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Effects of Some Lamiaceae Species Methanol Extracts on Potential Mycotoxin Producer Fungi

T. Askun,[1] G. Tumen,[1] F. Satil,[1] and T. Kilic[2]

[1]Balikesir University, Faculty of Science and Art, Department of Biology, 10145, Balıkesir-Turkey; [2]Balıkesir University, Faculty of Science and Art, Department of Chemistry, 10145, Balıkesir-Turkey

Abstract

In this study, antifungal effects of some Lamiaceae species (*Thymbra spicata* L.*Satureja hortensis* L., *Origanum onites* L., *O. vulgare* L. subsp. *hirtum* (Link) Iestswaart, *O. vulgare* L. subsp *vulgare*, *O. minutiflorum* O. Schwarz & P.H. Davis, *Sideritis vuralii* H. Duman & Baser and *S. caesarea* H. Duman, Aytaç & Baser) commonly used by people, were investigated. To determine the antifungal effects, the aerial parts of plant methanol extracts were tested against four fungal species, *Aspergillus flavus* Link., *A. niger* Raper, and Fennel, *A. ochraceus* K. Wilh., and *Fusarium proliferatum* (Matsushima) Nirenberg. Three plant species, *O. vulgare* subsp. *hirtum, O. minutiflorum,* and *T. spicata,* methanol extracts showed antifungicidal activity with a minimum inhibitory concentration (MIC) of 1.6 mg/ml against four potential mycotoxigenic fungi. The results were evaluated using statistical tests.

Keywords: Antifungal activity, fungi, *Origanum, Satureja, Sideritis, Thymbra.*

Introduction

Plants have the capability to produce secondary metabolites. Antifungal compounds are the products of the plant's secondary metabolism, and the action of these compounds can be used to inhibit the growth of spoilage and pathogenic microorganisms in food. These compounds may protect plants by inhibiting the growth of mycotoxigenic and pathogenic fungi.

Inhibitory effects of Lamiaceae plant extracts, using different plant parts such as bark, stem, root, leaves, and fruits against bacteria and fungi, have been investigated for biological activities. Lamiaceae plant extracts prepared by using different plant parts such as bark, stem, root, leaves, and fruits used in many biological activity studies. The extracts have been found to have antibacterial activity (Alma et al., 2003; Amanlou et al., 2004; Bozin et al., 2006; Digrak et al., 2001; Chorianopoulos at al., 2006), antifungal activity (Bouchra et al., 2003; Digrak et al., 2001; Gulluce et al., 2003; Guynot et al., 2003; Souza et al., 2005), antimycobacterial activity (Ulubelen et al., 1997), antioxidant activity (Alma et al., 2003; Bozin et al., 2006; Mosaffa et al., 2006; Gulluce et al., 2003) and anti-inflammatory activity (Alcaráz et al., 1989; Jiménez et al., 1986).

Kamatou (2005) studied some *Salvia* species for *in vitro* pharmacological activities and a chemical investigation. Boyraz and Özcan (2006) and Soylu et al. (2006) studied inhibition of phytopathogenic fungi. Inhibitory effects of oregano components on some foodborne fungi were reported (Akgul & Kivanc, 1988).

When plants are used for preservative aims, all plant parts or their water extracts, but not their essential oils, are used. For example, the fig, one of the most important export products of western Turkey, is left to dry after being soaked in boiled water with "kekik" (Tumen, 1989).

This study is a part of an ongoing biological activity investigation of species belonging to the Lamiaceae family used by local people in Turkey for different purposes.

Origanum, *Satureja*, *Thymbra*, and *Thymus* species are known as "kekik" in Turkey and are widely used for several purposes (Satil et al., 2005). Apart from their culinary usage, they are used to cure stomachaches and respiratory colds (Kahraman & Kocabas, 2001). They are also known to prevent fungus growth in dried food. The genus

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Address correspondence to: Dr. Tulin Askun, Balikesir University, Faculty of Science and Art, Department of Biology, 10145, Balıkesir-Turkey. E-mail: taskun@balikesir.edu.tr

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Origanum is represented by 32 taxa in Turkey (Ietswaart, 1982; Duman, 2000). Turkey exports over 7000 tons of dried oregano. *Origanum* species are used locally as herbal tea or condiment in Turkey (Baser, 2002; Honda et al., 1996). Dried *Origanum* species are also used to obtain essential oil (oregano) and aromatic water. *Origanum* water rich in carvacrol is taken orally to prevent gastrointestinal disorders, to reduce blood cholesterol and glucose levels. It is also antispasmodic (Baser, 2002) and antibacterial (Lambert et al., 2001). Their essential oils are used as pain killer in rheumatism by rubbing externally on the painful limbs (Baser, 2002). Recent research showed that an aqueous extract of *O. vulgare* exhibited an anti-hyperglycaemic activity in rats (Lemhandri et al., 2004) and natural products were enhancing insulin sensitivity (Talpur et al., 2005).

The genus *Sideritis* L., known as "dagcayı" in Turkey, grows worldwide with 150 species, is now represented in Turkey by 46 species and altogether 55 taxa, 42 taxa being endemic in Turkey (Huber-Morath, 1982; Duman, 2000).

Sideritis is used as an anti-inflammatory (treatment to reduce inflammation) (Yeşilada & Ezer, 1989), antispasmodic (Ezer et al., 1992), antibacterial (Ezer et al., 1994) and antimicrobial (Akcos et al., 1998). In herbal tea form, it is used as a folk medicine to cure colds (Kirimer et al., 1999; Tumen et al., 1995).

Thymbra L. is one of the aromatic genera. *Thymbra* (Lamiaceae) is represented by the following two species and four taxa, *T. spicata* L. (var. *spicata*, var. *intricata* P.H. Davis) and T. *sintenisii* Bornm. & Aznav. (subsp. *sintenisii*, subsp. *isaurica* P.H. Davis), in the Flora of Turkey (Davis, 1982). They are among the sources of commercial "Thyme" in Turkey. Dried leaves and inflorescences are known as "Zahter" or "Sater" in the southeastern Anatolia and are used as an antiseptic and stimulating herbal tea (Baytop, 1999).

The genus *Satureja* L. is represented by 15 species in Turkey (Davis, 1982a; Tumen et al., 2000). It is extensively used as digestive, diuretic, and throat curative (Satil et al., 2005; Baytop, 1999).

Aspergillus, Penicillium, Fusarium, and *Alternaria* are important contaminants of cereal grains (D'Mello et al., 1993). Aflatoxins are produced by closely related species *Aspergillus flavus*, *A. parasiticus*, *A. wentii* and *A. nomius* (Kaaya & Kyamuhangire, 2006; Samson & Pitt, 2000). Ochratoxin A is also known as an important mycotoxin produced by *A. ochraceus*, *A. carbonarius*, *A. tubingensis,* and *A. niger* (Almela et al., 2007; Medina et al., 2005; Pardo et al., 2006). Fumonisins are produced by *F. verticilloides*, *F. proliferatum*, *F. nygamai* (Chen et al., 1992), and *F. oxyporum* (Kpodo et al., 2000).

Based on the ethnobotany usages, it was decided to research the antifungal activity of some "kekik and dagcayı" species (Tumen et al., 1995). The target of our study was to show the activity of the methanol extracts from Lamiaceae family plants on mycotoxin producer fungi. These fungal contaminants cause fungal growth and have the ability to produce mycotoxin on some commodity. Lamiaceae family covers a wide range of compounds such as terpenoids and flavonoids (Richardson, 1992; Sezik et al., 1985; Ezer et al., 1996; Amanlou, 2004). The importance of methanol extracts is that it may contain some valuable compounds such as terpenes, glycosides, alkaloids, irridoids and flavones. Taking these useful utilities into consideration, antifungal activities of *O. onites*, *O. vulgare* subsp. *hirtum*, *O. vulgare* subsp. *vulgare*, *O. munitiflorum*, *T. spicata*, *S. caesarea*, *S. vuralii* and *Satureja hortensis* from different regions of Anatolia were tested. Methanol extracts of these plants were studied for antifungal activity against *Fusarium proliferatum*, *Aspergillus flavus*, *Aspergillus ochraceus* and *Aspergillus niger* were investigated and the correlation between *in vitro* activity and ethnobotanical usage were compared.

Materials and Methods

Plant materials

Aerial parts (Herba in flowering stage) of plants were collected in June-July 2005. Locality, altitude, collection time and herbarium number of species are given in Table 1. Plants were identificated by Prof. Dr. Gulendam Tumen.

Preperation of extracts

The air-dried plants (at room temperature) of *O. onites* (135 g), *O. vulgare* subsp *hirtum* (63 g), *O. vulgare* subsp. *vulgare*, (115 g), *O. munitiflorum* (115 g) *T. spicata* (70 g), *S. vuralii* (71 g), *S. caesarea* (135 g) and *S. hortensis* (110 g) were extracted with 1 L methanol (98%) at room temperature during ten days according to Seshandri (1962) method. The methanol extracts were dried *in vacuo* at 40°C. The total yield quantities were 2.80, 1.54, 2.05, 1.85, 1.37, 1.42, 2.24, and 1.17 g, respectively. All stocks were kept in a deep freezer at −20°C.

Microorganisms

F. proliferatum, *A. flavus*, *A. ochraceus* and *A. niger* were used as test organisms in the antifungal assay. These organisms were chosen as representative species for fungi producing mycotoxin.

Minimum Inhibitory Concentration (MIC)

Strains were incubated on potato dextrose agar slants for 7–10 days. Methanol extracts of plants were prepared in 4, 2, 1, and 0.5 mg/mL concentrations and mixed with sterile semi-solidifying PDA medium. Final concentrations were 1.6, 0.4, 0.1, and 0.025 mg/ml, consecutively. After pouring into sterile Petri dishes (10 ml of each plate) and solidifying, a 5 mm diameter disk was placed in the centre of the agar surface for each plate (Soliman & Badeaa, 2002). Conidial suspension was adjusted to the required concentration (10^5 cfu/ml) by counting in a hemacytometer. Each disk was

Table 1. Herbarium data of Lamiaceae family species.

Genus species authority	Locality	Herbarium data	Collection Date
Origanum onites L.	Balıkesir, Yaşyer Köyü	FS1435	15.06.2005
Origanum vulgare L. subsp *hirtum* (Link) Iestswaart	Balıkesir, Yaşyer Köyü	FS1436	15.06.2005
Origanum vulgare L. subsp *vulgare*	Erzurum, Pasinler	FS1039	08.08.2002
Origanum minutiflorum O. Schwarz & P.H. Davis	Antalya, Beydağı	FS1439	21.07.2005
Thymbra spicata L.	Balıkesir, Ivrindi	FS1437	14.06.2005
Sideritis vuralii H.Duman & Baser	Mersin, Anamur	FS1440	01.10.2005
Sideritis caesarea H.Duman, Aytaç & Baser	Kayseri, Sarız	FS1438	28.07.2005
Saturea hortensis L.	Erzurum, Horasan	FS1041	08.08.2002

inoculated with a spore suspension of fungi. Plates were incubated under 27 ± 1°C for 3 days. Two replicates were used for each treatment.

Determination of fungistatic activities

Diameters of growth zone were measured twice by forming different angels and mean was calculated. Inhibition of fungal colony in percent (I%) was calculated as below:

$$I\% = (Dc - Ds)/Dc^{*}100$$

where D_c is the diameter of control (mm), and D_s is the diameter of sample (mm).

Statistics

In this study, the results were evaluated using one-way ANOVA, Tukey's HSD test and univariate two way ANOVA test. All results were obtained through independent experiments and expressed as significance (Table 3).

Subsequently, means were separated using Tukey's Honestly Significant Difference (HSD) test. Data for the correlations between/among (fungus-plant), (concentration-plant) (fungus-concentration-plant), (fungus-concentration) were also subjected to univariat two way ANOVA test. Results were considered statistically significant when $p < 0.05$.

Results

In this study, eight plant methanol extracts, three of which (*O. minutiflorum,* S. caesarea and *S. vuralii*) are endemic to Turkey, were used (Table 1).

O. minutiflorum, *O. vulgare* subsp. *hirtum*, *T. spicata* possessed the most effective methanol extracts (MIC values = 1.6 mg/mL; $p < 0.001$). Their methanol extracts showed fungicidal activity with 100% inhibition against four potential mycotoxigenic fungi (Tables 2 and 3).

Whereas, *S. ceasarea*, *O. vulgare* subsp. *vulgare*, *S. vuralii,* and *S. hortensis* showed no fungicidal activity (MIC value > 1.6 mg/mL), they showed fungistatic activity at 0.1 mg/mL concentration and over ($p < 0.001$), *O. onites* showed good fungicidal activity (MIC value = 1.6 mg/mL, $p < 0.001$) against three fungi (*A. niger*, *A. ochraceus* and *F. proliferatum*). *A. flavus* was resistant to this extract in all concentrations (Tables 2 and 3).

Table 2. Antifungal activity (MIC) of plants methanol extracts.

		MIC (mg/ml)			
Plants	Fungi	1.6	0.4	0.1	0.025
Origanum onites	*A. niger*	−	+	+	+
	A. flavus	+	+	+	+
	A. ochraceus	−	+	+	+
	F. proliferatum	−	+	+	+
Origanum vulgare subsp. *hirtum*	*A. niger*	−	+	+	+
	A. flavus	−	+	+	+
	A. ochraceus	−	+	+	+
	F. proliferatum	−	+	+	+
Origanum vulgare subsp. *vulgare*	*A. niger*	+	+	+	+
	A. flavus	+	+	+	+
	A. ochraceus	+	+	+	+
	F. proliferatum	+	+	+	+
Origanum minutiflorum	*A. niger*	−	+	+	+
	A. flavus	−	+	+	+
	A. ochraceus	−	+	+	+
	F. proliferatum	−	+	+	+
Thymbra spicata	*A. niger*	−	+	+	+
	A. flavus	−	+	+	+
	A. ochraceus	−	+	+	+
	F. proliferatum	−	+	+	+
Sideritis vuralii	*A. niger*	+	+	+	+
	A. flavus	+	+	+	+
	A. ochraceus	+	+	+	+
	F. proliferatum	+	+	+	+
Sideritis ceasarea	*A. niger*	+	+	+	+
	A. flavus	+	+	+	+
	A. ochraceus	+	+	+	+
	F. proliferatum	+	+	+	+
Satureja hortensis	*A. niger*	+	+	+	+
	A. flavus	+	+	+	+
	A. ochraceus	+	+	+	+
	F. proliferatum	+	+	+	+

Table 3. Fungicidal and fungistatic effects of Lamiaceae family methanol extracts against *A. niger, A. flavus,* A. *ochraceus* and *F. proliferatum* and colony growth reduction rate.

			Concentrations							
			1.6 mg/ml		0.4 mg/ml		0.1 mg/ml		0.025 mg/ml	
	Fungi	Control	Colony diameter (mm)	Reduction of growth %	Colony diameter (mm)	Reduction of growth %	Colony diameter (mm)	Reduction of growth %	Colony diameter (mm)	Reduction of growth %
Origanum onites	*A. niger*	55.00	0.00	100.00	30.00	45.45	49.00	10.91	49.50	10.00
	A. flavus	45.00	11.00	75.56	30.00	33.33	42.00	6.67	43.30	3.78
	A. ochraceus	20.30	0.00	100.00	15.50	23.65	15.00	26.11	23.30	−14.78
	F. proliferatum	35.30	0.00	100.00	22.50	36.26	30.00	15.01	32.50	7.93
Origanum vulgare subsp. *hirtum*	*A. niger*	55.00	0.00	100.00	10.00	81.82	33.50	39.09	47.80	13.09
	A. flavus	45.00	0.00	100.00	13.50	70.00	33.00	26.67	40.80	9.33
	A. ochraceus	20.30	0.00	100.00	12.50	38.42	13.30	34.48	21.50	−5.91
	F. proliferatum	35.30	0.00	100.00	11.50	67.42	22.50	36.26	30.50	13.60
Origanum vulgare subsp. *vulgare*	*A. niger*	55.00	33.30	39.45	33.50	39.09	33.50	39.09	52.80	4.00
	A. flavus	45.00	31.30	30.44	38.50	14.44	43.00	4.44	43.50	3.33
	A. ochraceus	20.30	17.50	13.79	17.30	14.78	18.80	7.39	25.20	−24.14
	F. proliferatum	35.30	20.80	41.08	25.00	29.18	30.50	13.60	33.50	5.10
Origanum minutiflorum	*A. niger*	55.00	0.00	100.00	18.00	67.27	43.30	21.27	47.00	14.55
	A. flavus	45.00	0.00	100.00	16.00	64.44	36.50	18.89	41.80	7.11
	A. ochraceus	20.30	0.00	100.00	12.30	39.41	14.80	27.09	23.00	−13.30
	F. proliferatum	35.30	0.00	100.00	13.00	63.17	30.00	15.01	31.00	12.18
Thymbra spicata	*A. niger*	55.00	0.00	100.00	25.00	54.55	45.00	18.18	50.50	8.18
	A. flavus	45.00	0.00	100.00	23.80	47.11	42.00	6.67	42.80	4.89
	A. ochraceus	20.30	0.00	100.00	14.30	29.56	19.00	6.40	23.00	−13.30
	F. proliferatum	35.30	0.00	100.00	14.00	60.34	31.00	12.18	33.30	5.67
Sideritis vuralii	*A. niger*	55.00	29.30	46.73	45.00	18.18	49.50	10.00	52.00	5.45
	A. flavus	45.00	29.30	34.89	40.00	11.11	45.00	0.00	43.50	3.33
	A. ochraceus	20.30	16.50	18.72	18.50	8.87	19.50	3.94	22.50	−10.84
	F. proliferatum	35.30	22.00	37.68	29.30	17.00	34.00	3.68	35.00	0.85
Sideritis ceasarea	*A. niger*	55.00	28.30	48.55	27.00	50.91	28.30	48.55	30.00	45.45
	A. flavus	45.00	27.00	40.00	22.50	50.00	25.30	43.78	25.00	44.44
	A. ochraceus	20.30	16.80	17.24	16.00	21.18	19.30	4.93	17.30	14.78
	F. proliferatum	35.30	19.50	44.76	22.00	37.68	24.00	32.01	23.30	33.99
Satureja hortensis	*A. niger*	55.00	20.80	62.18	41.50	24.55	50.00	9.09	50.30	8.55
	A. flavus	45.00	21.00	53.33	33.50	25.56	44.00	2.22	43.50	3.33
	A. ochraceus	20.30	13.50	33.50	16.00	21.18	17.80	12.32	23.50	−15.76
	F. proliferatum	35.30	12.00	66.01	21.00	40.51	32.00	9.35	33.30	5.67

Discussion

Concentration-dependent differences were observed with various fungi based on their morphological characteristics ($p < 0.001$). Mycelial growth was decreased and pigmentation colour in all fungi was inhibited at concentrations higher than 1 mg/mL (Table 3).

The filamentous fungi showed variable sensitivity. Results showed that *O. vulgare* subsp. *hirtum*, *O. minutiflorum,* and *T. spicata* methanol extracts displayed strong activity with complete inhibition of fungi at 4 mg/mL extract concentration (MIC value = 1.6 mg/mL) (Table 2).

O. onites also showed good activity with inhibition at 4 mg/mL of extract concentration (MIC value = 1.6 mg/mL), except *A. flavus* (MIC value >1.6 mg/mL). *O. vulgare* subsp. *vulgare*, *S. hortensis*, *S. ceasarea*, and *S. vuralii* showed only fungistatic activity by reducing the appearance of pigmentation colour and the colony size when compared with control groups (MIC >1.6 mg/mL).

This study indicated that addition of methanol crude extracts from O. *vulgare* subsp. *hirtum*, *O. minutiflorum* and *T. spicata* to the medium inhibited the growth of filamentous fungi *F. proliferatum*, *A. flavus*, *A. ochraceus* and *A. niger*.

In the tests, antifungal effect changed according to plant species. When subgroups were evaluated statistically by Tukey's HSD Test, depending on mean sample size and alpha = 0.05, six subgroups in eight species was found

Table 4. The two way Anova test showing the interactions among the subjects.

Source	df	Mean Square	F	P
Fungus-Concentration	12	780.8	416.195	0.001
Fungus - Plant	21	105.243	56.098	0.001
Concentration Plant	28	580.867	309.624	0.001
Fungus-Concentration-Plant	84	58.716	31.298	0.001

R Squared = 0.994 (Adjusted R Squared = 0.992).

($p < 0.001$). Differences in the groups having the same letter are unimportant (Table 5).

When *in vitro* antifungal activity of methanol extracts, *O. minutiflorum*, *O. vulgare* subsp. *hirtum*, *T. spicata*, were compared with their ethnobotanical usage, parallelism was observed. Common usage of these plants is not only to prevent the growth of fungus in dried food but also to cure stomachaches, respiratory track infections, colds, and diabetes.

Tests between subject effects were evaluated by univariate ANOVA test. The correlations between/among (fungus-plant), (concentration-plant), (fungus-plant-concentration) and (fungus-concentration) were important ($p < 0.001$) (Table 4).

The results obtained from this study showed that the activities were closely related to concentration. *O. minutiflorum*, *O. vulgare* subsp. *hirtum*, *T. spicata* extracts had great fungicidal effects in high concentrations and fungistatic effects in lower concentrations. This property of the valuable compounds may help as a safer alternative to protect food from mycotoxigenic fungi.

After determining high antifungal activity of some of these methanol extracts, the next step might be the isolation of the effective pure compounds from the extracts.

Table 5. Tukey's HSD test depends upon antifungal effect of plants species.

Plants	Means
Origanum vulgare subsp. *hirtum*	22.8375a
Origanum minutiflorum	24.1000b
Thymbra spicata	25.9500c
Sideritis ceasarea	26.3375c
Origanum onites	27.3750d
Satureja hortensis	31.4500e
Origanum vulgare subsp. *vulgare*	31.4875e
Sideritis vuralii	34.3125f

Means for groups in homogeneous subsets are displayed; x Uses Harmonic Mean Sample Size = 80.000. y Alpha = 0.05; *Differences having the same letters are unimportant.

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