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Full Length Research Paper

Antifungal effects of the essential oil from *Thymus vulgaris* L. and comparison with synthetic thymol on *Aspergillus niger*

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The antifungal effects of the essential oil from *Thymus vulgaris* L. and comparison with synthetic thymol on *Aspergillus niger* growth was studied. The chemical composition of the essential oil of *T. vulgaris*, the aerial parts of this plant which is grown in a village in Kerman Province at full flowering stage in June 2012 were collected. The sample was cleaned, dried in the shade and hydro distillation method was performed for the extraction of essential oil. The main oil content from the plant of *T. vulgaris* was 2.25% (v/w). Essential oil was analyzed by capillary gas chromatography (GC) using flame ionization (FID) and capillary gas chromatography coupled mass spectrometry (GC/MS) for detection. Thirty-two compounds were identified in the essential oil of *T. vulgaris* that concluded 99.56% of the total oil. The major components were Thymol (32.67%), *P*-cymene (16.68%), γ-terpinene (12.65%) and Carvacrol (8.32%). The study of antifungal effects of the oil sample was tested against strain of *A. niger* (PTCC=5223) fungi by disc diffusion method via average inhibition zone. The results show that essential oil from *Thymus* plant at 1, 1/2 and 1/4 oil dilutions exhibits strong antifungal activity than Streptomycin sulphate (72% SP) and gentamycin (8 mg/ml) antibiotics on *A. niger* and that exhibited on strong synthetic thymol was at 10% dilution. The high percentage antifungal activities of *Thymus* oil are related with thymol is a natural monoterpene phenol as the main compound.

Key words: *Thymus vulgaris* L., *Aspergillus niger*, essential oil, antifungal activity, fungal growth, thymol.

INTRODUCTION

Aspergillus is a genus consisting of several hundred mold species found in various climates worldwide. *A. niger* is a fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil and is commonly reported from indoor environments, where its black colonies can be confused with those of *Stachybotrys* species which is also called black mould (Samson et al., 2001). Some strains of *A. niger* have been reported to produce potent mycotoxins called ochratoxins (Abarca et al., 1994) but other sources disagree, claiming this report is based upon misidentification of the fungal species. Recent evidence suggests that some *A. niger* strains produce ochratoxin A (Schuster et al., 2002). *A. niger* is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and in severe cases, damage to the ear canal and tympanic membrane.

The genus Thymus (thyme) contains about 350 species of aromatic perennial herbaceous plants and sub shrubs 40 cm tall in the family Lamiaceae, native to temperate regions in Europe, North Africa and Asia. Stems tend to be narrow or even wiry; leaves are evergreen in most species, arranged in opposite pairs, oval, entire, and small, 4 to 20 mm long, and usually aromatic. Thyme flowers are in dense terminal heads, with an uneven calyx, with the upper lip three-lobed, yellow, white or purple. Several members of the genus are cultivated as culinary herbs or ornamentals, when they are also called thyme after its best-known species, *T. vulgaris* or Thyme Green. *T. vulgaris* L. or common thyme is a low growing herbaceous plant, sometimes becoming somewhat woody. It is an evergreen shrub growing to 0.2 m (0 ft 8 in) by 0.3 m (1 ft). It is hardy to zone 7 and is not frost tender. It is in leaf 12 January it is in flower from June to August. The flowers are hermaphrodite (have both male and female organs) and are pollinated by Bees, flies, Lepidoptera. It is noted for attracting wildlife. Herbalists of the middle Ages regarded thyme as a stimulant and antispasmodic and recommended sleeping on thyme and inhaling it as a remedy for melancholy and epilepsy.

In 1725, a German apothecary discovered that the plant's essential oil contains a powerful disinfectant called thymol that is effective against bacteria and fungi. Thymol also acts as an expectorant, loosening phlegm in the respiratory tract so it can be coughed up. Later herbalists listed thyme for these uses and as remedy for numerous other complaints, including diarrhea and fever. They prescribed the oil externally as an antiseptic for fungal infections such as athlete's foot. Thymol (also known as 2-isopropyl-5-methylphenol, IPMP) is a natural monoterpene phenol derivative of cymene, $C_{10}H_{14}O$, isomeric with carvacrol, found in oil of thyme, and extracted from *T. vulgaris* (common thyme) and various other kinds of plants as a white crystalline substance of a pleasant aromatic odor and strong antiseptic properties. Thymol also provides the distinctive, strong flavor of the culinary herb thyme, also produced from *T. vulgaris*. 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. thymol has been shown to be an effective fungicide (Ahmad et al., 2010). Thymol has microbial activity because of its phenolic structure. There is evidence supporting the belief that thymol, when applied two to three time’s daily, can eliminate certain kinds of fungal infections that affect fingernails and toenails in humans. Regular application to the affected nail over periods of about three months has been shown to eliminate the affliction by effectively preventing further progress by simply cutting the nail as one normally would, all infected material is eventually eliminated.

The antifungal nature of thymol is caused by thymol'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 by permitting the loss of macromolecules (Segvic et al., 2007). Oil of thyme, the essential oil of common thyme (*T. vulgaris*), contains 20 to 54% thymol. Thyme essential oil also contains a range of additional compounds, such as *p*-cymene, myrcene, borneol and linalool. Thymol, an antiseptic, is the main active ingredient in various commercially produced mouthwashes such as Listerine (Pierce, 1999). Before the advent of modern antibiotics, oil of thyme was used to medicate bandages. Thymol has also been shown to be effective against various fungi that commonly infect toenails (Ramsewak et al., 2003). Thymol can also be found as the active ingredient in some all-natural, alcohol-free hand sanitizers. A tea made by infusing the herb in water can be used for coughs and bronchitis. One study by Leeds Metropolitan University found that thyme may be beneficial in treating acne. This study evaluated and identified the chemical compounds of *T. vulgaris* mainly. Also antifungal activity of *T. vulgaris* has been compared with synthetic thymol and Streptomycin sulphate (72% SP), gentamicin (8 mg/ml) antibiotics standard on culture of *A. niger* (PTCC=5223) fungi*.*

MATERIALS AND METHODS

Plant material collection and isolation of their essential oil

The aerial parts of *T. vulgaris* L. were obtained from this plant grown in a village in Kerman Province, Iran at full flowering stage in June 2012. This plant identify by the botany herbarium in university of Kerman. The sample was cleaned in shade condition to prevent volatility of the plant material constituents and to keep the natural color of the sample fixed. Then they were air-dried and were 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 three hours. Following the sample oils were dried with anhydrous sodium sulfate and kept in sterile sample tubes in refrigerator. The oil yield from aerial parts of *T. vulgaris* plant was calculated.

Analysis of essential oil

Gas chromatography

GC analysis was performed using a model HP-439 gas chromatograph equipped with column CP Sil. 5 CB in 25 m length, internal diameter of 0.25 mm and film thickness 0.39 µm. Oven temperature was from 60 to 220°C at a rate of 7°C slope per minute. Injector temperature was 280°C and detector (FID) temperature was 270°C and carrier gas was helium.

Gas chromatography / mass spectrometry

In order to analyze and identify the combinations forming the essential oil, the chromatograph gas set attached to a mass spectrometry, Model Hewlett Packard-5973 was used. The conditions of analysis and specifications of the GC/MC set were as follows: Capillary column HP 5MS in 60 m length, internal diameter of 0.25 mm and layer thickness of 0.25 µM, thermal program of oven (3 min) in 60°C, then 60 to 220°C with a 6°C slope per minute, then 3 min in 220°C, the temperature of place of injection 280°C, gas conveying helium, the speed of gas move 1.0 milliliter per minute, the ratio of fission 1 to 43, the rate of injection 0.1 µL, temperature of the reservoir of ionization 230°C, ionization mode EI, Ionization energy 70eV. The series of normal Alkans C8-C17 were also injected to the set under the same condition with that of

Table 1. Combinations identified in the essential oil of *Thymus vulgaris* L.

Compound name	Restrictive index (RI)	Percentage (%)
Tricyclen	925	0.68
α-thujene	928	1.35
α-pinene	935	2.24
Camphene	957	0.72
Sabinene	978	0.56
β-pinene	984	1.07
Myrcene	992	0.34
α-phellandrene	1021	0.18
α-terpinene	1025	1.45
P-cymene	1029	16.68
Limonene	1035	1.47
1,8-cineole	1038	1.43
γ-terpinene	1067	12.65
Terpinolene	1094	0.73
Linalool	1126	1.36
Camphore	1138	0.69
Borneol	1154	2.85
Terpinene-4-ol	1192	0.34
α-terpineol	1203	1.56
Methyl thymol	1218	0.37
Thymoquinone	1224	0.43
Methyl carvacrol	1228	0.62
Geraniol	1246	2.32
Thymol	1278	32.67
Carvacrol	1287	8.32
Thymyl acetate	1362	0.22
β-caryophyllene	1425	2.39
α-humulene	1466	0.2
Germacrene D	1482	1.46
γ-cadinene	1521	0.67
Spathulenol	1569	0.18
Caryophyllene oxide	1579	1.36
Total		99.56

essential oil injection to calculate restrictive index (RI) of components of essential oil. The Restrictive Index of components of the sample was calculated by using a computerized program. Finally, the components of essential oil was identified by comparing the mass spectrums obtained with the existing standard mass spectrums at electronic library of Wiley 2000 existing in Absolution software of GC/Ms set and calculation of standard restrictive index in accordance with C8-C17 Alkans and comparing them with the existing standard figurers in references (Adams, 2001).

Antifungal assay

The solvent showing no antifungal activity from DMSO was selected as a diluting medium for the oil. Undiluted oil was taken as dilution 1, 1/2, 1/4, 1/8 and 1/16 dilutions of the oil were made DMSO. For antifungal activity 50 μL of each dilution was used. The antifungal activity of the essential oil was evaluated by disc diffusion method using Mueller Hinton Agar (Baron and Finegold, 1995) and determination of inhibition zones at different oil dilutions against *A. niger* (PTCC=5223). The fungal strains under experiment were obtained from the Center for Fungi and Bacteria of Iranian Scientific and Industrial Researches Organization. The antifungal property of the oil was tested by agar well diffusion method using Sabouraud Dextrose Agar (SDA). Standard reference antibiotics were used in order to control the sensitivity of the tested fungi (Streptomycin sulphate 72% SP and gentamicin 8 mg/ml). The incubation conditions used was 48 to 72 h at 24°C for fungi. All the experiments were carried out in triplicate and averages were calculated for the inhibition zone diameters.

RESULTS

The study of the analysis of *T. vulgaris* L. essential oil under investigation showed that the output of essential oil is 2.25% (v/w). The identified combination in essential oil, restrictive index (RI), and quantitative percentage of the compounds. Thirty-two compounds being identified in the essential oil of this plant with 99.56%, the combinations of Thymol (32.67%), *P*-cymene (16.68%), γ-terpinene (12.65%) and Carvacrol (8.32%) with 70.32% constitute the highest percentage of essential oil (Table 1).

Table 2. The zone diameter of inhibition of antibiotics, thyme oil and synthetic thymol on *Aspergillus niger* (mm).

Antibiotics		Dilutions of thyme oil					Synthetic thymol	
Streptomycin sulphate (72% SP)	**Gentamycin (8 mg/ml)**	**1**	**1/2**	**1/4**	**1/8**	**1/16**	**1 %**	**10%**
20	18	32	27	22	18	12	16	26

The indexes of restrictive have been calculated by injecting the mixture of normal hydrocarbons (C8-C17) to HP-5MS column. The results of studying the antifungal impacts of the *T. vulgaris* 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 32, 27, 22, 18 and 12 mm, respectively. the results with standard antibiotics Streptomycin sulphate (72% SP) with a diameter of 20 mm and gentamicin (8mg/ml) with a diameter of 18 mm had inhibitory effects. Thymol synthetic in 1% dilution had moderate inhibitory (16 mm) effect on *A. niger* growth but at 10 % dilution had a strong inhibitory (26 mm) of fungi growth. The results showed essential oil from *Thymus* plant at 1, 1/2 and 1/4 oil dilutions exhibited strong antifungal activity than Streptomycin sulphate (72% SP) and gentamycin (8 mg/ml) antibiotics on *A. niger* and exhibited strong of thymol was at 10% dilution. The high percentage antifungal activities of fumaria oil are related with thymol is a natural monoterpene phenol as the main compound (Table 2).

DISCUSSION

Identify chemical composition and antifungal effect of *T. vulgaris* L. in this paper compared with other researcher. In comparison our results with other researchers the essential oil of *Thymus vulgaris* L. has been studied in Iran and world. Essential oil of the aerial parts of *Z. multiflora, T. vulgaris* and *T. kotschyanus* were collected from growing fields in their natural habitats in Iran surveyed the effectiveness, Minimum inhibitory concentration (MIC) and Minimum fungicide concentration (MFC) of three medicinal plant essential oils of *Zataria multiflora, T. vulgaris* and *T. kotschyanus* on the mycelial growth of four pathogenic fungi including *Pythium aphanidermatum, Rhizoctonia solani* (AG4), *Fusarium graminearum* and *Sclerotinia sclerotiorum.* Essential oils were very effective on the four studied plant pathogenic fungi with growth inhibition average of 100% at 200µl/l concentration. In this research MIC and MFC of the essential oils were variable depending to species of fungi. *P. aphanidermatum* and *S. sclerotiorum* were the most sensitive and most resistant to the studied essential oils with average growth inhibition 89.54 and 75.35%, respectively (Amini et al., 2012). The composition and antimicrobial activity of the essential oil of *T. daenensis* Wild, an endemic species from Iran, was studied. Thirty compounds, accounting for 97.5% of the total oil, were identified. The main constituents were thymol (29.8%) and carvacrol (13.6%), *p*-cymene (11.3), borneol (6.8%) and 1, 8-cineole (5.89%).

The antimicrobial activity of essential oil of *T. daenensis* was tested against two Gram-negative and Gram-positive bacteria. The results of the bioassays showed the interesting antimicrobial activity, in Gram-positive bacteria, *S. aureus*, was the most sensitive to the oil, and as well the oil exhibited a remarkable antifungal activity against all the tested fungi. (Teimouri, 2012). In a research *Thymus vulgari* and *Citrus aurantifolia* were found to inhibit *A. parasiticus* (Razzaghi-Abyaneh et al., 2009). Essential oils *T. eriocalyx* could be safely used as preservatives (Rasooli et al., 2006). It is concluded that **e**ssential oils of *Thymus* and *Mentha* species possess great antifungal potential and could be used as natural preservatives and fungicides In related report the potential antifungal effects of *T. vulgaris* L., *T. tosevii* L., *Mentha spicata* L., and *Mentha piperita* L. (Labiatae) essential oils thymol (48.9%) and *p*-cymene (19.0%) were the main components of *T. vulgaris,* while carvacrol (12.8%), α-terpinyl acetate (12.3%), *cis*-myrtanol (11.2%) and thymol (10.4%) were dominant in *T. tosevii. Thymus* species showed very strong antifungal activities. In *M. piperita* oil menthol (37.4%), menthyl acetate (17.4%) and menthone (12.7%) were the main components, whereas those of *M. spicata* oil were carvone (69.5%) and menthone (21.9%). *Mentha* sp. showed strong antifungal activities, however lower than *Thymus* sp. (Soković et al., 2009). The thyme oil absolutely inhibited the mycelial growth of *A. flavus* at 0.7 µL ml^{-1} (Kumar et al., 2008).

Thymol is one of main compound of *T.spicata*, *Satureja thymbra*, *Salvia fruticosa*, *Laurus nobilis*, *Mentha pulegium*, *Inula viscosa*, *Pimpinella anisum*, *Eucalyptus camaldulensis*, and *Origanum minitiflorum* plants growing wild in southern Turkey. (Muller-Riebau et al., 1995). 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 control of pre- and post harvest phytopathogenic fungi on fruits or vegetables (Numpaque et al., 2011). In a study thymol exhibited approximately three-times stronger inhibition than essential oil of thyme (Segvic et al., 2007). 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 and Badeaa, 2002). Essential oil of *T. vulgaris* thymol chemotype potentiates the antifungal action of amphotericin B suggesting a possible utilization of this essential oil in addition to antifungal drugs for the treatment of mycoses (Giordani et al., 2005). The essential oils of *T. vulgaris* and *T. zygis* showed similar antifungal activity, which was greater than *T. mastichina* (Pina-Vaz et al., 2004). The composition of the essential oil of *T. pulegioides* showed high contents of carvacrol and thymol. *T. pulegioides* essential oil exhibited a significant activity against clinically relevant fungi (Pinto et al., 2006). Thymol and carvacrol that are the two main constituents of *T. glandulosus* and *Origanum compactum* exhibited the strongest antifungal activity (Chebli et al., 2003).

The essential oil of *T. revolutus* C. exhibited a significant antibacterial and antifungal activity (Karaman et al., 2001). *P-cymene*, linalool, terpinen-4-ol and thymol were main content in essential oils from two clonal types of *T. vulgaris* (Bhaskara Reddy et al., 1998). The most effective oil was that of thyme, with a fungicidal activity attributable to thymol, found in a concentration of 50.06% in the oil tested (Zambonelli et al., 1996). The fungicidal activity of the oils was correlated with their thymol content (Zambonelli et al., 2004). *A. flavus* was more sensitive to thyme essential oil than *A. niger*. Clove essential oil was a stronger inhibitor against *A. niger* than against *A. flavus* (Viuda-Maartos et al., 2007). The chemical composition of *Cinnamomum zeylanicum* (bark), *Cinnamomum zeylanicum* (leaf), *Cinnamomum cassia*, *Syzygium aromaticum* and *Cymbopogon citratus* had most active essential oils (Pawar and Thaker, 2006). *A. niger* was shown to be more difficult to destroy of essential oils *Thymus broussonettii, T. zygis* and *T. satureioides* than the most resistant bacterium *E. coli* (Tantaoui-Elaraki et al., 1993). The essential oils extracted from thyme (*T. vulgaris* L.), basil (*Ocimum basilicum* L.), coriander (*Coriandrum sativum* L.), rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), fennel (*Foeniculum vulgare* L.), spearmint (*Mentha spicata* L.) and carraway (*Carum carvi* L.) were investigated for their antimicrobial activity against eleven different bacterial and three fungal strains *A. niger* ATCC 16404, *Penicillium* sp. CICC 251 and two *E. coli* and *Salmonella enterica* serovar Enteritidis clinical isolates. The majority of the tested essential oils exhibited considerable inhibitory capacity against all the organisms tested, as supported by growth inhibition zone diameters, MICs and MBC's. Thyme, coriander and basil oils proved the best antibacterial activity, while thyme and spearmint oils better inhibited the fungal species (Lixandru et al., 2010).

Conclusion

The thyme essential oil recommended for large scale application based its strong antifungal as well as on *A. niger* efficacy. In this study we find out that regard to the antifungal effects of *T. vulgaris* essential oil under investigation as compared with synthetic thymol on *A. niger* and observed exhibited strong of synthetic thymol. The high percentage antifungal activities of *T.*oil are related with thymol is a natural monoterpene phenol as the main compound. This essential oil can be used as a combination with antifungal effects and natural origin. The effectiveness oil concentration of the oil depends on the target pathogen and effects of natural compounds on fungus.

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