

Search for novel antifungals from 49 indigenous medicinal plants: *Foeniculum vulgare* and *Platycladus orientalis* as strong inhibitors of aflatoxin production by *Aspergillus parasiticus*

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Abstract In a search for novel antifungals from natural sources, essential oils (EOs) and extracts of 49 medicinal plants were studied against an aflatoxin (AF)-producing *Aspergillus parasiticus* using a microbioassay technique. AF levels were measured in culture broth by high performance liquid chromatography. The EOs were analyzed by gas chromatography/mass spectrometry (GC/MS). Based on the results obtained, *Achillea millefolium* subsp. *elborsensis*, *Ferula gummosa*, *Mentha spicata*, *Heracleum pubescens* and *Thymus fedtschenkoi* markedly inhibited *A. parasiticus* growth by IC₅₀ values of 35 to 1,815 µg/ml without affecting AF production by the fungus. The EOs of flowers and roots of *Foeniculum vulgare* significantly inhibited both fungal growth (~70.0%) and production of AFs B₁ and G₁

(~99.0%). The ethyl acetate extract of *Platycladus orientalis* leaves suppressed AFB₁ (~90.0%) but not fungal growth and AFG₁ production. This work provides evidence for the first time that *F. vulgare* and *P. orientalis* are strong inhibitors of aflatoxin biosynthesis in *A. parasiticus*. The antifungal activities of the bioactive plants introduced in the present study could make an important contribution to explaining the use of these plants as effective antimicrobial candidates to protect foods and feeds from toxigenic fungus growth and subsequent AF contamination.

Keywords *Aspergillus parasiticus* · Antifungal activity · Aflatoxin · Medicinal plant · *Platycladus orientalis* · *Foeniculum vulgare*

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Introduction

The members of *Aspergillus* section *Flavi* comprise an important group of human pathogens, mycotoxin producers and food contaminants all over the world (Samson et al. 2000; Hedayati et al. 2007). Two species of this section, i.e., *Aspergillus flavus* and *Aspergillus parasiticus* have received major attention, not only for their ability to contaminate a wide array of substrates including peanuts, corn, pistachio nuts and oil seeds, but also for their production of carcinogenic aflatoxins (AFs) (Payne 1998). AFs are a real public health hazard due to their carcinogenic, mutagenic, teratogenic and immunosuppressive effects on various biological systems (Hedayati et al. 2007). On the other hand, economic losses from AF contamination of food and feed are a worldwide problem, costing millions of US dollars each year.

Despite the existence of a lot of useful information on AF inhibitors from natural sources (Holmes et al. 2008; Razzaghi-Abyaneh, et al. 2008, 2009, 2010), fungal invasion and subsequent AF contamination of food and feed is not yet under adequate control. Chemicals are used widely to control the detrimental effects of AF-producing fungi. However, chemical treatments suffer from severe limitations, including adverse reactions on biological systems, development of resistance by fungal pathogens, and undesirable effects on non-target organisms sharing the ecosystem. Thus, there is a clear tendency towards optimization of environmentally friendly fungicides that cause minimal damage to human health and the surrounding ecosystem (Ghisalberti 2000).

In recent years, researchers have focused on finding novel antimicrobials from natural sources including higher plants, microorganisms, insects, nematodes and vertebrates. Plants are rich sources of beneficial secondary metabolites. Their essential oils (EOs) and extracts have a wide array of biological activities, especially antimicrobial effects on different groups of pathogenic organisms (Shams-Ghahfarokhi et al. 2006; Bakkali et al. 2008; Webster et al. 2008; Tolouee et al. 2010). Nowadays, an expanding list of plant EOs has been classified as generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA) as approved flavors or food additives (Tripathi and Dubey 2004).

Interestingly, production of an extremely wide array of bioactive compounds by medicinal plants is completely dependent on parameters such as plant characteristics (i.e., species, variety and growth cycle), geographic condition, soil composition, etc. Thus, screening of a large number of plants from different geographic locations may increase the chance of finding novel bioactive compounds inhibitory to AF-producing fungi.

With respect to seasonal variations, geographic conditions and unique ecosystems, a wide array of medicinal

plants are grown in Iran—some being quite specific at the genus or species level. As a continuation of our ongoing research on natural antifungals, EOs and extracts of 49 indigenous medicinal plants belonging to 21 major families were evaluated in relation to their ability to inhibit *A. parasiticus* growth and AF (AFB₁ and AFG₁) production. Inhibitory components of the bioactive plants determined by GC/MS were given special consideration.

Materials and methods

Fungal strain and growth media

Aspergillus parasiticus NRRL 2999, a known producer of AFs of the B and G series, was used throughout the study. The fungus was cultured on potato dextrose agar (PDA; Merck, Darmstadt, Germany) slants for 7 days at 28°C. Spore suspension was prepared by gently scraping the culture surface using a sterile glass rod in the presence of a 0.1% aqueous solution of Tween 80. Potato dextrose broth (PDB; Scharlau Chemie, Barcelona, Spain) was the medium used for submerged cultures of *A. parasiticus* for AF production.

Plant materials, EOs and extract preparation

As indicated in Table 1, a total of 49 plant species belonging to 21 different families was studied. The plants were collected during April–June 2009. Vouchers were stored in the herbarium of the Research Institute of Forest and Rangelands. Plant materials (leaves, seeds, aerial parts, roots, flowers) were steam distilled for 90 min in a fully glass apparatus. EOs were prepared by hydro-distillation of sterilized plant parts using a Clevenger-type apparatus during a 4-h time period (Bradley 1993). The extraction was carried out for 120 min in 500 ml water. The EO yields were in the range of 0.25–1.2% of total weight, and were kept at 4°C until use. To prepare extracts, plant materials were air-dried and then powdered using a homogenizer. Amounts of 10 g of each air-dried plant material were extracted separately with 100 ml ethyl acetate (EtOAc) and *n*-hexane in Erlenmeyer flasks for 24 h. The extracts were filtered through Whatman No.1 filter papers and evaporated to near dryness by a rotary evaporator. Extracts were kept at 4°C until use.

GC/MS analyses of EOs

GC/MS analyses were performed using a Varian 3400 GC/MS apparatus coupled to a Saturn II ion trap detector (<http://varianinc.com/>). Quantitation was performed using Euro Chrom 2000 software (<http://www.knauer.net>) by the

area normalization method neglecting response factors. GC analysis was carried out using a DB-5 fused silica capillary column (60 m×0.25 mm×film thickness 0.25 µm; J & W Scientific, Rancho Cordova, CA). The operating conditions were as follows: injector and detector temperature, 250°C and 265°C, respectively; helium as carrier gas. Oven temperature programme was 40°C–250°C at the rate of 4°C/min. Mass spectrometry conditions were: ionization potential of 70 eV and electron multiplier energy equal to 2,000 V. The identities of EOs components were established from their GC retention indices relative to C7–C25 n-alkenes, by comparison of their MS spectra with those reported in the literature, and by computer matching with the Wiley 5 mass spectra library (<http://eu.wiley.com/WileyCDA>) and whenever possible, co-injection with a standard available in the laboratory (Davies 1998).

Microbioassay

Aspergillus parasiticus NRRL 2999 was cultured on PDB in 6-well flat-bottom microplates (Greiner bio-one, <http://www.greinerbioone.com>; well diameter 36.0 mm) in the presence of plants EOs, EtOAc and/or *n*-hexane extracts using a microbioassay technique (Razzaghi-Abyaneh et al. 2007). Culture medium (5 ml/well) was added to the microplates, which were inoculated with fungal spore suspension (5×10^6 spores/well) prepared in an aqueous solution of 0.1% Tween 80. Serial two-fold dilutions of the EOs and/or extracts (from 15.62 to 2,000 µg/ml) prepared in methanol (final concentration 1.0%) were added separately to the test wells. The control wells were treated in the same manner except that they did not contain plant EOs and extracts. Triplicate microplates were incubated for 96 h at 28°C under static conditions in two separate experiments.

Fungal dry weight determination

The total contents of each well including culture medium and fungal biomass were filtered through a thin layer of cheese cloth and then thoroughly washed with distilled water. A known weight of mycelium was placed in a stainless steel container and allowed to dry at 80°C to constant weight. The net dry weight of mycelium was then determined.

HPLC assay of AFs

AFs were first qualitatively detected by observing the thin layer chromatography (TLC) pattern of cultures spotted on Silica gel 60 F₂₅₄ plates under UV light (365 nm). The AF content of cultures was measured using HPLC (Knauer D-14163 UV-VIS system, Berlin, Germany) according to Razzaghi-Abyaneh et al. (2007) with some modifications.

A 50-µl aliquot of each sample (culture filtrate) was injected into the HPLC column (TSKgel ODS-80TS; 4.6 mm ID×15.0 cm, Tosoh Bioscience, Japan) and eluted at a flow rate of 1 ml/min. using water/acetonitrile/methanol (60:25:15, v/v/v) as mobile phase. The amounts of AFB₁ and AFG₁ were measured at a wavelength of 365 nm by comparison of the area under the curve (AUC) of unknown samples with authentic standards treated in the same manner. The retention times of AFB₁ and AFG₁ were 11.3 and 9.2 min, respectively.

Statistical analysis

Data on fungal growth and AF content were subjected to analysis of variance (one-way ANOVA) in Tukey range using a SPSS Version 10.0 Programme for Windows (<http://www.spss.com/>). Differences with $P < 0.05$ were considered significant.

Results

Plant characteristics and chemical composition of EOs

The general features of plants used in the present study are summarized in Table 1. A total of 49 medicinal plants belonging to 21 different families was evaluated in relation to their antifungal activities. The main EO constituents of the bioactive plants, i.e., *Foeniculum vulgare*, *A. millefolium* subsp. *elborsensis*, *Ferula gummosa* and *M. spicata* identified by GC-MS are summarized in Table 2 according to their retention indices (RI) and percentage composition. A total of 23 compounds were identified in the flower EOs of *A. millefolium* subsp. *elborsensis*, of which chamazulene (48.9%) was the main substance, followed by isoborneol (10.2%) and camphor (9.5%). Twelve compounds were identified in the leaves EO of *F. gummosa* with β-pinene (54.4%), guaiyl acetate (11.6%) and guaiol (9.1%) as the main constituents. Dillapiol (90.1%) was the main component of *F. vulgare* root EO, while *trans*-anethole (68.4%) was the principle substance of the plant flower EO. Among the 18 compounds identified in the leaf EO of *M. spicata*, piperitenone oxide (34.7%) was the main constituent, followed by *cis*-carveol (21.7%) and 1,8-cineole+limonene (11.3%).

Effect of plant EOs and extracts on *A. parasiticus* growth

Figure 1 shows the inhibitory effects of bioactive plant species on *A. parasiticus* growth without affecting AF production by the fungus. All the plant EOs and extracts inhibited fungal growth in a dose-dependent manner in the range of 4.21% to 100%. The maximum growth inhibition

Table 1 General features and preliminary data of antifungal and antiaflatoxigenic potential of the essential oils and extracts of 49 medicinal plants belonging to 21 different families. *AFB₁* Aflatoxin B₁, *AFG₁* aflatoxin G₁

Family	Plant species	Common name	Part used ^a	Antifungal activity ^b	Antiaflatoxigenic activity ^b	
					AFB ₁	AFG ₁
Apiaceae	<i>Heracleum pubescens</i>	Downy cow parsnip	Seeds (Et)	+	–	–
	<i>Ferula gummosa</i>	Galbanum	Aerial parts (EO)	+	–	–
	<i>Foeniculum vulgare</i>	Fennel	Roots/flowers (EO)	+	+	+
Araliaceae	<i>Hedera helix</i>	Common ivy	Leaves	–	–	–
Asteraceae	<i>Achillea biebersteinii</i>	Milfoil	Leaves	–	–	–
	<i>Achillea callichora</i>	Milfoil	Flowers	–	–	–
	<i>Sonchus oleraceus</i>	Common sowthistle	Flowers	–	–	–
	<i>Artemisia annua</i>	Sweet wormwood	Leaves	–	–	–
	<i>Centaurea iberica</i>	Iberian starthistle	Flowers	–	–	–
	<i>Achillea millefolium</i> subsp. <i>elborsensis</i>	Yarrow	Flowers (EO)	+	–	–
	<i>Tanacetum balsamita</i>	Costmary	Flowers	–	–	–
	<i>Artemisia sieberi</i>	Absinthium	Leaves	–	–	–
	<i>Artemisia absinthium</i>	Grand wormwood	Leaves	–	–	–
	Bignoniaceae	<i>Campsis radicans</i>	Trumpet	Flowers	–	–
<i>Catalpa bignonioides</i>		Southern catalpa	Flowers	–	–	–
Caprifoliaceae	<i>Lonicera caprifolium</i>	Perfoliate honeysuckle	Leaves	–	–	–
Celastraceae	<i>Euonymus japonicus</i> var. <i>argentovariegata</i>	Japanese spindle	Leaves	–	–	–
Cupressaceae	<i>Platyclusus orientalis</i>	Chinese arborvitae	Leaves (Et)	–	+	–
	<i>Cupressus arizonica</i>	Arizona cypress	Leaves	–	–	–
Elaeagnaceae	<i>Elaeagnus angustifolia</i>	Russian silverberry	Leaves	–	–	–
Euphorbiaceae	<i>Ricinus communis</i>	Castor oil plant	Seeds	–	–	–
Fabaceae	<i>Wisteria sinensis</i>	Chinese wisteria	Flowers	–	–	–
	<i>Sophora alopecuroides</i>	Sophora root	Flowers	–	–	–
	<i>Amorpha fruticosa</i>	Desert false indigo	Flowers	–	–	–
	<i>Gleditsia caspica</i>	Caspian honeylocust	Flowers	–	–	–
Juglandaceae	<i>Juglans regia</i>	Persian walnut	Leaves	–	–	–
Lamiaceae	<i>Thymus fedtschenkoi</i>		Aerial parts (Et)	+	–	–
	<i>Stachys lavandulifolia</i>	Woundwort stachys	Leaves	–	–	–
	<i>Mentha pulegium</i>	Pennyroyal	Leaves	–	–	–
	<i>Lavandula angustifolia</i>	Common lavender	Leaves	–	–	–
	<i>Vitex negundo</i>	Five-leaved chaste tree	Leaves	–	–	–
	<i>Rosmarinus officinalis</i>	Rosemary	Aerial parts	–	–	–
	<i>Mentha spicata</i>	Spearmint	Leaves (EO)	+	–	–
	<i>Mentha piperita</i>	Peppermint	Leaves	–	–	–
	<i>Melissa officinalis</i>	Balm	Leaves	–	–	–
	<i>Perovskia artemisioides</i>	Perovskia	Leaves	–	–	–
Malvaceae	<i>Malva neglecta</i>	Common mallow	Flowers	–	–	–
Meliaceae	<i>Melia azedarach</i>	Persian lilac	Seeds	–	–	–
Moraceae	<i>Morus alba</i>	White mulberry	Leaves	–	–	–
Myrtaceae	<i>Eucalyptus camaldulensis</i>	River red gum	Leaves	–	–	–
	<i>Eucalyptus rostrata</i>	Red gum	Leaves	–	–	–
Oleaceae	<i>Fraxinus excelsior</i> subsp. <i>coriifolia</i>	Common ash	Leaves	–	–	–
	<i>Fraxinus angustifolia</i>	Narrow-leafed ash	Leaves	–	–	–
Poaceae	<i>Polypogon maritimus</i>	Mediterranean beard grass	Aerial parts	–	–	–

Table 1 (continued)

Family	Plant species	Common name	Part used ^a	Antifungal activity ^b	Antiaflatoxigenic activity ^b	
					AFB ₁	AFG ₁
	<i>Poa annua</i>	Annual meadow grass	Aerial parts	–	–	–
Rosaceae	<i>Amygdalus scoparia</i>	Mountain almond	Leaves	–	–	–
Rutaceae	<i>Citrus sinensis</i>	Sweet orange	Leaves	–	–	–
	<i>Ruta graveolens</i>	Common rue	Aerial parts	–	–	–
Urticaceae	<i>Urtica urens</i>	Annual nettle	Leaves	–	–	–

^a Effective plant preparations: Et ethyl acetate extract, EO essential oil

^b + present; – not present

observed was at a final concentration of 2,000 µg/ml for all plants in the order of *M. spicata* (100%), *T. fedtschenkoi* *n*-hexane extract (99.58%), *H. pubescens* (99.10%), *A. millefolium* subsp. *elborsensis* (93.03%), *T. fedtschenkoi* EtOAc extract (92.04%), and *F. gummosa* (53.45%). The IC₅₀ values for these plants were reported as 35, 125, 370, 580, 720 and 1815 µg/ml, respectively. For all the plants except *A. millefolium* subsp. *elborsensis*, growth inhibitory activity was significant at concentrations higher than 31.25 µg/ml compared to appropriate controls (ANOVA, $P < 0.05$).

Inhibition of AFs B₁ and G₁ by *F. vulgare* EO

As shown in Table 3, EOs from the flowers, roots and stems of *F. vulgare* suppressed AF production by the fungus. The root EO inhibited both AFB₁ and AFG₁ production in parallel with a marked retardation in fungal growth. The maximum inhibition rates of fungal growth, and of AFB₁ and AFG₁ production were 65.66%, 99.48% and 99.62%, respectively. The growth inhibitory activity was significant at all concentrations except 15.62 µg/ml in comparison with controls (ANOVA, $P < 0.05$). Besides plant roots, a concentration of 2000 µg/ml of the plant flower and seed EOs was also examined. As indicated in Table 3, flower EO significantly inhibited fungal growth (68.58%) and production of AFB₁ (98.95%) and AFG₁ (99.81%) in comparison with controls (ANOVA, $P < 0.05$). The stem EO inhibited AFG₁ production significantly while not affecting fungal growth and AFB₁ production (Table 3).

Inhibition of AFB₁ production by the EtOAc extract of *P. orientalis* leaves

Table 4 presents the biological activity of the EtOAc extract of *P. orientalis* leaves on *A. parasiticus* growth and AF production. The plant extract markedly suppressed AFB₁ production, while it did not inhibit fungal growth

and AFG₁ production (data not shown), even at the highest concentration of 2,000 µg/ml. The inhibition of AFB₁ production was significant for all plant concentrations except 15.62 µg/ml, with a maximum of 89.48% at 2,000 µg/ml (ANOVA, $P < 0.05$). The IC₅₀ value for AFB₁ inhibition was calculated as 55.0 µg/ml. Surprisingly, fungal growth was enhanced by the plant extract at concentrations greater than 125 µg/ml and reached a significant level at 2,000 µg/ml, despite the potent inhibition of AFB₁ production by the fungus (Table 4).

Discussion

In the present study, a total of 49 medicinal plants belonging to 21 different families were evaluated for their antifungal activities. Antifungal activity of the EOs from *A. millefolium* subsp. *elborsensis*, *Ferula gummosa*, *Foeniculum vulgare*, and *M. spicata*, EtOAc extract of *H. pubescens*, and EtOAc and *n*-hexane extracts of *T. fedtschenkoi* against aflatoxigenic *A. parasiticus* was shown. The plants *F. vulgare* and *P. orientalis* were identified as potent inhibitors of fungal AF biosynthesis. All the bioactive plants except *P. orientalis* inhibited *A. parasiticus* growth in a dose-dependent manner to different extents. Based on the IC₅₀ values of the plant preparations, *M. spicata* was the most effective fungal growth inhibitor followed by *T. fedtschenkoi*, *H. pubescens*, *A. millefolium* subsp. *elborsensis*, *F. vulgare* and *F. gummosa*. To the best of our knowledge, this is the first report of antifungal activity of *T. fedtschenkoi* and *A. millefolium* subsp. *elborsensis* against aflatoxigenic *A. parasiticus*.

Plant EOs are composed of a wide array of chemicals that are characterized by two or three major components at high concentrations (20–70%) and other components present in trace amounts. Generally, the major components determine the biological properties of EOs (Bakkali et al. 2008). In the present study, β-pinene, guaiyl acetate

Table 2 Chemical composition of *Ferula gummosa*, *Achillea millefolium* subsp. *elborsensis*, *Foeniculum vulgare* and *Mentha spicata* essential oils

No.	<i>F. gummosa</i> (aerial parts)			<i>A. millefolium</i> subsp. <i>elborsensis</i> (flowers)			<i>F. vulgare</i> roots			Flowers			<i>M. spicata</i> (leaves)		
	Compound	RI	%	Compound	RI	%	Compound	RI	%	Compound	RI	%	Compound	RI	%
1	α -Pinene	934	2.4	Tricyclene	924	0.8	Myrcene	985	0.7	α -Pinene	929	0.4	α -Pinene	933	0.2
2	β -Pinene	970	54.4	α -Thujene	930	0.6	1,8-Cineole	1024	0.7	Myrcene	978	0.5	Sabinene	968	0.5
3	Myrcene	977	1.8	Heptanol	963	0.7	Fenchone	1053	2.2	Limonenone+1,8-Cineole	1,024	17.3	β -Pinene	973	0.6
4	δ -3-Carene	998	1.2	3-Octanone	985	0.4	α -Fenchol	1083	4.9	γ -Terpinene	1,048	1.3	Myrcene	984	2.4
5	(Z)- β -Ocimene	1,018	1.4	p-Cymene	1,020	4.0	Elemicin	1519	0.8	Fenchone	1,067	4.6	1,8-Cineole+ Limonene	1,026	11.3
6	1,3,5-Undecatriene	1,153	0.5	β -Phellandrene	1,030	0.1	Dillapiol	1612	90.1	Estragole	1,176	2.5	(Z)- β -Ocimene	1,028	0.6
7	Terpinenyl acetate	1,327	0.5	Camphor	1,134	9.5				cis-carveole	1,215	0.8	γ -Terpinene	1,057	2.1
8	Elemol	1,530	0.6	Isoborneol	1,155	10.2				cis-anethole	1,224	1.4	α -Thujone	1,095	0.2
9	Germaacrene B	1,547	0.6	Pinocarvone	1,160	5.1				trans-anethole	1,265	68.4	Isomenthol	1,176	0.6
10	Guaiol	1,580	9.1	cis-pinocarveol	1,181	4.0				Piperitenone oxide	1,348	0.4	cis-dihydrocarvone	1,181	1.80
11	Guaiyl acetate	1,649	11.6	trans-piperitol	1,203	0.8				Germaacrene D	1,476	0.4	cis-carveol	1,224	21.7
12	β -Guaiene	1,706	0.5	Carvenone	1,250	0.7				Carvone			Carvone	1,231	2.6
13				α -Copaene	1,372	0.6				Piperitone			Piperitone	1,314	0.6
14				Longifolene	1,400	1.2				Piperitenone oxide			Piperitenone oxide	1,341	34.7
15				β -Caryophyllene	1,416	1.3				β -Elemene			β -Elemene	1,390	0.6
16				γ -Gurjunene	1,470	0.5				β -Caryophyllen			β -Caryophyllen	1,447	4.5
17				Pentadecane	1,502	1.5				Germaacrene B			Germaacrene B	1,483	2.9
18				Germaacrene B	1,555	0.9				α -Farnesene			α -Farnesene	1,498	0.6
19				γ -Eudesmol	1,635	1.2									
20				α -Bisabolol	1,678	0.5									
21				Chamazulene	1,714	48.9									
22				Heneicosane	2,110	0.7									
23				Ethyl octadecanoate	2,182	0.4									
	Total		84.6	Total		94.6	Total		99.4	Total		98.0	Total		88.5

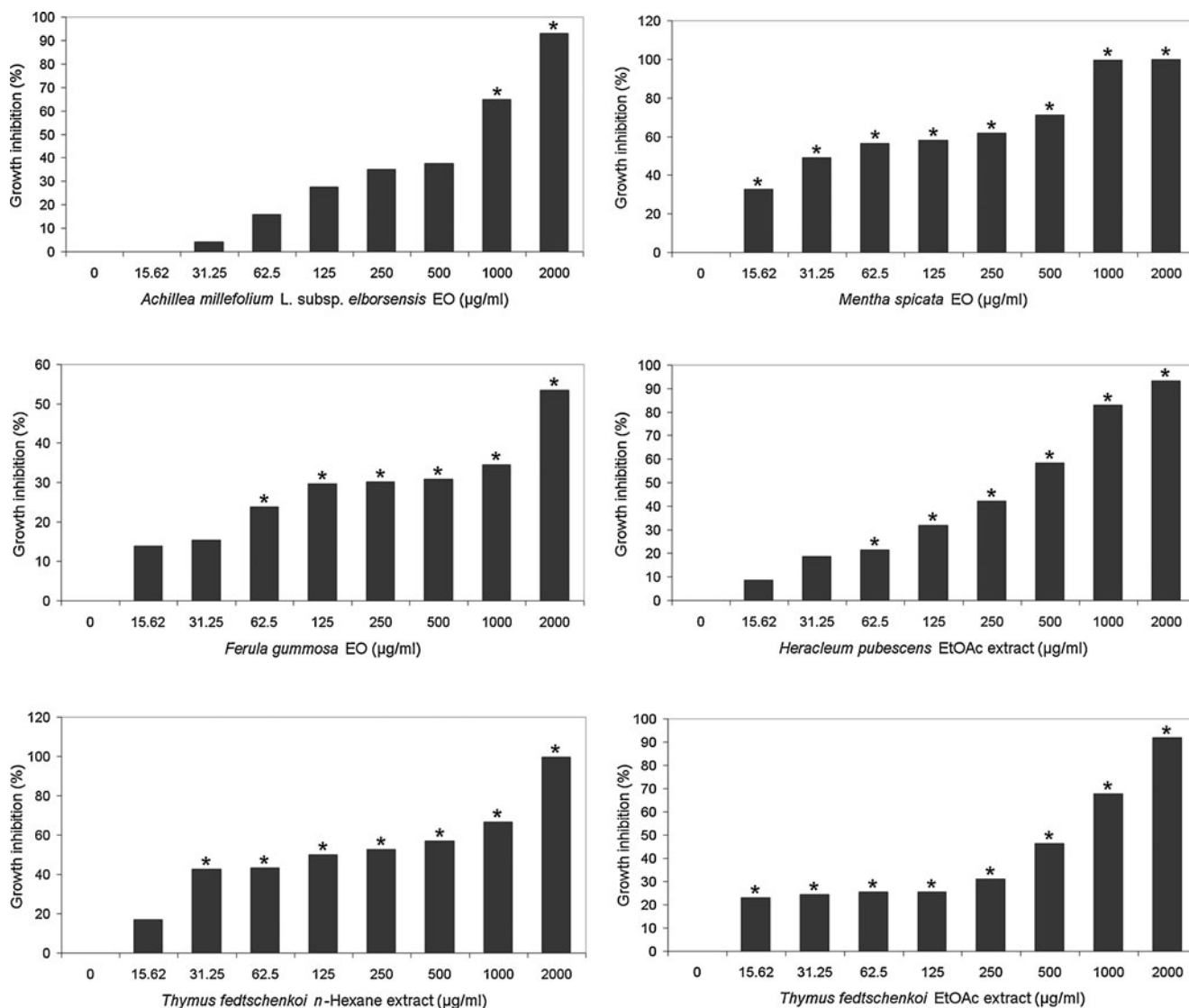


Fig. 1 Inhibitory effects of essential oils (EOs) and extracts from bioactive plants on *Aspergillus parasiticus* NRRL 2999 growth in microbioassay. Results are the mean±SD obtained from two separate

experiments in triplicate. * Statistically significant differences with a control (ANOVA, $P < 0.05$)

and guaiol were identified as major components of *F. gummosa* EO; chamazulene, isoborneol and camphor as components of *A. millefolium* subsp. *elborsensis* EO; piperitenone oxide, cis-carveol and 1,8-cineole+limonene as components of *M. spicata* EO, and dillapiol (roots) and trans-anethole (flowers) as components of EO of *F. vulgare*. The majority of these compounds are monoterpenes (cineole, camphor, β -pinene, cis-carveol, piperitenone oxide, isoborneol) and the others belong to phynelpropanoids (trans-anethole, dillapiol), terpenoids (chamazulene) or cyclic terpenes (limonene). All the monoterpenes, trans-anethole and limonene are known for their inhibitory effects on the growth of various fungal species to different extents (Bakkali et al. 2008). Thus, they might be responsible for the antifungal activity of the

corresponding plants against *A. parasiticus* demonstrated in the present study.

The results of the AF assay in the presence of plant EOs and extracts revealed more interesting data than that of fungal growth. Two species, i.e., *F. vulgare* and *P. orientalis* were found to be potent inhibitors of AF production by *A. parasiticus*. The EOs from *F. vulgare* roots and flowers inhibited both AFB₁ and AFG₁ production dose dependently, consistent with their inhibitory effects on fungal growth. *F. vulgare* Mill. (Fennel) is a perennial herb belonging to the Apiaceae family that is distributed widely throughout the world both as wild and cultivated species. Despite the existence of interesting data on the antifungal activity of different plant preparations (Kwon, et al. 2002; Mimica-Dukic et al. 2003; Singh et al. 2006; Napoli et al.

Table 3 Inhibition of fungal growth and production of AFs B₁ and G₁ in *A. parasiticus* NRRL 2999 exposed to the EOs prepared from *Foeniculum vulgare* roots, flowers and stems

Plant part	EO concentration (µg/ml)	Fungal dry weight (DW; mg)		AFB ₁ (µg/mg fungal DW)		AFG ₁ (µg/mg fungal DW)	
		Mean±SD	Inhibition (%)	Mean±SD	Inhibition (%)	Mean±SD	Inhibition (%)
Roots	0.0	12.20±0.70	0.00	3.063±0.130	0.00	0.532±0.037	0.00
	15.62	12.93±0.76	0.00	1.865±0.384*	39.11	0.351±0.036*	34.02
	31.25	10.37±0.51*	15.00	1.806±0.116*	41.04	0.150±0.007*	71.80
	62.5	9.77±0.21*	19.92	1.701±0.227*	44.47	0.042±0.002*	92.10
	125	9.50±0.30*	22.13	1.50±0.238*	51.03	0.038±0.005*	92.86
	250	9.43±0.75*	22.70	0.982±0.153*	67.94	0.036±0.002*	93.23
	500	8.34±0.42*	31.64	0.167±0.028*	94.55	0.003±0.000*	99.44
	1,000	4.98±0.55*	59.18	0.017±0.005*	99.44	0.002±0.000*	99.62
	2,000	4.19±0.67*	65.66	0.016±0.007*	99.48	0.002±0.000*	99.62
Stems	2,000	11.17±1.04	8.44	2.483±0.45	18.93	0.378±0.054*	28.95
Flowers	2,000	3.83±0.52*	68.61	0.032±0.002*	98.95	0.001±0.000*	99.81

*Statistically significant difference from the control (ANOVA, $P < 0.05$); there was no further significant inhibition of fungal growth and production of aflatoxins B₁/G₁ at plant EO concentrations > 2,000 µg/ml

2010), no data is available about their effects on AF production by the producing fungi. Our results demonstrate for the first time a strong inhibitory activity of the EOs of both roots and flowers on production of AFs B₁ and G₁ by *A. parasiticus* in parallel with a marked suppression of fungal growth. The antifungal activity of *F. vulgare* toward *A. parasiticus* demonstrated in the present work may be accounted for by the presence of 1,8-cineol, limonene, trans-anethole and fenchone—the major constituents of *F. vulgare* roots and flowers, which have been reported

previously to have similar activities. The main component of *F. vulgare* roots identified in the present study, i.e., dillapiol, is a phenylpropanoid responsible for pathway-specific inhibition of AFG₁ as previously reported by Razzaghi-Abyaneh et al. (2007). Limonene—a major constituent of *F. vulgare* flower EO—was previously identified as an inhibitor of AF production by *A. parasiticus* (Greene-McDowelle et al. 1999). However, it was not identified as a constituent of *F. vulgare* root EO in the present work. Hence, the active component(s) of root EOs of the plant causing AFB₁ inhibition require further characterization.

Table 4 Inhibition of AFB₁ production in *A. parasiticus* NRRL 2999 by the ethyl acetate (EtOAc) extract of *Platycladus orientalis* leaves. Fungal growth was stimulated by the plant extract to a significant degree at the highest concentration of 2,000 µg/ml

Plant EtOAc extract concentration (µg/ml)	Fungal dry weight (DW; mg)	AFB ₁ (µg/mg fungal DW)	
	Mean±SD	Mean ±SD	Inhibition (%)
0.0	11.03±1.25	1.88±0.44	0.00
15.62	9.23±0.31	1.39±0.27	26.06
31.25	9.20±0.90	1.29±0.12*	31.38
62.50	9.57±0.81	0.85±0.03*	54.79
125	10.15±1.09	0.76±0.08*	59.57
250	13.06±1.40	0.67±0.10*	64.36
500	13.10±0.85	0.67±0.09*	64.36
1,000	13.20±0.87	0.30±0.04*	84.04
2,000	18.00±1.87*	0.19±0.02*	89.89

*Statistically significant difference with a control (ANOVA; $P < 0.05$); there was no further significant inhibition of AFB₁ production at plant extract concentrations > 2,000 µg/ml

The potent inhibition of AF production by the EtOAc extract of *P. orientalis* leaves was interesting in relation to the fact that it significantly suppressed AFB₁ production with no effect on AFG₁ synthesis, and had a remarkable stimulatory effect on fungal growth. *Platycladus orientalis* is an evergreen coniferous tree in the cypress family Cupressaceae that is distributed widely from the West to the North of Iran. Leaves of this plant are commonly used in oriental traditional medicine. Although a wide array of biological activities have been reported for extracts prepared from different parts of the plant, little has been documented about plant antimicrobial activities (Lu et al. 2006; Chen et al. 2007; Wang et al. 2008). It has been shown that the flavonoid constituents, such as pinusolide, rutin, quercitrin, quercetin, myricetin, aromadendrin, amantoflavone and hinokiflalone, of *P. orientalis* leaves are responsible for its biological activities (Lu et al. 2006). The majority of these compounds are known as potent antioxidants in the sense that they are active toward free radicals such as reactive oxygen species. Thus, they may be responsible for the plant-induced AFB₁ inhibition observed

in the present study by a mechanism of suppressing the oxidative stress response to the toxigenic fungus. The growth stimulatory effect of *P. orientalis* for toxigenic *A. parasiticus* in parallel with the marked suppression of AFB₁ but not AFG₁ production reported here is a very promising result. This finding further substantiates the complex nature of the relationship between fungal growth as an index of primary metabolism and AF production as a hallmark of secondary metabolism in toxigenic fungi.

Overall, the results of the present study show clearly that bioactive plants, especially *F. vulgare* and *P. orientalis*, with strong inhibitory activity toward *A. parasiticus* growth and/or AF production, are potential targets for use as natural preservatives to control toxigenic fungal growth and subsequent AF contamination of foods, feeds and agricultural commodities. Further identification of the inhibitory compounds of these bioactive plants and a comprehensive ecological study to evaluate their effectiveness in the field are recommended.

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References

- Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008) Biological effects of essential oils—a review. *Food Chem Toxicol* 46:446–475
- Bradley P (1993) The British herbal compendium: vol 1: A handbook of scientific information on widely used plant drugs. British Herbal Medicine Association, London
- Chen L, Ding L, Yu A, Yang R, Wang X, Li J, Jin H, Zhang H (2007) Continuous determination of total flavonoids in *Platycladus orientalis* (L.) Franco by dynamic microwave-assisted extraction coupled with on-line derivatization and ultraviolet-visible detection. *Anal Chim Acta* 596:164–170
- Davies NW (1998) Gas chromatographic retention index of monoterpenes and sesquiterpenes on methyl silicone and carbowax 20 M phases. *J Chromatogr* 503:1–24
- Ghisalberti EL (2000) Bioactive metabolites from soilborne fungi: natural fungicides and biocontrol agents. In: Atta-ur-Rahman (eds) *Studies in natural products chemistry*, vol 21. Part 2: bioactive natural products (Part B). Elsevier, Amsterdam, pp 181–250
- Greene-McDowelle DM, Ingber B, Wright MS, HJJr Z, Bhatnagar D, Cleveland TE (1999) The effects of selected cotton-leaf volatiles on growth, development and aflatoxin production of *Aspergillus parasiticus*. *Toxicon* 37:883–893
- Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P, Denning DW (2007) *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. *Microbiology* 153:1677–1692
- Holmes RA, Boston RS, Payne GA (2008) Diverse inhibitors of aflatoxin biosynthesis. *Appl Microbiol Biotechnol* 78:559–572
- Kwon YS, Choi WG, Kim WJ, Kim WK, Kim MJ, Kang WH, Kim CM (2002) Antimicrobial constituents of *Foeniculum vulgare*. *Arch Pharm Res* 25:154–157
- Lu YH, Liu ZY, Wang ZT, Wei DZ (2006) Quality evaluation of *Platycladus orientalis* (L.) Franco through simultaneous determination of four bioactive flavonoids by high-performance liquid chromatography. *J Pharm Biomed Anal* 42:1186–1190
- Mimica-Dukic N, Kujundzic S, Sokovic M, Couladis M (2003) Essential oil composition and antifungal activity of *Foeniculum vulgare* Mill. obtained by different distillation conditions. *Phytother Res* 17:368–371
- Napoli EM, Curcuruto G, Ruberto G (2010) Screening the essential oil composition of wild Sicilian fennel. *Biochem Syst Ecol* 38:213–223
- Payne GA (1998) Process of contamination by aflatoxin-producing fungi and their impact on crops. In: Sinha KK, Bhatnagar D (eds) *Mycotoxins in agricultural and food safety*. Dekker, New York, pp 279–306
- Razzaghi-Abyaneh M, Yoshinari T, Shams-Ghahfarokhi M, Rezaee MB, Nagasawa H, Sakuda S (2007) Dillapiol and apiol as specific inhibitors for the biosynthesis of aflatoxin G₁ in *Aspergillus parasiticus*. *Biosci Biotechnol Biochem* 71:2329–2332
- Razzaghi-Abyaneh M, Shams-Ghahfarokhi M, Yoshinari T, Rezaee MB, Jaimand K, Nagasawa H, Sakuda S (2008) Inhibitory effects of *Satureja hortensis* L. essential oil on growth and aflatoxin production by *Aspergillus parasiticus*. *Int J Food Microbiol* 123:228–233
- Razzaghi-Abyaneh M, Shams-Ghahfarokhi M, Rezaee MB, Jaimand K, Alinezhad S, Saberi R, Yoshinari T (2009) Chemical composition and anti-aflatoxinogenic activity of *Carum carvi* L., *Thymus vulgaris* and *Citrus aurantifolia* essential oils. *Food Control* 20:1018–1024
- Razzaghi-Abyaneh M, Shams-Ghahfarokhi M, Rezaee MB, Sakuda S (2010) Natural aflatoxin inhibitors from medicinal plants. In: Rai M, Varma A (eds) *Mycotoxins in food, feed and bioweapons*. Springer, Berlin, pp 329–352
- Samson AR, Hoekstra ES, Frisvad JC, Filtenborg O (2000) Introduction to food and airborne fungi, 6th edn. CBS, Utrecht
- Shams-Ghahfarokhi M, Shokohamiri MR, Amirrajab N, Moghadasi B, Ghajari A, Zeini F, Sadeghi G, Razzaghi-Abyaneh M (2006) In vitro antifungal activities of *Allium cepa*, *Allium sativum* and ketoconazole against some pathogenic yeasts and dermatophytes. *Fitoterapia* 77:321–323
- Singh G, Maurya S, de Lampasona MP, Catalan C (2006) Chemical constituents, antifungal and antioxidative potential of *Foeniculum vulgare* volatile oil and its acetone extract. *Food Control* 17:745–752
- Tolouee M, Alinezhad S, Saberi R, Eslamifard A, Zad SJ, Jaimand K, Taeb J, Rezaee MB, Kawachi M, Shams-Ghahfarokhi M, Razzaghi-Abyaneh M (2010) Effect of *Matricaria chamomilla* L. flower essential oil on the growth and ultrastructure of *Aspergillus niger* van Tieghem. *Int J Food Microbiol* 139:127–133
- Tripathi P, Dubey NK (2004) Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. *Postharvest Biol Technol* 32:235–245
- Wang YZ, Tang CP, Ke CQ, Weiss HC, Gesing ER, Ye Y (2008) Diterpenoids from the pericarp of *Platycladus orientalis*. *Phytochemistry* 69:518–526
- Webster D, Taschereau P, Belland RJ, Sand C, Rennie RP (2008) Antifungal activity of medicinal plant extracts; preliminary screening studies. *J Ethnopharmacol* 115:140–146