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ORIGINAL ARTICLE

Antifungal Activities of the Essential Oils on Post-harvest Disease Agent *Aspergilus flavus*

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ABSTRACT

Antifungal activities of the essential oils obtained from *Hyssopus officinalis, Cuminum cyminum, Thymus vulgaris* and cones of *cupressus arizonica* were investigated against *Aspergilus flavus*. Different concentrations of the essential oils on conidial germination and germ tube elongation were determined *in-vitro*. Essential oils applied in 5 levels, included 0 (as control), 0.125, 0.25, 0.375 and 0.5 %. The antifungal activities of the these essential oils were evaluated by disc diffusion method on PDA medium. The results showed that the essential oil of all plants affected the growth of *Aspergillus flavus* under *in-vitro* conditions. *Aspergillus flavus* sopres in control treatment filled the petridish medium on second day of experiment, but essential oils treatments in some levels inhibited and in others decreased the growth of *Aspergillus flavus*. GC–MS analysis of the chemical composition of essential oils were investigated to determine their different component. Data showed essential oil of *Cuminum cyminum* was more effective in comparison whit others. Furthermore, the study suggests that these essential oils can be used as preservatives in foods.

Key words: Essential oils, Antifungal activity, *Aspergilus*

Introdution

Fungi are significant destroyers of foodstuffs during storage, rendering them unfit for human consumption by retarding their nutritive value and sometimes by producing mycotoxins.

In spite of the introduction of new antifungal drugs, they are limited in number. The increase of fungal resistance to classical drugs, the treatment costs, and the fact that most available antifungal drugs have only fungistatic activity, justify the search for new strategies [1]. Aromatic plants have been widely used in folk medicine. It is known that most of their properties are due to their volatile oils. They have been empirically used as antimicrobial agents [2], but only limited information exists of action are still unknown. According to our preliminary results [3,4], some essential oils show an important antifungal activity against yeasts, dermatophyte fungi and Aspergillus strains, which could predict therapeutic benefits, mainly for diseases with mucosal, cutaneous and respiratory tract involvement.

Essential oils as antimicrobial agents present two main characters: their natural origin generally means more safety to people and environment; and they can be considered at low risk for development of microbial resistance since they are mixtures of compounds which may present different mechanisms of antimicrobial activity [17,18]. Application of essential oil is a very attractive method for controlling postharvest diseases. Production of essential oils by plants is believed to be

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predominantly a defense mechanism against pathogens and pests and indeed, essential oils have been shown to possess antimicrobial and antifungal properties [5]. Essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers and their exploitation for potential multi-purpose functional use [6].

Essential oils are made up of many different volatile compounds and the composition of the oil quite often varies between species [7]. It seems that the antifungal and antimicrobial effects are the result of many compounds acting synergistically [8]. There would be negligible chance of development of resistant races of fungi after application of essential oils to fruit and vegetables. As a consequence essential oils are one of the most promising candidate groups of natural compounds for the development of safer antifungal agents [9].

Aflatoxins have also been identified as a potential biological weapon for food and water contamination [13]. Various agricultural commodities have been found to be contaminated with either aflatoxin producing fungi or aflatoxins [14]. Aflatoxins are secondary metabolites produced by *Aspergillus flavus, A. parasiticus, A. nomius, A. tamarii* and *A. bombycis* [10]. These toxins are acutely and chronically toxic to both humans and animals [11] and they are among the most potent mutagenic and carcinogenic compounds known to be produced in nature [12].

Aspergillosis generally rises as a respiratory disease characterized for the presence of granulomatous lesions in lungs or bronchi, followed by dissemination to other organs throughout the bloodstream [15,16]. The incidence of aflatoxin contamination in tree nuts is low, but aflatoxin levels can be quite variable and high levels can develop in a small percentage of nuts [1920]. The drupaceous pistachio fruit consists of an edible kernel (seed) and a seed coat (testa) encased in a hardened shell (endocarp), all of which are surrounded by a fleshy hull (mesocarp and epicarp) which is removed during processing [24]. A single pistachio with an aflatoxin concentration of 60,000 ppb can contaminate an otherwise aflatoxin-free 10-lb (ca. 4.5-kg) test lot (approximately 3,000 nuts) at the U.S. Food and Drug Administration action level of 20 ppb. Thus, it is of interest to determine why certain nuts are susceptible to developing high levels of aflatoxin. it has been estimated that only 1 in 28,250 walnuts [21], 1 in 26,500 almonds [22], and 1 in 25,000 pistachios [23] develop high levels of aflatoxin contamination. The pistachio shell splits as the nut matures, a desirable feature for marketing and consumption; however, the hull remains intact in the majority of mature pistachios at harvest [25].

Aspergilus flavus is a weak plant pathogen (a wound pathogen), and aflatoxin is rarely detected in pistachio kernels with intact hulls [26]. In a small percentage (1 to 5%) of pistachios, the shell and the still adhering hull split together [27].

The aim of the present investigation was to find a suitable antifungal agent, capable of inhibiting pathogens which cause vaginitis. Essential oils are not as broad spectrum as synthetic pesticides, but their effectiveness can be improved by using them in conjunction with carefully designed packaging. The presence of free moisture in a package provides the ideal environment for the growth of many post-harvest pathogens. The aim of this study was to assess the antifungal activity of *Hyssopus officinalis, Cuminum cyminum, Thymus vulgaris* and cones of *cupressus arizonica* essential oils against *Aspergillus flavus*.

Materials and methods

Plant material and hydrodistillation

Air dried aerial parts of *Hyssopus officinalis, Cuminum cyminum, Thymus vulgaris* and cones of *cupressus arizonica* were hydrodistilled for 3 h using a Clevenger-type apparatus. these plants were collected from the garden of Horticultural Department of Ferdowsi University of Mashhad (Iran) (November 2006). The air-dried aerial parts (50 g) were hydro distilled in a Clevenger apparatus (sigma chemical company) for 5 h. in accordance with the British pharmacopoeia. The aqueous phase was extracted with dichloromethane (Qualigens) (3 x 50 mL). The organic phase was dried with sodium sulphate (Bio-RAD), filtered and the solvent evaporated until dryness by air-dry. These all oils of above plants were screened for their antimycotic activity.

Assay of antifungal activity

Antifungal activity of the essential oil was evaluated. The test was performed in sterile Petri dishes (90 mm diameter) containing potato-dextrose-agar (PDA) medium (20 mL).

Different concentrations: 0.125% , 0.25% 0.375% and 0.5% of essential oils first added to 2.5 CC Twin 20 and then mixed with water . 2.5 CC Twine 20-20 used as control with no essential oils. The fungus (*Aspergilus flavus)* has been isolated from rotted and injured fruits and purified for several times.

These concentrations added to PDA medium .All tests were performed in triplicate. Amycelial disc (5-mm diameter) taken from the 7-day-old culture of the test fungus was inoculated to each Petri dish. Plates containing nonpoisoned medium served as control. Fungal colony surface, in control as well as in treatment sets, were recorded after incubation for 7

days at 25 ± 2°C.

Gas chromatography-mass spectrometry

A mass spectrometer whit an ion trap detector in full scan mode under electron impact ionization (70 e V) used. The chromatographic column used for the analysis was HP-5 capillary column (30m × 0.25mm, film thickness 0.25µm). the carrier gas was helium, at a flow rate of 1 mL min^{-1} . the injection were performed in split less mode at 230°C. one micro litter of essential oil in hexan (HPLC grade) injected an analyzed whit the column held initially at 60 °C min^{-1} heating ramp and subsequently kept at 260 °C for 13 min. the relative percentages amounts of separated compounds calculated from total ion chromatograms by computerized integrator.

Results

Antifungal activity of *Hyssopus officinalis, cupressus arizonica, Cuminum cyminum* and *Thymus vulgaris* were determined against *Aspergillus flavus*. These essential oils were tested by agar diffusion plate method caused significant reduction in the growth of *Aspergillus flavus*. The rate of growth reduction was directly proportional to the concentration of tested material in the medium. In Table.1disc surface were shown in 2nd day after inoculation.

The oil of *Cuminum cyminum* strongly inhibited the growth of *A.flavus* in all concentrations in 2nd day. In *Hyssopus officinalis* and *cupressus arizonica* only high concentrations suppressed this growth. In Table 2. Disc surface of *A.flavus* in 2nd, 4th and 6th day showed same trend as Table 1. The highest concentration of *Cuminum cyminum* suppressed *A.flavus* in all days. Essential oils of *Hyssopus officinalis, Thymus vulgaris* and *cupressus arizonica* respectively inhibited the growth of *A.flavus*. when the concentration rose the growth plunged significantly. Fig.1-4 show disc growth of *A.flavus* in 6th day. The thing that is clear is inhibitory property of *Cuminum cyminum* essential oil in comparison with others. In sum up all essential oils in all concentrations inhibit the growth of *A.flavus* while in the control with no essential oil all petri dish surface filled in 2nd day (Table.1).

All petri dish surface in control whit no essential oil filled in this stage. Results showed that *Hyssopus officinalis* oil can't suppress mycelium growth in all concentrations as others.*Cuminum cyminum* oil possesses a remarkable antifungal activity against *Aspergillus flavus*.

Results of GC-MS analyses of *Hyssopus officinalis, Cuminum cyminum,Thymus vulgaris* and cones of *Cupressus arizonica* in tables 3-6 indicates there are many different components in all essential oils. In our studies, only those components which were present in the oils in amounts higher than 0.1% have been taken into consideration. All oils tested exhibited different degrees of antifungal against *Aspergillus flavus*.

The essential oil from *Hyssopus officinalis* differed markedly from distillated,*Thymus vulgaris, Cuminum cyminum* and cones of *Cupressus arizonica*. On the contrary, *Cuminum cyminum* oil p-mentha-1,4-dien-7-al (27.4%) was the dominant compound next to -terpinene (12.8%). Moreover, constituents such as: o-cymene, p-cymene, limonene, β-pinene and... were found in higher amounts. in *Cupressus arizonica*, α-pinene (69.0%) and in *Hyssopus officinalis* cis-pinocamphone (44.7%) and trans-pinocamphone (14.1%) and in *Thymus vulgaris* thymol (18.12%) and carvacrol (12.11%) were dominant components.

Discussion

The traditional use of plants as medicines provide the basis for indicating which essential oils and plant oils may be useful for specific medical conditions. It is important to investigate scientifically those plants which have been used in traditional medicines as potential sources of novel antimicrobial compounds [32].

Soliman & Badea [28] reported completeinhibition of *A. flavus, A. parasiticus* and *A. ochraceus* by cinnamon oil. It has been reported that the inhibition of mould mycelial growth (fungistatic or fungicidal effect) in solid or liquid medium by essential oils over a wide range of concentrations has been accompanied by concomitant decease or total inhibition of mycotoxins production (e.g. by *Aspergillus parasiticus, A. ochraceus, Fusarium graminearum, F.proliferatum*) [29]. Although, the antimicrobial activity of an essential oilis attributed mainly to its major components, the synergistic or antagonistic effect of compounds in minor percentage in the mixture has to be considered [30]. Rasooli and Owlia [31] observed that thyme oils provided irreversible damage to cell wall (degenerative changes), cytoplasm membrane (irregular, dissociated from cell wall, invaginated) and nuclear membrane (folding) of *Aspergillus parasiticus*.

When comparing data obtained in different studies most publications provide generalizations about whether or not plant oil possesses activity against fungi. However, the not all provide details about the extent or spectrum this activity. Some publications also show the relative activity of plant oils and extracts by comparing results from different oils tested against the same organism(s).

Comparison of the data obtained in this study with previously published results is problematic. First, the composition of plant oils and extracts is

known to vary according to local climatic and environmental conditions [33]. Furthermore, some

Table 1: Antifungal activity of 4 essential oils of medicinal plants against *Aspergillus flavus* in second day

plant oil	*Aspergilus flavus* disc surface (mm^2) in different concentration % (0.125, 0.25, 0.375, 0.5)				Control/ no oil
Hyssopus officinalis	260.28±0.5	173.52±0.5	173.52±0.5	129.53±0.5	6358.50±0.5
Cupressus arizonica	273.18±0.5	222.68±0.5	198.47±0.5	1019.68±0.5	
Cuminum cyminum	19.62±0.5	19.62±0.5	19.62±0.5	19.62±0.5	
Thymus vulgaris	106.26±0.5	87.90±0.5	74.95±0.5	26.38±0.5	

Table 2: disc surface(mm^2) of mycelium in 2,4 and 6th day

Plant	*Hyssopus officinalis*				*Cupressus arizonica*			
Concentration(%)	0.125	0.25	0.375	0.5	0.125	0.25	0.375	0.5
2nd day	260.28±0.5	173.52±0.5	173.52±0.5	129.53±0.5	273.18±0.5	222.68±0.5	198.47±0.5	1019.68±0.5
4th day	1711.06±0.5	2418.65±0.5	1976.76±0.5	1474.75±0.5	648.93±0.5	399.04±0.5	231.44±0.5	103.31±0.5
6th day	5204.55±0.5	3002.36±0.5	2534.25±0.5	1516.52±0.5	1496.73±0.5	417.62±0.5	252.11±0.5	117.38±0.5
Plant		Cuminum cyminum				Thymus vulgaris		
Concentration(%)	0.125	0.25	0.375	0.5	0.125	0.25	0.375	0.5
2nd day	19.62±0.5	19.62±0.5	19.62±0.5	19.62±0.5	106.26±0.5	87.90±0.5	74.95±0.5	26.38±0.5
4th day	157.01±0.5	22.23±0.5	24.07±0.5	19.62±0.5	1329.38±0.5	1291.35±0.5	928.95±0.5	302.50±0.5
6th day	176.62±0.5	26.16±0.5	25.02±0.5	19.62±0.5	2298.06±0.5	1829.24±0.5	1495.68±0.5	729.80±0.5

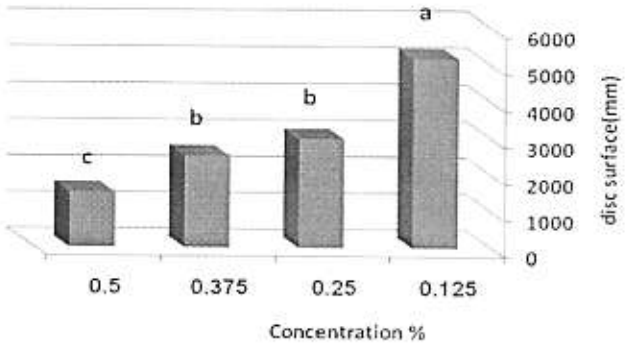

Fig. 1: Disc growth of mycelium in 6th day of *Hyssopus officinalis* essential oils.

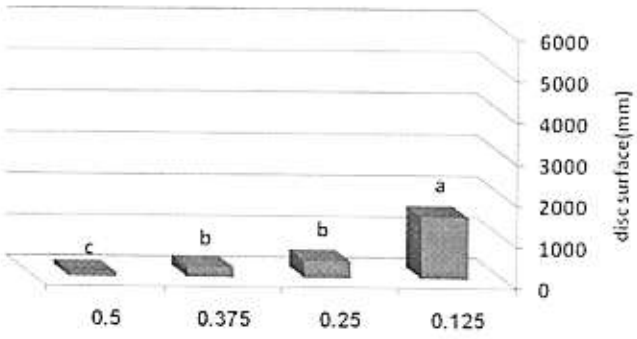

Fig. 2: Disc growth of mycelium in 6th day of *cupressus arizonica* essential oils.

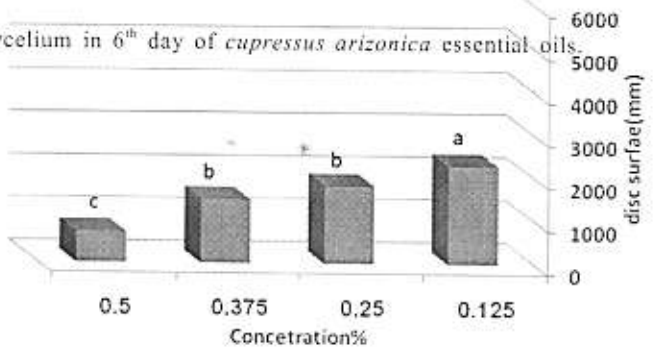

Fig. 3: Disc growth of mycelium in 6th day of *Thymus vulgaris* essential oils.

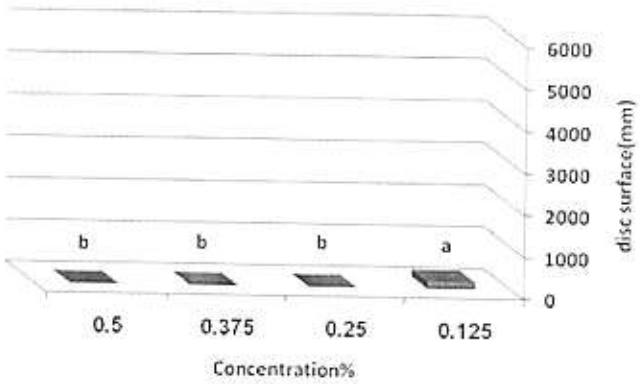

Fig. 4: Disc growth of mycelium in 6th day of *Cuminum cyminum* essential oils.

Table 3: Chemical composition of *cuminum cyminum*

component	RI	Composition%
Tricyclene*	925	0.1
α-pinene*	936	0.6
sabinene	974	0.5
β-pinene	977	11.4
Myrcene*	991	0.9
β-phellandrene*	1011	1.3
α-cymene	1026	3.1
p-cymene*	1036	5.7
limonene	1039	3.1
β-phellandrene	1039	2.2
-terpinene*	1068	12.8
cumin aldehyde	1239	16.1
cumin alcohol	1251	0.4
p-mentha-1,3-dien-7-al	1256	8.7
p-mentha-1,4-dien-7-al	1280	27.4
perillaldehyde	1291	0.6
perilla alcohol	1299	0.3
Eugenol*	1355	0.7
geranyl acetate*	1379	1.7
Caryophyllene*	1414	1.3
trans-thujone	1114	0.1
myrcenol	1105	0.32
camphor	1145	1.64
borneol	1167	2.28
-terpineol	1188	0.77
thymol methyl ether	1235	0.1
carvacrol methyl ether	1244	1.42
geraniol	1253	9.13
geranial	1266	0.33
thymol	1300	18.12
carvacrol	1318	12.11
α-terpynyl acetate	1348	0.75
geranyl acetat	1380	3.14
β-caryophllene	1436	0.51
spathulenol	1582	0.18
Total		93.62

RI: Kovats Retention Indices. Other components were found at <0.1%

Table 4: Chemical composition of *Thymus vulgaris*

Component	RI	Composition%
tricyclene	926	0.13
α-thujene	929	0.58
α-pinene	936	6.45
camphene	945	4.31
β-pinene	983	0.76
1-octen-3-ol	980	2.1
3-octanone	985	0.45
3-octanal	995	0.12
myrecene	997	1.37
α-phellandrene	1002	0.1
α-terpinene	1017	1.07
1,8-cineole	1029	0.54
p-cymene	1032	15.12
limonene	1038	0.98
cis-b-ocimene	1040	0.28
trans-b-ocimene	1050	0.31
-terpinene	1059	1.63
terpinolene	1088	1.73
cis-thujone	1101	4.28

Table 5: Chemical composition of *Hyssopus officinalis*

Component	RI	Composition %
sabinene	976	5.2
beta-myrcene	991	0.8
beta-phellandrene	1031	2.4
linalool	1098	1.1
trans-pinocamphone (pinocamphone)	1160	14.1
cis-pinocamphone (isopinocamphone)	1173	44.7
terpin-4-ol	1177	1
myrtenol	1194	2.8
methyleugenol	1401	0.4
cis-alpha-bergamotene	1415	1.4
beta-caryophyllene	1418	1.4
alpha-caryophyllene	1454	0.9
germacrene D	1480	1.6
germacrene D-11-ol		5.7
elemol	1549	5.6
spathulenol	1576	2.8
caryophyllene oxide	1581	1.6
delta-selinene		0.4

RI: Kovats Retention Indices. Other components were found at <0.1%

oils with the same common name may be derived from different plant species [34]. Secondly, the

method used to assess antimicrobial activity and the choice of test organism(s), varies between publications [32].

In summary, this study confirms that many essential oils possess *in vitro* antifungal activity. However, if plant issues of safety and toxicity will need to be addressed. Our data on the antifungal properties of oils suggest that these oils should be examined further to evaluate its potential as a natural fungicide.

In conclusion, our results indicate that essential oils could find a practical and rational use in the inhibition of mould growth. Particularly, *Hyssopus officinalis, Cuminum cyminum,Thymus vulgaris* and cones of *Cupressus arizonica* essential oils possess strong anti-*Aspergillus* activity inhibiting the growth. The broad inhibition of fungal growth by these essential oils, in addition to its availability as natural volatile product, justifies its possible rational use as an alternative antifungal compound to control the growth and dissemination of pathogen *Aspergillus falvus*.

Table 6: Chemical composition of cones of *Cupressus arizonica*

Component	RI	Composition(%)
α-pinene	937	69.9
β-pinene + sabinene	973	2.3
Δ-3-carene	1007	11.7
β-myrcene	987	2.5
limonene	1025	1.4
α-terpinolene	1081	3.6
α-cubebene	1342	0.6
bornyl acetate	1268	0.5
terpinene-4-ol	1168	0.7
caryophyllene	-	0.4
α-humulene	1438	0.3
α-terpineol	1184	0.6
β-cubebene	1464	1.9
Δ-cadinene	1504	0.3
α-cedrol	1580	1.5
Total		98.2

RI: Kovats Retention Indices. Other components were found at <0.1%

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