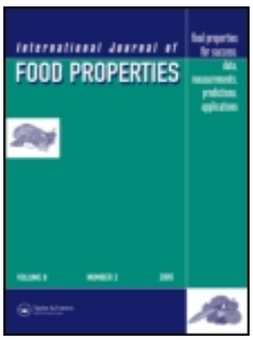

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ANTIFUNGAL ACTIVITY AND CHEMICAL COMPOSITION OF ESSENTIAL OIL OF *ORIGANUM HYPERICIFOLIUM*

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Endemic oregano's,* Origanum hypericifolium *O. Schwartz and P.H. Davis, essential oil was extracted to exert its biological activity in vitro. Fifteen components in its extracts performed by hydro distillation. The major components in the fruit and flower volatiles of* O. hypericifolium *were p-cymene (34.33 g/100 g oil), carvacrol (21.76 g/100 g oil), thymol (19.54 g/100 g) and γ-terpinene (13.91 g/100 g oil). The antifungal activity of* O. hypericifolium*'s oil was evaluated against 14 fungi isolated from hazelnut and walnut. Nuts are capable of harboring toxigenic fungi and the threat of mycotoxin contamination on them exists. Essential oil of* O. hypericifolium *was found to be active both in contact and headspace assays in vitro producing hyphal growth inhibition. In the contact assay,* P. frequentans *was found to be the least sensitive species. The more sensitive species were* P. castellonense, P. verrucosum. *var.* cyclopium*,* C. globosum*, and* A. kiliense*. Their growth was completely (100%) inhibited at days 3 and 6. In the volatile assay, all the mycelial growth of all tested fungi was completely inhibited at day 3. The volatile activity was found to be highly efficient than that of contact activity assay. This could be because of the aromatic contents of Origanum, such as monoterpenes, carvacrol, thymol, and p-cymene.

Keywords: *Essential oil,* Origanum hypericifolium*, Chemical composition, Antifungal activity, Endemic.*

INTRODUCTION

The antimicrobial properties of essential oils from a variety of plants have been assessed previously, and these studies reported that these plant metabolites had potentials as natural and alternative antimicrobial substances in food conservation.[1,2] Members of the genus *Origanum*, Lamiaceae, have recently been of great interest, in both academia and the food industry as potential natural additives to replace synthetic products.[3–5] Due to

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their antibacterial, antifungal, insecticidal, antioxidant, and anti-carcinogenic activities,[6] the essential oil and its chemical composition of *Origanum* species is now being studied intensively.[7–15]

Turkey is regarded as an important gene-centre for the family Lamiaceae. The leafy parts of plants, such as oregano, thyme, and savory, have been added to meat, chicken, and food products for many years.[16] Members of the genus *Origanum* (Lamiaceae) are among the most important aromatic plants worldwide. Twenty-four species and 27 taxa are found in the flora of Turkey and the East Aegean Islands, 16 of them being endemic.[17] *Origanum hypericifolium* O. Schwartz & P.H. Davis is known as "Çökelek kekiği" or "Kekik" by locals and is endemic for Denizli province. This plant is used for treatment of some diseases, especially for diabetes, as an herbal tea.[18,19]

An increased interest in natural alternatives has focused on the potential applications of plant essential oils due to their antimicrobial properties against a wide spectrum of microorganisms, including bacteria, yeasts, and fungi, that are well established.[7,20,21] As is well known, in food treating, fungal product spoilage causes both severe economic losses and potential health hazards due to mycotoxins.[22,23]

Tree nuts are important components of the Mediterranean diet, but they can be exposed to infection by a variety of micro-organisms that can induce spoilage or produce toxic metabolites to living things. Although the sources of infections are not known in many cases, they are deteriorated by factors, such as insect damage, drought, and high temperatures. A frequency survey reported that the most prevalent genera found were *Aspergillus*, *Rhizopus*, and *Penicillium*.[24] Mycotoxigenic fungi of particular concern are *Aspergillus* species that produce hepatotoxic aflatoxins and nephrotoxic ochratoxins.[25]

From 2000 to 2007, the annual unshelled hazelnut production in Turkey varied between 350,000 and 661,000 tons. This accounts for 68.20% of the total world unshelled hazelnut production.[26] Walnuts (*Juglans regia* L.) are widely distributed all over the world. As walnuts are harvested during the rainy season, i.e., September–October, its deterioration is caused by insects, fungi, and moisture. Therefore, fungal infection is one of the main causes of damage leading to the production of mycotoxins. In this manner, Aflatoxins, produced by *Aspergillus* spp., are one of the most potent carcinogens.[27] The aims of the present study were to analyze the chemical composition and to characterize the antifungal activity of essential oils extracted from an endemic Oregano species, *Origanum hypericifolium* O. Schwartz & P.H. Davis, against fungal species isolated from both hazelnuts and walnuts.

MATERIALS AND METHODS

Plant Materials

The flowers and fruits of *O. hypericifolium* were collected during its flowering stage, July–August 2007, on Mount Sandras (elevation 1860 m), Beyağaç-Denizli. The voucher specimen is deposited at the herbarium of Pamukkale University, Faculty of Science & Art, Biology Department (herbarium no. AÇE 2545). The samples were air-dried and stored in a polyethylene bag until use. In the drying procedure, plant samples were spread out on a paper sheet at a shadowy part of the room, kept from direct contact of the sun light, until their weight became constant. They were kept at room temperature, 22 $\pm$ 3.5°C, up to extracting their oil contents.

Steam Distillation (SD) and Analyzing Oil by GC-MS

The flowers and fruits parts of *O. hypericifolium* were steam distilled separately for 3 h using a Clevenger-type apparatus according to the European Pharmacopoeia.[28] The essential oil obtained was dried over anhydrous sodium sulphate and stored at 4°C until analysis. The essential oil was analyzed by gas chromatography-mass spectroscopy (Shimadzu GC-MS-QP2010 Plus Shimadzu Scientific Instruments, Columbia, MD, USA) attached to a TRB-5MS capillary column; Varian CPWAX 52CB, 50m, 0.32 mm i.d., film thickness 1.2μm; Interlink Scientific Services Ltd., Dartford, Kent, UK. Helium was used as the carrier gas. The oven temperature was programmed from 50 to 300°C at a rate of 5°C/min. Diluted samples (1/100 in hexane, v/v) of 1.0 μL were injected by an auto-sampler in the split mode (1/100). Essential oil components were identified by comparing to MS library. The relative percentage of the essential oil constituents was calculated from the GC peak areas.

Obtaining Identification and Maintaining of the Mycoflora

The seed kernels and nuts were surface-sterilized with a 1% solution of sodium hypochlorite and then rinsed with sterile distilled water. Seed kernels were then placed in Petri dishes containing potato dextrose agar (PDA) medium. The plates were kept for 7 days at room temperature (23°C). The developing fungal colonies were isolated, identified and maintained on PDA and Czapek's-Dox agar media.[29]

Contact Assay

PDA plates were prepared using 9-cm glass Petri dishes containing 20 mL of PDA. A 1-cm disc of agar was removed from the centre of the plates. Twenty mL of water (control) or undiluted oil was pipetted into these wells. Two 5-mm diameter discs of the test species were cut from the periphery of less than 1-week-old cultures on PDA plates and placed mycelial surface down on opposite edges of the test plates against the sides of the dishes. The plates were incubated in the dark at 20°C. After 3 and 6 days of inoculation, extension of hyphae towards the central well was measured from the inner edge of the inocula discs to the leading edges of colonies at a point nearest the well. Mean growth measurements were calculated from eight replicates of each of the fungal species.

Volatile Assay

PDA plates were prepared using 8.2-cm plastic Petri dishes containing 20 ml of PDA. A 5-mm diameter disc of the test species was cut from the periphery of the actively growing culture of the PDA plates and placed mycelial surface down on the centre of the dish. The Petri dishes were placed with the lid upside down. A 10-μl aliquot of water (control) or undiluted oil was pipetted on the lid without agar. The plates were incubated in the dark at 20°C. Mean growth measurements were calculated from eight replicates of each fungal species. Since positive control assays are mainly used in the determination of minimum inhibitory concentration, these tests were not employed in this study.

Calculation and Statistics

Growth inhibition of the treatment against the control was measured by percentage, using the formula (C-T/C) × 100, where C is hyphal extension (mm) of controls and T is hyphal extension (mm) of oil-treated plates. A *t*-test was also computed for statistical significance of the results in the contact and volatile assays.

RESULTS

Chemical Composition of the Essential Oils

The yields of *O. hypericifolium* on a dry weight basis were 2.9 g/100 g dry solids (v/w). The water contents of the fresh plant samples were ca 47.5%. Table 1 shows the percentages of the main components present in the essential oils extracted from the fruits and flowers of *O. hypericifolium* collected from Sandras Mount. Fifteen components in *O. hypericifolium* were identified from hydro distillation. It was observed that the essential oil was containing monoterpenes as well as the sesquiterpenes. The major constituents were p-cymene (34.33 g/100 g oil), carvacrol (21.76 g/100 g oil), thymol (19.54 g/100 g oil) and γ-terpinene (13.91 g/100 g oil).

Fungi Isolated from Hazelnut and Walnut

Acremonium kiliense Grüts, *Alternaria alternata* (Fr.) Keissl., *Aspergillus flavus* Link, *Aspergillus niger* van Tiegh, *Aspergillus terreus* Thom, *Chatomium globosum* Kunz., *Cladosporium oxisporum*, *Penicillium frequentans* Westling, *Penicillium griseum* Bonorden, *Penicillium castellonense* C. Ramirez & A.T. Martinez, *Penicillium estinogenum* A Komatsu & S. Abe ex G. Sm., *Penicillium simplicissimum* (Oudemans)

Table 1 Percentage compositions of *O. hypericifolium* volatile components isolated by using hydrodistillation technique.

Compound[a]	RT (min.)	%[b]
α-Pinene	7.2	1.83
Camphene	8.5	0.17
β-Pinene	10.1	0.09
Myrecene	12.3	0.90
α-Terpinene	13.3	1.75
γ-Terpinene	16.6	13.91
p-Cymene	18.1	34.33
1-Octen-3-ol	25.8	1.78
Terpineol	29.9	0.35
Caryophyllene	38.5	1.07
Terpinene-4-ol	38.9	0.76
Borneol	44.4	0.52
Spathulenol	68.2	0.11
Thymol	70.1	19.54
Carvacrol	71.6	21.76
Unknown		1.13

[a]As identified by GC-MS software; names according to NIST mass spectral library.

[b]Percentage of each component is calculated as peak area.

Thom., *Penicillium zacinthae* C. Ramirez & Martinez, were isolated from hazelnut, and *Penicillium verrucosum.* var. *cyclopium* (Westling) Samson, Stolk & Hadlok was isolated from walnut seeds.

Inhibitory Effect of Essential Oil on the Mycelial Growth of Fungi in Agar Diffusion Plate

The flower head oil of *O. hypericifolium* was seen to be active both in contact and on exposure to the headspace volatiles, since they both significantly reduced the growth of fungi in comparison with the control. It should be mentioned, though, that results from the agar diffusion plate assay might also include some effects of the oil vapors besides the contact action. The inhibitory effects of the essential oil on the mycelial growth of 14 fungal species isolated from nuts in agar diffusion plate assay are shown in Table 2. The results indicated that the growth of fungal species, except of *P. frequentans*, were highly reduced (>80%) at the third day of the experiments. *P. frequentans* was found to be the least sensitive species. The mycelial growth of *A. flavus* was minimal inhibited by the oil at the 6th day. Although no clear differences in the activity could be observed for many fungi, the more sensitive species were *P. castellonense*, *P. verrucosum.* var. *cyclopium*, *C. globosum*, *A. kiliense* (100%) at days 3 and 6.

Inhibitory Effect of the Essential Oil on the Mycelial Growth of Fungi in Volatile Assay

In the volatile assay, as seen in Table 3, all the mycelial growth of all tested fungi was completely inhibited at day 3. In the mean time, inhibition percentages were the highest at the 6th day. *P. verrucosum* var. *cyclopium* was observed to be the least sensitive species

Table 2 Growth inhibition of fungal species in agar diffusion plate assay by oil (20 μl/petri dish) of *Origanum hypericifolium*.

	Day 3			Day 6		
Fungal species	Treated (mm)	Control (mm)	Inhibition[a] (%)	Treated (mm)	Control (mm)	Inhibition[a] (%)
A. kiliense	0	2.25	100*	0	11.72	100*
A. alternata	1.5	25	94*	3.25	50	93.5*
A. flavus	0.62	3.25	80.92*	2.62	7	62.57**
A. niger	2.8	34	91.76*	8.5	70	87.85*
A. terreus	1.25	14	91.07*	3	35	91.42*
C. globosum	0	30	100*	2.12	63	96.63*
C. oxisporum	1	16	93.75*	8.75	37	76.35*
P. castellonense	0	17	100*	0	38	100*
P. estinogenum	0.62	8	92.25*	2.5	17.5	85.71*
P. frequentans	5.37	19	71.73*	15.25	38	59.86*
P. griseum	0.87	13	93.30*	3.25	34	90.44*
P. simplicissimum	0	12	100*	0	19	100*
P. verrucosum. var. cyclopium	0	15	100*	0	29	100*
P. zicinthae	1.37	15	90.86*	2.62	33	92.06*

[a]*t*-Test for comparison between treatment and control.

*Significantly different ($P < 0.05$, $P < 0.01$, and $P < 0.001$); **Not statistically different ($P < 0.001$).

Table 3 Growth inhibition of fungal species by volatile oil (20 μl/petri dish) of *Origanum hypericifolium*.

	Day 3			Day 6		
Fungal species	Treated (mm)	Control (mm)	Inhibition[a] (%)	Treated (mm)	Control (mm)	Inhibition[a] (%)*
A. kiliense	0	10	100*	0	18	100*
A. alternata	0	25	100*	0	49	100*
A. flavus	0	10	100*	0	15	100*
A. niger	0	26	100*	0	55	100*
A. terreus	0	18	100*	0	25	100*
C. globosum	0	29	100*	0	58	100*
C. oxisporum	0	15	100*	0	35	100*
P. frequentans	0	18	100*	2	29	93.10*
P. griseum	0	19	100*	0	21	100*
P. castellonense	0	18	100*	0	38	100*
P. estinogenum	0	11	100*	0	20	100*
P. simplicissimum	0	22	100*	0.5	32	98.43*
P. verrucosum. var. *cyclopium*	0	15	100*	3.25	18	81.94*
P. zicinthae	0	15	100*	0	31	100*

[a]*t*-Test for comparison between treatment and control.
*Significantly different ($P < 0.05$, $P < 0.01$, and $P < 0.001$).

in these conditions. The volatile activity was found to be highly significant than that of contact activity assay.

DISCUSSION

The number of components identified in *O. hypericifolium* using SD was 15. As can be seen from Table 1, the major components in the fruit and flower volatile of *O. hypericifolium* were p-cymene, carvacrol, thymol and γ-terpinene. Baser et al.[30] also found that carvacrol, *p*-cymene and γ-terpinene were among the major contributors to essential oils of *O. hypericifolium.*

Aspergillus flavus, *A. niger*, *Penicillium* spp., and *Alternaria alternata* were isolated from hazelnut samples in this research. *A. flavus* is a common filamentous fungi and a major threat to agriculture and human health due to its mycotoxin production.[23,31] Aflatoxins are widely distributed toxins produced by strains of *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*.[32] *A. flavus* produces only B aflatoxins.[33] *Alternaria alternata*, *Aspergillus niger*, and mycotoxigenic *Fusarium* are other common and well-characterized species that produce the mycotoxins roquefortine C, PR-toxin, alternariol, ochratoxin A, and fumonisin.[23,34]

Mycotoxin contamination in some edible dry fruits and nuts has been reported previously,[27,35] It was also reported that molds and mycotoxins in almonds, peanuts, hazelnut, and pistachio nuts and detected aflatoxins were up to 95 mg/kg in these samples.[38] The predominant fungi presented in these samples were *A. flavus*, *A. niger*, *A. glaucus* Link ex Grey, and *Penicillium* spp.[27,37] It was demonstrated that oregano essential oils inhibited *A. niger*, *Aspergillus ochraceus*, and *A. flavus*.[38] Our findings (Tables 2 and 3) were in agreement with earlier investigations regarding the antifungal activity of essential oils by exhibiting the highest antifungal activity against soil borne

and foliar pathogenic fungi, such as *Satureja thymbra*, *Micromeria fruticosa*, *Majorana syriaca*, *Origanum syriacum*, and *Thymus vulgaris.*[39]

A literature survey showed that a variety of micro-organisms, such as *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., and *Rhizopus* spp. can lead to food spoilage in the food industry.[42] Recently, there has been considerable interest expressed in extracts and essential oils from aromatic plants with antimicrobial activities for controlling pathogens and toxin-producing micro-organisms in foods.[41–45] These properties could be because of many active phytochemicals, including flavonoids, terpenoids, carotenoids, coumarins, and curcumines.[44] Since both health and economic considerations, researches are extensively paid attention to nowadays.[38] It is thought that natural plant extracts might provide an alternative way to protect nutritions from fungal contamination.[40]

The antimicrobial nature of *O. hyprerifolium* essential oils investigated in this study was apparently related to its phenolic components, such as thymol, carvacrol, and its precursors for *p*-cymene and *c*-terpinene.[6,7,46] A study carried out on *O. acuditens*'s essential oil reported similar results with our findings.[47] It was also reported that the essential oils from *Cinnamomum zeylanicum* (cinnamon), *Mentha piperita* (peppermint), *Ocimum basilicum* (basil), *Origanum vulgare* (origanum), *Teloxys ambrosioides* (the flavoring herb epazote), *Syzygium aromaticum* (clove), and *Thymus vulgaris* (thyme) had a potent inhibitor on *Aspergillus flavus* growing on maize seeds.[8]

It was reported that *Origanum glandulosum*'s oil had the highest inhibitory effect on *Penicillium expansum*, and it was followed by *Fusarium solani* and *P. expansum*.[9] Another study expressed that standard essential oils of *Origanum* species, *O. bilgeri*, and *O. solymicum* had an antifungal effect much more that used for certain antifungal compounds on *Candida albicans.* The first oil was much more effective than that of the latter. It was also suggested that this could be because of their carvacrol and *p*-cymene contents.[10]

It was established that although the essential oil of *Origanum vulgare* had the highest potent effect on *Fusarium avenaceum*, *Paecilomyces variotii*, *Rhizopus stolonifer*, and *Scopulariopsis brevicaulis*, the following species were observed to be the most resisted organisms: *Candida glabrata*, *Saccharomyces cerevisiae*, *Geotrichum candidum*, *Aureobasidium pullulans*, and *Acremonium furcatum*.[11] As it is known, *O. vulgare*'s carvacrol and thymol contents are very low. It is thought that its inhibitory effect could be chemicals other than carvacrol and thymol. Similarly, 39 essential oils from different plants were examined on *Bauveria. cinerea*, *Cladosporium. gloeosporioides*, *Fusarium oxysporum*, *Penicillium ultimum*, and *Rhizoctonia solani*, and found that *O. vulgare*'s oil was the most potent inhibitors on the growth of fungi mentioned.[12] It was also reported that the oil of *O. vulgare* had a very effective inhibitory effect on *Aspegillus flavus*, *A. parasiticus*, *A. terreus*, *A. ochraceus*, *A. fumigates*, and *A. niger*.[13]

The essential oil of *Origanum minutiflorum* had more antifungal activity than that of *Laurus nobilis*, *Foeniculum vulgare*, and *Schinus molle*'s oils.[14] The researchers also observed that 1% of this oil had an inhibitory effect on *Penicillium rubrum* and *Alternaria alternate. Origanum heracleoticum*'s essential oil had a potent inhibitor for *Cladosporium. fulvum*, *C. cladosporioides*, *Penicillium helianthi*, *P. magdonaldii*, and *Trichophyton mentagraphytes* but *Trichoderma viride*, *Fusarium sporotrichoides*, *Penicillium*, and *Aspergillus* sp. were found to be resisted species.[15] *Origanum onites*'s essential oil had a strong antifungal activity on *Alternaria alternata*, *Aspergillus flavus* (two strains), *Aspergillus niger* (two strains), *Aspergillus parasiticus*, *Fusarium semitectum*, *Fusarium oxysporum*, *Mucor racemosus*, and *Penicillium roqueforti*.[23]

In general, major components of essential oils of *Origanum* species contain mainly aromatic monoterpenes, such as carvacrol, thymol, and *p*-cymene, and their activity are often associated with these compounds.[7–15,19,39,43–45] Our findings (Table 1) showed that these effective compounds are present at high levels in the studied essential oil extracted from *Origanum hypericifolium.* As can be seen in Table 1, besides the aromatic monoterpenes, some acyclic monoterpenes, such as linalool, were also present.

The antimicrobial activity of essential oils or their constituents, such as thymol, carvacrol, and vanillin, could be the result of damage to the enzymatic cell system, including those connected to energy production and synthesis of structural compounds.[48] It was indicated that phenolics could denature the enzymes responsible for spore germination or interfere with the amino acid involved in germination or interfere with the amino acids involved in germination.[37,49] Some scientists showed irreversible damage in cell walls, cell membranes, and cellular organelles when *A. parasiticus* and *A. flavus* were exposed to different essential oils.[50,51] In addition to this, it is important to recognize that there are complex interactions with environmental factors, such as water availability and efficacy of essential oils. It could be possible to use a combination of these to reduce the growth and aflatoxin production of *A. flavus* and *A. parasiticus*.[19,31] Oregano and its essential oils are effective against molds, especially aflatoxigenic strains. Since the antimicrobial effects of spice and herb essential oils are of interest regarding their possible usage as alternative food preservatives.[52]

Due to the increasing consumer demand for more natural foods and the increasing microbial resistance of pathogenic micro-organisms against antibiotics, natural protective substances isolated from plants are considered as promising sources of food preservatives.[12,13] In this context, aromatic plants, especially spices of *Origanum* genus, have appeared as effective compounds to provide microbiological safety of foods.[6,7,16,53] Antiradical activity of *O. vulgare*, antioxidant, antimicrobial, and radical scavenging capacity of *Cyclotrichium niveum*, belonging to the same family with Origanum, were also reported previously.[54,55] Earlier findings clearly indicated that essential oils should find practical application in the inhibition of mycotoxin production by mycotoxigenic fungi. Essential oils, such as anise and boldo, could be safely used as a preservative material on some foods because they stopped fungal growth and AFB1 accumulation. These oils could also be added to nuts in storage to protect them from fungal infection, and could be used as a substitute for chemical fungicides.[37,41]

CONCLUSION

The major constituent of SD was *p*-cymene followed by cymene followed by carvacrol, thymol, and γ-terpinene. The essential oils from *O. hypericifolium* showed a highly potent antifungal activity on fungi isolated from hazelnut and walnut. The volatile activity was found to be highly effective than that of contact activity assay. The more sensitive species examined in the study were *P. castellonense*, *P. verrucosum.* var. *cyclopium*, *C. globosum*, and *A. kiliense* in the contact assay. In the volatile assay, all the mycelial growth of all tested fungi was completely inhibited at the 3rd day of tests. On the other hand, *P. verrucosum* var. *cyclopium* was observed to be the least sensitive species in these conditions. Origanum's antifungal activity could be because of its aromatic contents, such as monoterpenes, carvacrol, thymol, and *p*-cymene. The isolated fungi, *Aspergillus flavus*, *A. niger*, and *Alternaria alternata*, are known as store molds which were isolated from hazelnut

and walnuts, and were reported to be mycotoxigenic species. In this circumstance, the oils studied could be used to prevent the harmful effects of these kinds of fungi.

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