also, fungus strain is important for effectiveness of eucalypt essential oil. Low inhibitory effect of eucalypt oil on blue mould may be due to different fungus strain.

The results of present and previous researches about secondary metabolites using for fruit post harvest rots control show that these compound have high potential in biological control. These compounds have synthetic fungicides effect but no destroyer effect on human and environment. Therefore, identification of natural compounds includes fungicide and bactericide effects are necessary.

In conclusion, the present results show that hydrodistillation oils of eucalypt have antifungal activity. We can suggest application of eucalypt oil as floating method (200 and 400 µl for 10 min) for reduce of P. *digitatum* growth on Washington Navel orange under storage condition.

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