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Antimicrobial activity of the essential oil of *Thymus kotschyanus* grown wild in Iran

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Abstract

The aims of this study were to extraction and identify the compounds formed and antimicrobial properties of essential oil on Iranian *Thymus kotschyanus*. Plant essential oil was prepared by a hydro-distillation method using Clevenger-type apparatus and analyzed by gas chromatography coupled with mass spectrometry (GC/MS) for determining their chemical composition. 49 compounds consisting 99.94% of the total components were identified from the essential oil of *Thymus kotschyanus* obtained. Among those, carvacrol (50.40%), 1, 8 cineole(8%), thymol (6.78%), borneol (6.46%) and E-Caryophyllene (4.35%) were the major oil components. Antimicrobial activity of the essential oils was investigated by micro broth dilution and disc diffusion methods. The results of this study shows that *Thymus kotschyanus* essential oils have inhibitory effect respectively on yeast, Gram-positive and Gram-negative pathogenic bacteria(yeast> Gram-positive bacteria> Gram-negative bacteria).Also, essential oil showed high antimicrobial activity against *Candida albicans* and *Bacilluscereus* two medically important pathogens.

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Introduction

The use of medicinal plants in the world and especially in Asian countries, contributes significantly to primary health care. Researchers and pharmaceutical industries are considering medicinal plants as a good choice, because these natural resources have ordinarily fewer side effects (Zargari, 1996). Also they are costless and effective against a broad spectrum of antibiotic resistant microorganisms. In many parts of the world, the extracts and essential oil of medicinal plants are used in folk medicine for their antimicrobial and antiviral properties (Hassawi and Kharma, 2006), that have been used. The increasing occurrence of antimicrobial resistance represents a worldwide major concern for both human and veterinary medicine (Lorian, 1996). For this reason, there is a growing interest in the antimicrobial screening of extracts and essential oils from plants in order to discover new antimicrobial agents.

Essential oils as antimicrobial agents present two main characters: their natural origin generally means more safety to people and environment; and they can be considered at low risk for development of microbial resistance since they are mixtures of compounds which may present different mechanisms of antimicrobial activity (Karbin *et al.*, 2009).

Nowadays, about 25% of the drugs prescribed worldwide came from plants and 252 of them are considered as basic and essential by the World Health Organization (WHO).

Infectious diseases are the second leading cause of death worldwide. Treatment of infections continues to be problematic in modern time because of the severe side effects of some drugs and the growing resistance to antimicrobial agents. Hence, search for newer, safer and more potent antimicrobials is a pressing need. Herbal medicines have received much attention as a source of new antibacterial drugs since they are considered as time-tested and comparatively safe both for human use and the environment (Fazly-Bazzaz *et al*., 2005).

Iran has a great deal of ecological diversity and has a rich herbal flora which is still much unstudied regarding phytochemistry and bioactivity. Lamiaceae (formerly Labiatae) is one of the most important plant families in which *Thymus* with about 215 species, is a significant genus (Zaidi and Crow, 2005). From the time of the ancient Iranian, the plants were considered to protect against diseases. Iran has a very honorable past in traditional medicine, which goes back to the time of Babylonian-Assyrian civilization. One of the most significant ancient heritages is sophisticated experience of people who have tried over millennia to find useful plants for health improvement, with each generation adding its own experience to this tradition (Naghibi *et al.*, 2005). Based on literature search, 18% of the plant species are used for medicinal purposes in Iran. The medicinal properties of the genus *Thymus* have made it one of the most popular medicinal plants (Nickavar *et al.*, 2005).

The widespread use of antibiotics both inside and outside of medicine is playing a significant role in the emergence of bacterial resistant (Goossens *et al.*, 2005). Although, there were low levels of preexisting antibiotic resistant bacteria before the widespread use of antibiotics evolutionary pressure from their use has played a role in the development of multidrug resistance varieties and the spread of resistance between bacterial species (Hawkey and Jones, 2009). The genus *Thymus* L., known as “Avishan” in Persian, is a well-known aromatic perennial herb originated from Mediterranean region. Among 215 species of this genus grown in the world, 18 species are distributed in Iranian flora, and 4 of these species are endemic (Jalas, 1982; Stahl-Biskup & Saez, 2002). *Thymus* species are well known as medicinal plants because of their biological and pharmacological properties. In traditional medicine, leaves and flowering parts of *Thymus* species are used (Amin, 2005; Zargari, 1990). *Thymus* oils and extracts are widely used in pharmaceutical, cosmetic and perfume industry also for flavoring and preservation of several food products (Bauer, Garbe, & Surburg, 1997). *Thymus kotschyanus* Boiss. and Hohen. common

name: (thyme) belongs to the Lamiaceae family.

In Iraqi folk medicine, the whole plant is anthelmintic, antioxidant, strongly antiseptic, antispasmodic, carminative, deodorant, diaphoretic, disinfectant, expectorant, sedative and tonic. Internally, it is taken in the treatment of bronchitis, catarrh, laryngitis, flatulent indigestion, painful menstruation, colic and hangovers. Externally, it is applied to minor injuries(wound-healing), mastitis, mouth, throat and gum infections(Al-Rawi and Chakravarty, 1988).

The objectives of this study were (i) to investigate the antimicrobial activity of the essential oil of *T. kotschyanus*, and (ii) to determine the chemical composition of its hydro-distilled essential oil by GC/MS.

Materials and methods

Plant Materials

The aerial parts of *Thymus kotschyanus* were collected from experimental field of Islamic Azad University, Estahban Branch, Iran (29°632' N, 54°142' E; 1760 m above sea level). The plant material used for the extraction of the essential oil was air-dried at room temperature (20–25◦C) in the shade.

Essential oil extraction

The essential oil of all air-dried samples (25 grams) was isolated by hydro-distillation for 3 h, using a Clevenger-type apparatus according to the method recommended in British Pharmacopoeia (British Pharmacopoeia, 1988). The distillated oils were dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4 ◦C until analysis. The oils were yellow in color and had distinct sharp odor.

Essential oil analysis

GC–MS analysis was carried out by use of Agilent gas chromatograph equipped with fused silica capillary HP-5MS column (30 m ×0.25 mm i.d.;film thickness 0.25 micron) coupled with 5975-C mass spectrometer. The injector and detector and MS temperatures were kept at 280◦C.Helium was used as carrier gas at a flow rate of 1 ml/min; with ionization voltage of 70eV. Ion source and interface temperatures were 230◦C and 280◦C, respectively. Mass range was from 50 to 480 amu. Oven temperature program was 60–210◦C at the rate of 3◦C/min and then programmed to 240◦C at the rate of 20◦C/min and the final temperature kept for 8.5 min; split ratio was 1:50.

Antimicrobial activity

Microorganisms

Standard strain of *Candida albicans* (ATCC 10231 BBL) and three standard strains of Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (PTCC1435) and *Bacillus cereus* (PTCC1247) also standard strain of Gram-negative bacteria *Escherichia coli* (PTCC1399) were obtained from Microbiology Laboratory, Shiraz University of Medical Sciences.

Determination of antifungal/antibacterial activity by the disc diffusion method

In vitro antimicrobial activity of the essential oil of *Thymus kotschyanus* was evaluated by disc diffusion method, with determination of inhibition zones (IZ), according to the National Committee for Clinical Laboratory Standards(NCCLS, 2001) using 100 µl of each suspension of the tested microorganisms containing 10^8 CFU/ml for microorganisms strains that was measured using the spectrophotometer. The bacteria inoculate was prepared by suspending overnight colonies from Nutrient agar (NA) media, and the *C. albicans* was prepared by suspending colonies from 48 h old potato dextrose agar (PDA) cultures respectively. Fungal or bacterial suspension were seeded into Petri dishes (9 cm) containing 20 ml sterile Nutrient agar (NA) or potato dextrose agar (PDA) using a sterile cotton swab. Essential oils were diluted in DMSO to different test concentrations. The sterile paper discs (Whatman, 6 mm in diameter) containing approximately 20 µl of the essential oils were impregnated with different amount of essential oils (20, 10, 5, 2.5, 1.25, 0.63, 0.31 and 0.16 µl/disc), then placed on the surface of Nutrient agar plates which had previously been inoculated with the tested

microorganisms. The plates were inoculated with bacteria incubated at 37°C for 24 h and at 24°C for 48 to 72 h for the *C. albicans* strain. After incubation, bacteria growth inhibition as the mean inhibition zone diameter around the discs for each concentration was measured in millimeters using vernier calipers. All the experiments were carried out in triplicate. Blank discs containing 20 µl DMSO were used as negative controls.

Determination of the minimum inhibition concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/MFC)

Initially is removed 100µl of nutrient broth, and were dumped in to small Welles of the first six rows of micro plate. Then essential oil was added to the first small well of each row. After mixing the contents of the first small wells, 100µl was removed and added to the next small well and so. A serial dilution twice of essential oil was prepared to twelfth small wells and was discarded 100µl of twelve of the small wells. For test of each row were considered a row control and essential oil was not added to control lines. Then was added 100µl of bacteria suspension to the test small wells of all rows (Alipour and Khanmohammadi, 2011).

To the determination the MBC, of all small wells without turbidity were cultured in NA medium. Then the medium at the proper temperature for each bacterium was placed inside the incubator. After the time required for bacterial growth. The lowest concentration of essential oil that 99.9% of bacteria have not growth, were considered as bactericidal concentration (Alipour and Khanmohammadi, 2011). The experiment was repeated three times.

Statistical analysis

Analysis of variance was performed by NOVA by the software SAS (version 9.2 for windows). Significant differences between means were determined by Duncan's new multiple-range test. A significant difference was considered at the level of $P < 0.01$.

Result and discussion

Chemical composition of the essential oil

The essential oil isolated by hydro distillation of the aerial part of *Thymus kotschyanus* were found to be pale yellow oil and analyzed by GC and GC-MS for determining their chemical composition. In total, 49 compounds consisting 99.94% of the total components were identified from the essential oil of *Thymus kotschyanus* obtained. Among those, carvacrol(50.40%), 1, 8 cineole (8%), thymol (6.78%), borneol (6.46%), E- Caryophyllene (4.35%), Carvacrol methy ether (3.65%), Eugenol (3.06%) and cis-Sabinene hydrate (2.12%) were the major oil components (Table 1).

The composition of essential oil in *Thymus kotschyanus* has been examined previously by other researchers. Habibi *et al.*, (2006) reported, linalool and α-Terpinene were the highest amount in species *Thymus kotschyanus* (45 and 39.5 percent respectively). Rasooli and Mirmostafa (2003**),** characterized for *T. kotschyanus* essential oil with carvacrol (35.06, 22.75%), thymol (26.60, 16.52%), γ-terpinene (7.81, 0.34%), borneol (2.29, 4.52%), myrcene (0.26, 12.65%), thymolquinone (0, 11.39%), nerol (0, 6.10%), and β-caryophyllene (0, 5.54%), respectively as the major components before and at the flowering stages. In another study by Sefidkon *et al.*, (1999) the essential oils were isolated from the aerial parts of *Thymus kotschyanus* in three stages of plant growth (before, at the beginning of and at complete flowering). The main constituents in all of the oils were carvacrol (40.74–61.23%), thymol (7.51–26.92%), γ-terpinene (3.72–8.25%), *p*-cymene (3.28–6.74%) and borneol (1.33–4.52%).Also Nickavar *et al.*, (2005) identified thirty one components accounting for 98.7% of *T. kotschyanus* oil. The major constituents were thymol (38.6%), carvacrol (33.9%), γ-terpinene (8.2%) and *p*-cymene (7.3%).Another study concerning the *T. kotschyanus* showed that, the major components were pulegone (18.7%), isomenthone (17.8%), thymol (14.9%), 1,8-cineole (9.0%), piperitenone (6.3%) and carvacrol (5.5%) (Morteza-Semnani *et al.*, 2006). These differences in the essential oil compositions can be attributed to several environmental factors such as climatic,

seasonal and geographical or ontogenesis variations (Alizadeh *et al.*, 2011; Alizadeh *et al.*, 2013).

Variation in chemical composition of essential oils, in particular, and extracts of medicinal plants may be observed due to the originand the developmental stage of collected plant materials (Burt, 2004). Also essential oil yield and their components in plants is related to genetic (Mohammed and Al-Bayati, 2009), elevation, topography (Pourohit and Vyas, 2004; Rahimmalek *et al.*, 2009) and genotype (G), growing conditions (E) and their interaction (G × E) (Basu *et al.*, 2009; Shafie *et al.*, 2009).

Table 1. Essential oil constituents in *Thymus kotschyanus*.

No	Compound	RI[a]	Percentage in oil
1	□-Thujene	926	0.008
2	□-Pinene	932	0.02
3	Camphene	947	0.02
4	Sabinene	971	0.03
5	1-Octen-3-ol	975	0.12
6	3-Octanone	983	0.05
7	dehydro-1,8-Cineole	989	0.13
8	3-Octanol	993	0.03
9	□-Phellandrene	1004	0.01
10	□-Terpinene	1015	0.07
11	p-Cymene	1023	1.35
12	Limonene	1028	0.07
13	1,8-Cineole	1031	8.00
14	(Z)-□-Ocimene	1035	0.15
15	Benzene acetaldehyde	1041	0.07
16	(E)-□-Ocimene	1045	0.96
17	□-Terpinene	1056	0.92
18	cis-Sabinene hydrate	1066	2.12
19	Terpinolene	1086	0.07
20	Linalool	1098	0.72
21	n-Nonanal	1102	0.16
22	trans-Pinocarveol	1137	0.23
23	Camphor	1142	0.52
24	Borneol	1166	6.46
25	Terpinene-4-ol	1176	1.36
26	□-Terpineol	1189	1.06
27	Carvacrol methy ether	1243	3.65
28	Thymol	1293	6.78
29	Carvacrol	1301	50.40
30	□-Terpinylacetate	1350	0.05
31	Eugenol	1359	3.06
32	□-Copaene	1374	0.06
33	□-Bourbonene	1383	0.15
34	(Z)-Jasmone	1398	0.19
35	(Z)-Caryophyllene	1405	0.06
36	(E)-Caryophyllene	1419	4.35
37	□-Copaene	1427	0.06
38	Aromadendrene	1437	0.06
39	□-Humulene	1451	0.19
40	Germacrene D	1479	0.48
41	Bicyclogermacrene	1494	0.24
42	□-Bisabolene	1508	1.46
43	□-Cadinene	1512	0.45
44	□-Cadinene	1522	0.24
45	(E)-□-Bisabolene	1539	0.12
46	Spathulenol	1576	0.33
47	Caryophyllene oxide	1581	1.63
48	epi-□-Cadinol	1639	1.08
49	□-Cadinol	1652	0.15
	□otal		99.94

[a]RI, retention indices in elution order from HP-5 column

Data expressed as percentage of total.

Table 2. The interactionofmicroorganismsand concentration of*Thymuskotschyanus* essential oilonthesize of theinhibition zone.

Oil concentration (µl/disc) Microorganisms	20	10	5	2.5	1.25	0.63	0.31	0.16
Escherichia coli	16.3±0.723^{f}	7.9±0.941^{jkl}	7.2±1.103^{kl}	7.8±1.211^{jkl}	8.3±0.173^{ijkl}	7.7±0.377^{jkl}	8.1±0.940^{ijkl}	7.6±0.665^{jkl}
Candida albicans	34.7±4.443^{a}	16.6±1.818^{f}	16.4±1.198^{f}	15.1±1.253^{fg}	8.7±0.410^{hijkl}	7.1±0.482^{kl}	9.3±1.123^{hijkl}	9.3±1.600^{hijkl}
Staphylococcus aureus	22.0±0.950^{cd}	20.3±0.848^{cde}	15.2±3.483^{fg}	9.9±2.346^{hijkl}	7.8±0.752^{jkl}	7.2±0.230^{kl}	7.3±0.736^{kl}	6.5±0.510^{kl}
Staphylococcus epidermidis	26.8±2.444^{b}	25.4±0.973^{bc}	12.3±0.770^{gh}	7.6±0.523^{jkl}	7.9±0.845^{ijkl}	7.6±0.741^{e}	6.3±0.201^{l}	6.2±0.057^{l}
Bacillus cereus	34.7±5.122^{a}	26.3±0.414^{b}	15.3±0.900^{fg}	7.9±0.333^{jkl}	7.5±1.296^{hijkl}	6.8±0.480^{kl}	6.9±0.687^{kl}	6.3±0.150^{l}

Each value in the table was obtained by calculating the average of three experiments ± standard deviation.
In the table, means with the same letters are not significantly different at 1% level of Duncan,s new multiple range test.

Table 3. Antimicrobial activity of *Thymus kotschyanus* essential oil.

Microorganisms	MIC	MBC/MFC
Escherichia coli	6.25 µl/ml	6.25 µl/ml
Candida albicans	3.645 µl/ml	3.125 µl/ml
Staphylococcus aureus	1.562 µl/ml	3.645 µl/ml
Staphylococcus epidermidis	0.097 µl/ml	0.195 µl/ml
Bacillus cereus	1.562 µl/ml	50 µl/ml

MIC: Minimum Inhibitory Concentration
MBC: Minimum Bactericidal Concentration
MFC: Minimum Fungicidal Concentration.

Antibacterial activity

The antibacterial activity of *T. kotschyanus* essential oils against microorganisms which are considered in this study was assessed by evaluating the presence of IZ and MIC values. Results (Table 2), showed that the essential oils of *T. kotschyanus* have great potential of antibacterial activity against all of the five bacteria tested. The most marked effect was observed against *Candida albicans* and *Bacillus cereus*. The IZ and MIC values for bacterial strains, which were sensitive to the essential oils of *T. kotschyanus*. The results of this study show that *Thymus kotschyanus* essential oils were more effective against yeast than Gram-positive and Gram- positive than Gram- negative strains. Essential oils rich in phenolic compounds, such as carvacrol, are widely reported to possess high levels of antimicrobial activity (Baydar, Sagdic, Ozkan, & Karadogan, 2004). Several studies have focused on the antimicrobial activity of the essential oils of thyme in order to identify the responsible compounds (Burt, 2004; Crespo, Jimenez, Gomis, & Navarro, 1990; Nelson, 1997). Carvacrol, which is the main component of *T. kotschyanus* essential oils, has been considered as a biocidal, resulting in bacterial membrane perturbations that lead to leakage of intracellular ATP and potassium ions and ultimately cell death (Helander *etal*., 1998; Juven, Kanner, Schued, &Weisslowicz, 1994; Ultee, Kets, & Smid, 1999). The effect of carvacrol on *Staphylococus* was investigated by Knowles, Roller, Murray, and Naidu (2005). However, it was also considered that minor components, as well as a possible interaction between the substances could also affect the antimicrobial activities. In fact, other constituents, such as γ-terpinene, have been considered to display relatively good activity due to their possible synergistic or

antagonistic effects (Didry, Dubreuil, & Pinkas, 1993; Vardar- Unlu *etal*., 2003). Antimicrobial activity of an essential oil is attributed mainly to its major components which is determined by the genotype and influenced by environmental and agronomic conditions (Baydar *etal*., 2004; British pharmacopoeia, 1988), although the synergistic or antagonistic effect of one compound in minor percentage of mixture has to be considered (Burt, 2004). Therefore, antimicrobial, antioxidant, and other biological activities may vary, based on the variations in the chemical composition (Chorianopoulos *et al*., 2004; Leung & Foster, 1996).

Conclusion

The data presented confirm the antibacterial potential of *T. kotschyanus* essential oil. The essential oils tested represent an inexpensive source of natural antibacterial substances, and it can be used in aromatherapy and pharmacy, and also in pathogenic systems to prevent the growth of microbes. Also, the results of investigated antimicrobial activity determined by the paper disc diffusion method showed the higher resistance of Gram-negative bacteria to the oil. The antimicrobial properties of the oil could be associated with the high percentage of phenolic components such as thymol and carvacrol which are known to possess strong antimicrobial activities. Further studies are needed to determine the antibacterial activities of the compounds for the observed potential value. Suggesting that, the essential oils of the selected plants could be a possible source to obtain new and effective herbal medicines to treat infections and also in the search for novel antibacterial agents with the potential application of some major or minor constituents alone, mixed of presented essential oils or in combination with antibiotics for the treatment and prevention of pathologies associated with multi resistant bacteria. However, the mechanism of inhibitory effects of these plant's oils against infectious bacteria is still unclear, and further investigations regarding the in vitro and in vivo should be conducted in order to clear mechanisms pathway and develop such products. This study is part of a continued search for new drugs with high activity and few side effects that can be used to treat diseases associated with pathogen bacteria strains. More studies are needed to determine the substances are selective for certain bacterial species.

It seems that the activity of the essential oil of the plant is due to the interactions of their components. However, more studies are needed to be done on antimicrobial effect of the essential oil of these species.

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