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Chemical Composition of Essential Oils of Iranian *Pimpinella anisum* L. and *Foeniculum vulgare* Miller and their Antifungal Activity Against Postharvest Pathogens

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Abstract: Increasing attentions have been paid on the application of essential oils and plant extracts for control of postharvest pathogens due to their natural origin and less appearance of resistance in fungi pathogens. In this study, in addition to the chemical analysis of Iranian anise and fennel essential oils, their fungicidal and/or fungistatic effects on two postharvest pathogens were investigated. Essential oils were extracted by means of hydro-distillation and afterwards GC-MS analysis was performed to identify their components. The main constituents were E-anethol (92.9 %), p-alylanisol (2.2 %), Z-α-biosabolene (1.8 %) for anise oil and E-anethol (71.2 %), limonene (8.2 %), Fenchone (8.53 %), Methylchavicol (7.01 %) for fennel oil. Both essential oils exhibited fungistatic effect on *Aspegillus niger* but fennel was more effective. Satisfactory fungistatic effects against *Rhizopus stolinifer*, a stubborn pathogen, were observed for fennel oil. Anise oil did not show significant inhibitory effect on *R. stolinifer* in used concentrations. It was shown that presence of E-anethol as the major component of both oils is not responsible for antifungal activity and especial antifungal activities arise from other reasons such as interaction of components or complexity of the oils.

Keywords: Antifungal activity; essential oil; *Foeniculum vulgare*; *Pimpinella anisum*; E-anethol.

Introduction: Fungi are significant destroyers of food stuffs during storage, rendering them unfit for human consumption by retarding their nutritive value and sometime by producing mycotoxins [1,2]. Storage fungi are commonly controlled by synthetic chemicals. However, these agents will be phased out in the near future due to their potential adverse impact on the environment [3]. Furthermore, the use of fungicides is more harmful in the post

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harvest period because of the short time between treatment and consumption. Some fungi have shown resistance against broad spectrum fungicides, such as Benzimidazoles, Imazalil and Prochloraz due to repeated usage and some of them such as *Mucor* and *Rhizopus* are not sensitive and need especial fungicides to be controlled [4, 5]. Benzimidazoles, the widest applied fungicides in postharvest period, have no effect on Dematiaceous fungi (dark spore fungi), Oomysets, *Mucor* and *Rhizopus* (two later are major postharvest pathogens of numerous products). In addition, Imazalil and Prochloraz are ineffective against *R. Stolonifer* [6].

Markets are desperately looking for new products with fewer residues in order to comply with the food safety standards. Various industries are now looking into sources of alternative, more natural and environmentally friendly antimicrobials, antibiotics, antioxidants and crop protection agents [7]. Therefore, biodegradable alternatives should be developed worldwide for reducing postharvest losses [2]. Vigorous plant tissues contain many natural antifungal compounds, and these compounds defend plants against disease. Plants exhibit non-host resistance against most of potential pathogens by producing these compounds [8]. Selected plants and their essential oils have been evaluated as natural sources for controlling storage fungi [1]. Essential oils are volatile materials containing complex mixture of compounds. The constituents of the oils are mainly monoterpenes, sesquiterpines and phenylpropanoids [9, 10]. Acquired resistance is to be less arising from complex nature of the oils and different antifungal components existing in extracted oils [10]. Generally, their action is resulted from combined effect of both their active and inactive compounds. Inactive compounds might influence resorption, rate of reactions and bioavailability of the active compounds [7, 10].

Anise (*Pimpinella anisum* L.), a grassy annual plant with white flower and small green to yellow seed grows in Iran, Turkey, India, Egypt, and other worm regions throughout the word [11]. According to the study by Askari *et al.*[12] anise seed contains 1.5-3.5 % mass of volatile oil consisting primarily of *trans*-anethole and *cis*-anethole. The major component of anise, *trans*-anethole, is largely used as a substrate for synthesis of various pharmaceutical substances [13]. Anise and specifically its essential oil have been used in Iranian folklore medicine for many years [11]. Volatile compounds of the essential oil obtained from seeds have exhibited *in vitro* activity against *Saccharomyces cerevisiae* and some clinical yeast isolates [9, 13]. To our knowledge, no report has been published on application of anise essential oil as a controlling agent of plant pathogenic fungi so far.

Fennel (*Foeniculum vulgare* Miller) is an annual, biennial or perennial plant, depending on the variety, belonging to Apiaceae family and is native to the Mediterranean area. Fennel has been known, since antiquity, as a medicinal and aromatic herb, commonly used to flavor liqueurs, breads, fishes, salads and cheeses [14]. Some reports have been published on anti-fungal activity of fennel against some plant pathogenic fungi [15, 16, 17].

The aim of the present study was to determine the chemical composition of the essential oils obtained from seeds of *P. anisum* and *F. vulgare* by GC-MS analysis and evaluate their eventual antifungal activity on two important postharvest fungi, *Aspergillus niger* and *Rhizopus stolinifer*.

Materials and methods

Extraction of essential oils: Seeds of *Pimpinella anisum* and *Foeniculum vulgare*

prepared from medical plant and drugs research institute (Shahid Beheshti University, Tehran, Iran) in summer 2007 and powdered by blender. Cultivation conditions were selected as Omidbeygi [18]. The essential oils were isolated by means of hydrodistillation, using Clevenger-type apparatus. The extraction procedure was done for 3 h. Distilled oils were dried by anhydrous sodium sulfate, poured in opaque vials and stored at 4°C till GC-MS analysis. Three replications with 50g of each sample were performed and essential oil yields were investigated.

GC analysis: GC analysis performed with a Thermoquest gas chromatograph with a flame ionization detector (FID). Analysis carried out using fused silica capillary DB-1 column (60 m × 0.25 mm i.d.; film thickness 0.25 μm). Operating conditions were as follows: injector and detector temperatures were 250°C and 300°C, respectively. Nitrogen was used as carrier gas at a flow rate of 1 ml/min; oven temperature programmed, 60°C-250°C at the rate of 5°C/min, and finally held isothermally for 10 min.[19].

GC-MS analysis: GC-MS analysis was also performed by using Thermoquest-Finnigan gas chromatograph equipped with column described above and coupled with a TRACE mass quadrupole detector. Helium was used as carrier gas with ionization voltage of 70 ev. Ion source and interface temperatures were 200°C and 250°C, respectively. Mass range was from *m/z* 43-456. Gas chromatographic conditions were as given for GC.

Identification of compounds: The retention indices were calculated for all volatile constituents using a homologous series of *n-alkanes* C_6 - C_{24}. Identification of individual compounds carried out by comparison of their mass spectra with those of similar compounds from a database (Wiely/NBS library) or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those of reported in the literature [15]. For quantification purpose, relative area percentages obtained by FID, were used without using correction factors.

Fungal strains and media: Strains of postharvest fungi, *A. niger* and *R. stolinifer,* were isolated from strawberry store. Cultures of the organisms were maintained on potato dextrose agar (PDA) medium.

Toxicity study: The toxicity of the essential oils against tested fungi was studied by poisonous medium technique [2] using potato dextrose agar medium. Six concentrations (0, 125, 250, 500, 100 and 2000 μL /L) were mixed with sterile molten PDA (cooled to 40°C), also a surfactant (Tween 80) was added (0.02 % v/v) to facilitate dispersion oil. Two control samples including a plate containing the medium without any surfactant and essential oil content and the other with surfactant were also prepared. The PDA with added oils was then poured into 9 cm Petri dishes. Thiabendazol fungicide used, regarding that this fungicide haven't effect on *R. stolinifer* [5], in the same conditions to make comparison with oils. For inoculation, mycelium was taken from the periphery of 4-day old stock cultures. Plugs of mycelium were removed with a 7 mm diameter cork borer, inverted and placed in the center of each Petri dish. Plates sealed by parafilm to prevent realize of volatile compounds. Four

replicate plates were sited up for each concentration and plates were incubated in the dark at 27°C. According to Cakir *et al*.[4] Growth inhibition of treatment against control was calculated by percentage, using the following formula:

$$\%\text{Inhibition} = \frac{C - T}{C} \times 100$$

Where C is an average of 3 replicates of hyphal extension (mm) of controls and T is an average of 3 replicates of hyphal extension (mm) of plates treated with essential oils. For detection of fungistatic or fungitoxic effect in which oil inhibited, fungal disc were reinoculated onto the fresh medium and revival of fungal growth was recorded in 27°C after 10 day.

Data analysis: Data were analyzed for significance by analysis of variance, followed by the student's *t*-test (α = 0.001, 0.01, 0.05), with SAS 9.1 software (SAS institute, Cary, NC). Probit analysis was used to measure EC 50 and MIC with SPSS 9. MIC assumed as minimum level of essential oil concentration with 95 % reduction of the fungi growth [7].

Result and Discussion: Isolated essential oil of anise had pale yellow in color and its yield (v/w) was 3.3 %. This yield was similar to other Iranian reports, but significantly more than of other countries [12]. According to Zehtab-Salmani *et al*.[20] environmental and cultural factors such as light, date of sowing and irrigation, increase essential oil yield and amount of anethole in this plant. Essential oil yield from fennel seeds was 2.6 % (v/w).

The GC and GC-MS analyses of the essential oil of *P. anisum* permitted the identification of 17 principal constituents making a total of 99.9 % of the oil (Tables 1). E-*anethol* (92.9 %), p-Allylanisole (2.2 %) and Z-α-bisabolene (1.8 %) were among the main ones. These results were different from the chemical composition previously reported for this species. Rodrigues *et al*.[21] reported that methylchavicol and eugenol were the major component of this oil. These compounds were not detected in our study, while E-anethol existed at higher concentrations comparing with other reports [12]. So it could be prospected that Iranian cultivars would be excellent sources of anethole.

Carvone and *cis*-dihydrocarvone were among detected components in Iranian anise oil confirming previous reports published in Iran [12]. These compounds are rare in literatures have been marked on anise oil analysis [21]. Yamini *et al*.[22] showed that Iranian fennel composition is also different from others possessing much higher amounts of *trans*-anetole. Major component of Fennel seed essential oil observed in our study were E-anethole (71.2 %), Limonene (8.2 %), Fenchone (8.53 %) and Methylchavicol (7.01 %) (Tables 1) confirming different composition of this type of fennel [14, 15, 22].

Results obtained by *in vitro* studies showed that at concentration of 2000 µL/L, the radial growth of *A. niger* was completely inhibited by anise essential oil, whereas for *R. stolinifer* relative fungistatic effect observed with 65.3 % of growth inhibition (figures 1. and 2). Fungistatic effect of anise on *A. niger* had MIC of 2000 µL/L and EC 50 = 400 µL/L, MIC was more than 2000 µL/L with EC 50 = 1517 µL/L for *R. stolinifer*. Fennel essential oil exhibited satisfactory inhibitory effect against *A. niger* with MIC = 1388 µL/L and EC 50 =5 30 µL/L. *R. stolinifer* showed similar *A. niger* susceptibility (MIC = 1256 µL/L and EC 50 = 487 µL/L).

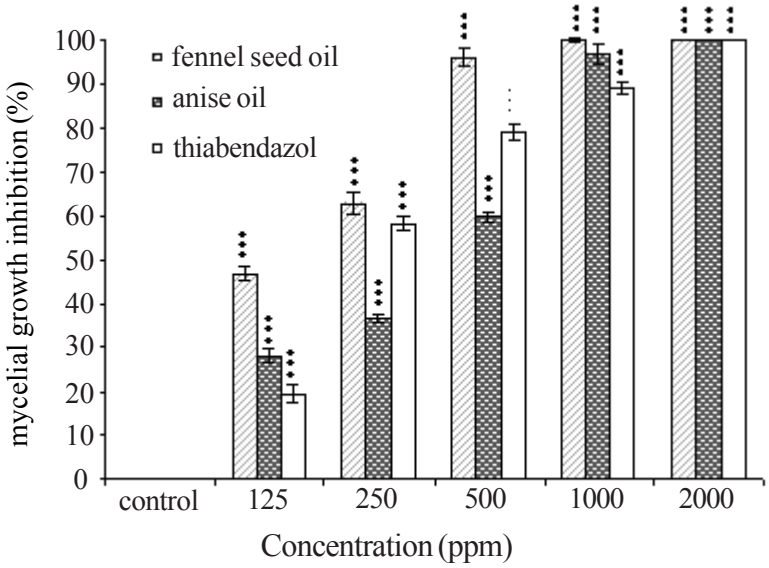

Fig. 1. Effects of various concentration (0-2000 ppm) of fennel seed and anise essential oils and Thiabendazol on mycelial growth inhibition of *Aspergillus niger***.** Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as *P< 0.05; ** P< 0.01; *** P< 0.001.

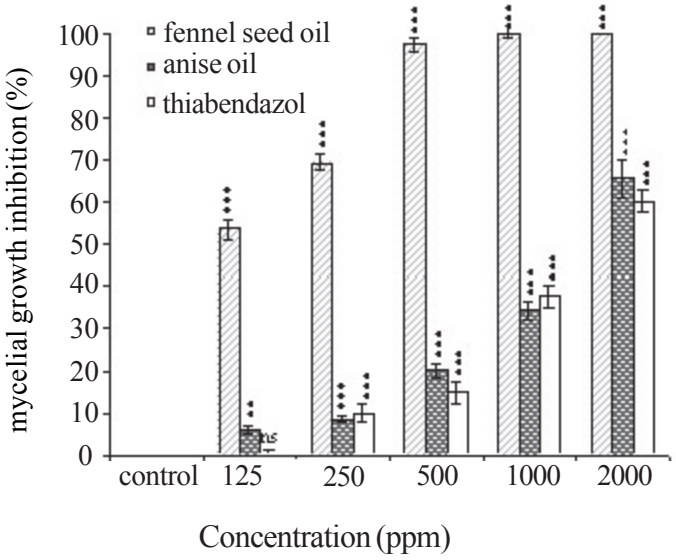

Fig. 2. Effects of various concentration (0-2000 ppm) of fennel seed and anise essential oils and Thiabendazol on mycelial growth inhibition of *Rhizopus stolinifer*. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as *P< 0.05; ** P< 0.01; *** P< 0.001.

Thiabendazol fungicide did not show fungicidal activity against *R. stolinifer*. It should be mentioned that *R. stolinifer* is a resistant fungi and many of broad-spectrum chemical fungicides do not have satisfactory effect on this fungi [5]. However, some reports have been published on excellent inhibition with some essential oils such as *Dennetia tripetala* [23], *Cympopogon citrates* [24] and *Thymus vulgaris* [25].

Regarding to the similarity observed in chemical composition, it was expected that antifungal activity would be alike, if *trans*-anethole was the effective ingredient. Vice versa, anise oil did not show desirable antifungal activity, at least for *R. stolonifer*, despite having high amounts of *trans*-anethole. It could be concluded that strong fungicidal activity of fennel should be arise from other ingredients; however synergistic effects of its components should be considered [7]. Also different sensitivity of fungi can be related to action mechanism of compounds and activity of applied oils.

Methylchavicol, one of major components of fennel, and E-anethole, major component of both of fennel and anise, are phenylpropanoids and act in the fermentative stages of fungi as antifungal agents with a similar effect [9]. It is well known that the phenolic components of essential oils show the strongest antimicrobial activity, followed by aldehydes, ketones, and alcohols [15]. It is shown that in conditions with the lack of oxygen, E-anetole has a significant fungicidal effect against fungi growing. Regardless of aeration, *rho* mutants, which are possessing fermentative activity even in presence of oxygen, are sensitive against Anethole [9]. Anethole does not have fungicidal effect of resting spores. Alike storage conditions presented work performed at aerobic status and it could be proposed that E-anethole could be evaluated under anaerobic conditions to make comparsions.

Presumptively resistance of *R. stolonifer* against anise essential oil and Thiabendazol was alike. Such resistance was not observed for essential oil of fennel probably because of a different mode of action or active compounds. It should be mentioned that good antifungal activity of fennel oil is to be deduced from components other than E-anethole or unknown mechanism arising from fennel composition. This can be the object of other studies to identify and find the reasons. According to the former publications, fennel oil has a wide range of antifungal activity and is able to control different storage [16], soil borne [15] and air born fungi [17].

Conclusion: During past time, frequent applications and higher doses of fungicides have been used. However, this can cause negative effects on the environment and food safety and also resistances may be appeared for pathogenic fungi. So substitution of these materials with more safe and effective substances especially with natural derivatives, which have a complex composition resulting lower probability of fungi resistance and originated from nature resulting more safe properties, should be considered seriously.

Anise and fennel are commercially cultivated in Iran and have been used in medicinal applications. Therefore, it could be inferred that anise and fennel do not have toxicity for human and their application as antifungal agents in storage period seems not to be adverse. Essential oil of fennel had fungistatic effect against both of fungi in MIC concentration. This oil is to be an effective plant originated toxicant for postharvest fungi, and is potentially suitable for protection of fresh fruits facing storage fungi. Also because of its effective

action on both examined fungi and other published data, essential oil of fennel can be considered as a potential, broad spectrum and safe substitute of chemical agents.

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Table 1. Composition of the essential oil isolated from *Pimpinella anisum* and *Foeniculum vulgare*

Pimpinella anisum			*Foeniculum vulgare*		
Compound	**RI[a]**	**%**	**Compound**	**RI[a]**	**%**
α-Phellandrene	1001	-	α-Thujene	924	0.1
Limonene	1025	0.8	α-Pinene	931	0.9
Geyrene	1140	-	Camphene	943	0.1
p-Allylanisole	1180	2.2	Sabinene	965	0.2
cis-Dihydrocarvone	1185	0.1	β-Pinene	972	0.2
Carvone	1221	0.8	Myrcene	978	0.5
p-Anisaldehyde	1229	0.1	p-Cymene	1013	0.1
Z-Anethole	1233	0.1	Limonene	1024	8.2
E-Anethole	1281	92.9	(Z)-β-Ocimene	1031	0.9
δ-Element	1338	0.1	γ-Terpinene	1050	0.6
(Z)-β-Farnesene	1446	-	Fenchone	1073	8.5
Aromadendrene	1454	0.1	Camphor	1125	0.1
ar-Curcumene	1473	0.2	Methylchavicol	1178	7.0
(Z)-α-Bisabolene	1483	1.8	Fenchyl acetate	1230	0.4
Zingiberene	1489	0.4	Z-Anethole	1243	0.3
β-Bisabolene	1506	0.2	E-Anethole	1269	71.2
β-Sesquiphellandrene	1521	0.1			
Total		99.9			99.7

[a]RI= retention indices relative to C_6–C_{24} *n-alkanes* on the DB-1 column (Cakir *et al*., 2005)