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In vitro antifungal effects of *Fumaria vaillantii* Loisel. essential oil on *Aspergillus flavus*

M. Moghtader

Department of Biodiversity, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran. E-mail:moghtader18@yahoo.com.

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In order to identify the chemical composition of essential oil of *Fumaria vaillantii*, the leaves with young branches of this plant which grows in a village in Kerman Province at full flowering stage in May 2012 were collected. The sample was cleaned and then dried in the shade, and essential oil hydrodistillation method was performed. The main oil content from the plant of *F. vaillantii* was 0.25% (v/w) and that essential oil was analyzed by capillary gas chromatography (GC) using flame ionization (FID) and capillary gas chromatography coupled mass spectrometry (GC/MS) for detection. Eighteen compounds were identified in the essential oil of *F. vaillantii* that included 99.62% of the total oil. The major components were Parfumidine (18.94%), Fumaricine (16.30%), Thymol (12.45%) and Fumaritine (10.78%). The study of the antifungal effects of the oil sample was carried against strain of *Aspergillus flavus* (PTCC=5004) by disc diffusion method via average inhibition zone. The results show that the essential oil from fumaria plant at 1 and 1/2 oil dilutions exhibited strong antifungal activity than gentamycin antibiotic on *A. flavus* and synthetic thymol exhibited good inhibition at 10% dilution. Large percentage antifungal activities of fumaria oil are related with thymol as a natural monoterpene phenol is the main compound.

Key words: *Fumaria vaillantii* Loisel. essential oil, *Aspergillus flavus*, aflatoxin, thymol.

INTRODUCTION

Aspergillus is a genus of moulds reproduced only asexually. Some *Aspergillus* species function as plant and/or animal pathogens (Bennett, 2010; Geiser, 2009). More than 60 *Aspergillus* species are medically relevant pathogens (Thom, 1926). The most common pathogenic species are *Aspergillus fumigatus* and *Aspergillus flavus*. *A. flavus* produces aflatoxin which is both a toxin and a carcinogen, and which can contaminate foods such as nuts. The most common that cause allergic disease are *A. fumigatus* and *Aspergillus clavatus*. Other species are important as agricultural pathogens. *Aspergillus* spp. cause disease on many grain crops, especially maize, and synthesize mycotoxins including aflatoxin. Thymol (2-isopropyl-5-methylphenol) is a natural monoterpene phenol extracted from *Thymus vulgaris* (common thyme) and various other kinds of plants as a white crystalline substance of a pleasant aromatic odor and strong antiseptic properties. Thymol is part of a naturally occurring class of compounds known as biocides, with strong antimicrobial attributes when used alone or with other biocides such as carvacrol (Ahmad et al., 2010). The antifungal nature of thymol is caused by thyme's ability to alter the hyphal morphology and cause hyphal aggregates, resulting in reduced hyphal diameters and lyses of hyphal wall (Numpaque et al., 2011). Additionally, thymol is lipophilic, enabling it to interact with the cell membrane of fungus cells, altering cell membrane permeability permitting the loss of macromolecules (Segvic et al., 2007). This study evaluated and identified the chemical compounds of *F. vaillantii*. Also, antifungal activity of *F. vaillantii* has been compared with synthetic thymol and standard gentamicin

antibiotic on culture of *A. flavus.*

MATERIALS AND METHODS

Plant material collection and isolation of their essential oil

The leaves and young branches of *F.vaillantii* were collected at Kerman Province (Iran) at full flowering stage in May 2012. The samples were air-dried and powdered using a milling machine and kept in a cool dry place until ready for extraction of the essential oil. Afterwards, essential oil was taken from 150 g of the powdered sample in hydro distillation method with the help of Clevenger set for 3 h. The sample oils were dried with anhydrous sodium sulfate and kept in sterile sample tubes in a refrigerator.

Analysis of essential oil

Gas chromatography

GC analysis was performed using a HP-439 gas chromatograph equipped with a CP-Sil 5CB capillary column (25 m × 0.25 mm id, 33 µm film thickness). Oven temperature was from 60 to 220°C at 7°C min. Injector temperature 280°C detector (FID) temperature 270°C and carrier gas was helium (ml/min).

Gas chromatography/mass mass spectrometry

GC-MS analyses were carried out using a Hewlett Packard-5973 apparatus which was equipped with a MS reference library and a HP 5MS cross linked fused-silica capillary column (60 m × 0.25 mm i.d., 0.25 µm phase thickness). The oven temperature program was 60°C for 3 min, rising to 220°C at 6°C/min, 220°C at 20°C/min, 220°C (3 min); the injector temperature was 280°C; the carrier gas was helium at 1 mL7min; the injection mode was split with a split ratio of 1:43; the sample volume injected was 0.1 µL; the interface temperature 230°C. Identification and quantification of oil components, the components of the oil were identified by comparison of their linear retention indices (LRIs) on the CP-Sil 5CB column (determined in relation to a homologous series of n-alkanes, C8-C17) with those of pure standards or as reported in the literature (Adams, 2001). The percentages of each component were reported as raw percentages without standardization.

Antifungal assay

Antifungal activity of the essential oil was assayed using the agar disc diffusion method using Mueller Hinton Agar (Baron and Finegold, 1995) and the measure of inhibition zones at different oil dilutions against *A. flavus* (PTCC=5004) from Center for Fungi and Bacteria of Iranian Scientific and Industrial Researches Organization was done. A sample of 50 µL of a suspension of the tested microorganism was spread onto the surface of Mueller-Hinton agar plates. Filter paper discs (5 mm in diameter) were placed on the surface of inoculated plates, and then they were soaked with 50 µL of essential oil dilutions in DMSO (1, 1/2, 1/4, 1/8 and 1/16). Gentamicin (8 mg/ml) was used as positive control. After incubation at 24°C for 48 and 72 h, the diameters of the inhibition zones were measured in millimeters. Each test was carried out in triplicate.

RESULTS AND DISCUSSION

The *F. vaillantii* essential oil yield was 0.25% (v/w). 18 compounds were identified in the essential oil of this plant with 99.62%; the combinations of Parfumidine (18.94%); Fumaricine (16.30%), Thymol (12.45%) and Fumaritine (10.78%) with 58.47% constitute the highest percentage of essential oil (Table 1).

The results of studying the antifungal impacts of the *F. vaillantii* essential oil shows that the oil of this plant has an inhibitory effect in 1, 1/2, 1/4, 1/8 and 1/16 dilution with average diameter growth of 28, 22, 15, 12 and 8 mm respectively. The results of standard antibiotic gentamicin (8mg/ml) with a diameter of 19 mm had inhibitory effect. Synthetic thymol in 1% dilution had no inhibitory effect on *A. flavus* growth but at 10% dilution had a good inhibition (18 mm) of the fungi growth (Table 2). The results show that the essential oil from fumaria plant at 1 and 1/2 oil dilutions exhibited strong antifungal activity than gentamycin antibiotic on *A. flavus* and thymol exhibited good inhibition at 10% dilution. The large percentage antifungal activities of fumaria oil is related to thymol a natural monoterpene phenol which is the main compound.

The essential oil of *F.vaillantii* plant has been studied less in Iran and in the world. In a report, Eugenol (1) and thymol (2) exhibited excellent fungicidal activity against pathogenic yeasts, including isolates resistant to azoles. The rapid irreversible action of compound-1 and compound 2 on fungal cells suggested a membrane-located target for their action (Ahmad et al., 2010). *Fumaria officinalis* is approved for the indication of colicky pain affecting the gallbladder and biliary system, together with the gastrointestinal tract (Hentschel et al., 1995). In a report, the essential oil was classified into three groups. The first group, composed of citron, lavender and tea tree oils, stopped the apical growth in a loading dose of 63 µg ml^{-1} air, but allowed the regrowth of the hyphae after removal of the vapor, indicating fungi static action. The second group, consisting of perilla and lemon-grass oils, stopped the apical growth in a loading dose of 6.3 µg ml^{-1} air, and did not allow the regrowth after gaseous contact at 63 µg ml^{-1} air, indicative of fungicidal action. The third group, consisting of cinnamon bark and thyme oils, retarded the growth in a dose of 6.3 µg ml^{-1} air, stopped it in a dose of 63 µg ml^{-1} air, and incompletely suppressed regrowth of the hyphae (Inouye et al., 2000). In a research, minimum inhibitory concentration (MIC) of thymol on *A. flavus* was highly effective at doses as low as 250 ppm (Mahmoud, 1994). In a paper, total quinolizidine alkaloid contents were 426 mg/100 g (*F. capreolata)* and 521 mg/100 g (*F. bastardi)*. The isoquinoline alkaloids, stylopine, protopine, fumaritine, fumaricine, fumarophycine, fumariline and fumarofine were determined. In the first species, an ester of phtalic acid was identified, and in the second species a peak seems to be a benzophenanthridine was identified, probably dehydro derivative and three other peaks were identified as phtalidisoquinoline; one of them seems to be dihydrofumariline. The chemotaxonomic significance of

Table 1. Compounds identified in the essential oil of *Fumaria vaillantii* Loisel.

Compound Name	Restrictive Index (RI)	Percentage (%)
Cryptopine	1093	0.29
Corydamine	1135	0.82
Fumarophycine	1176	2.96
α-terpineol	1185	1.89
Hydrastine	1206	5.06
Unknown	1252	0.16
Unknown	1287	0.22
Thymol	1287	12.45
Carvacrol	1306	8.53
Parfumine	1342	1.68
Stylopine	1474	5.73
Sinactine	1488	2.32
Fumariline	1492	3.42
Protopine	1502	4.29
Fumaritine	1516	10.78
Parfumidine	1533	18.94
Fumaricine	1575	16.3
Adlumine	1589	0.63
Bicuculline	1607	0.3
Dihydrofumariline	1622	3.23
Total		99.62

The indexes of restrictive have been calculated by injecting the mixture of normal hydrocarbons (C8-C17) to HP-5MS column.

Table 2. The zone diameter (mm) of inhibition of antibiotic, fumaria oil and synthetic thymol on *aspergillus flavus* (mm)

Antibiotic	Dilutions of fumaria oil					Synthetic thymol (%)	
Gentamycin (8 mg/ml)	**1**	**1/2**	**1/4**	**1/8**	**1/16**	**1**	**10**
19	28	22	15	12	8	0	18

the results is discussed (Maiz-Benabdesselam et al., 2007). In a report, thymol and carvacrol inhibited the radial growth of *Colletotrichum acutatum* and *Botryodiplodia theobromae* completely and this effect remained for 240 h. Furthermore, thymol and carvacrol were metabolized by the plant pathogenic fungi in low proportion to several compounds, including thymoquinone, thymohydroquinone, thymyl and carvacryl acetate, thymyl and carvacryl methyl ether. The transformations affect the structural requirements of thymol and carvacrol related to their antimicrobial activity and mode of action. The relatively high antifungal activity of thymol and carvacrol against *C. acutatum* and *B. theobromae* and the low levels of microbial transformation indicate that both compounds could be an alternative to traditional chemical fungicides for the control of pre- and postharvest phytopathogenic fungi on fruits or vegetables (Numpaque et al., 2011). The essential oil of the aerial parts of *Rosmarinus officinalis* collected from Konya, Turkey was analyzed by gas chromatography and gas chromatography mass spectrometry. The oil yield of dried plant (volume/dry weight) obtained by hydro distillation was 1.9%. 20 compounds representing 99.93% of the oils were identified. The main constituents of the oils were *p*-cymene (44.02%), linalool (20.5%), γ-terpinene (16.62%), thymol (1.81%), β-pinene (3.61%), α-pinene (2.83%) and eucalyptol (2.64%). The oil consisted of monoterpenic hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons. Also, the inhibition effect of rosemary oil was investigated against *Alternaria alternata*, *Botrytis cinerea* and *Fusarium oxysporum*. The experiment was carried out *in vitro* using disc diffusion to investigate the antifungal action of the oil. Oil tested on potato dextrose agar plates exhibited an inhibitory effect. The extent of inhibition of fungal growth varied depending

on the levels of essential oil used in experiment (Ozcan *et al.,* 2008). In a research, the 50% ethanolic extract of *Fumaria indica* was investigated for its anti-inflammatory and antinociceptive potential in animal models. The extract (400 mg kg^{-1}) exhibited maximum anti-inflammatory effects of 42.2 and 42.1% after 3 h with carrageenean and histamine, respectively. The same dose of extract showed 38.9% reduction in granuloma mass in a chronic condition. A significant anti-nociceptive activity was evidenced in mice; 6.6 to 67.7% ($p < 0.01$) protection in mechanical, 33.9 to 125.1% ($p < 0.05$) protection in thermal induced pain and 22.2 to 73.9% ($p < 0.05$) protection in acetic acid-induced writhing (Rao et al., 2007). In a study, minimum inhibitory concentrations (MIC) of both thymol and essential oil (*Thymus vulgaris* L.) were below 20 µg ml^{-1}, except for Mucor spp. (µg ml^{-1}). Thymol exhibited approximately three-times stronger inhibition than essential oil of thyme. The vaporous phase of the thyme essential oil (82 µg l^{-1}) in glass chambers strongly suppressed the sporulation of moulds during 60 days of exposure. The thyme essential oil possesses a wide range spectrum of fungicidal activity. The vaporous phase of the oil exhibited long-lasting suppressive activity on moulds from damp dwellings. Essential oil of thyme and thymol could be used for disinfection of mouldy walls in the dwellings in low concentration (Segvic *et al.,* 2007). In a report, essential oils of 12 medicinal plants were tested for inhibitory activity against *A. flavus*, *A. parasiticus*, *A. ochraceus* and *Fusarium moniliforme*. The oils of thyme and cinnamon (< or = 500 ppm), marigold (< or = 2000 ppm), spearmint, basil, and quyssum (3000 ppm) completely inhibited all the test fungi. Caraway was inhibitory at 2000 ppm against *A. flavus*, *A. parasiticus* and 3000 ppm against *A. ochraceaus* and *F. moniliforme*. *A. flavus*, *A. ochraceus*, *A. parasiticus* and *F. moniliforme* were completely inhibited by anise at< or = 500 ppm. However, chamomile and hazanbul at all concentrations were partially effective against the test toxigenic fungi. The results indicate that the test toxigenic fungi are sensitive to the 12 essential oils, and particularly sensitive to thyme and cinnamon. The results also show that the essential oils of thyme, cinnamon, anise and spearmint have more effect on fungal development and subsequent mycotoxin production in wheat grains. The extent of inhibition of fungal growth and mycotoxin production was dependent on the concentration of essential oils used (Soliman et al*.,* 2002). In a research, the isoquinoline alkaloids protopine, cryptopine, sinactine, stylopine, bicuculline, adlumine, parfumine, fumariline, fumarophycine, fumaritine, dihydrofumariline, parfumidine and dihydrosanguinarine were determined in *Fumaria agraria, F. bastardii, F. capreolata, F. sepium, F. densiflora, F. faurei, F. officinalis* subsp. *officinalis, F. parviflora, F. petteri* subsp. *calcarata* and *F. macrosepala* (Suau et al*.,* 2002). Essential oil of white wood (*Melaleuca cajeputi*) gave the highest inhibition followed by the essential oils of cinnamon (*Cinnamomum cassia*) and lavender (*Lavandula officinalis*), respectively. Furthermore, the inhibitory effects of these three essential oils at different concentrations were examined. It was found that the essential oil of white wood at 1.5625% (v/v) and of cinnamon and lavender at 50% (v/v) were the optimum concentrations for fungal growth inhibition. The essential oil of white wood at 25% (v/v) completely inhibited the growth of *A. flavus* IMI 242684 on PDA for 28 days (Thanaboripat et al., 2007). In this study, we find out that regarding the antifungal effects of *F.vaillantii* essential oil under investigation as compared with synthetic thymol and gentamycin antibiotic, this essential oil can be used as a combination with antifungal effects of natural origin.

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