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Full Length Research Paper

Harmful activities of Micro organisms and essential oil composition of *Thymus daenensis* Celak from Iran

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The composition and antimicrobial activity of the essential oil of *Thymus daenensis* Wild, an endemic species from Iran, was studied. The volatile oil obtained by hydrodistillation was characterized by the physico-chemical properties, gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) techniques. 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 and three fungi by disc diffusion method. The results of the bioassays showed the interesting antimicrobial activity, in which the Gram-positive bacteria, *Staphylococcus aureus*, was the most sensitive to the oil, as well the oil exhibited a remarkable antifungal activity against all the tested fungi. These results confirm the possibility of using *T. daenensis* in food system, medicine and pharmacy.

Key words: Antimicrobial activity, crop, essential oil, gas chromatography/mass spectrometry (GC/MS), *Thymus daenesis.*

INTRODUCTION

The antimicrobial properties of plant volatile oils and their constituents from a wide variety of plants have been assessed and reviewed (Baydar et al., 2004; Burt, 2004; Simoes et al., 2004; Zakaraya et al., 1993). It is clear from these studies that these secondary plant metabolites have potential uses in medical procedures and applications in the cosmetic, pharmaceutical and food industries (Baydar et al., 2004; Burt, 2004; Bagci et al., 2005). Also, increasingly adverse drug reaction to the synthetic antibiotics and the increasing resistance of some pathogens to synthetic antibiotics, has been another argument against the use these chemicals as therapeutics (Abusage et al., 2002; Bagci et al., 2005). Biological activity of essential oils depends on their chemical composition which is determined by the genotype and influenced by environmental and agronomic conditions (Baydar et al., 2004; British pharmacopoeia, 1988). The genus *Thymus* has numerous species and varieties and their essential oil composition have been studied earlier (Baydar et al., 2004; Burt, 2004, Bagci et al., 2005), from *Thymus daenensis* grown in Iran and in other countries (Mozaffarian, 1996). The *Thymus* genus belonging to the Lamiaceae family includes approximately 350 species, existing mainly in Europe, Western Asia and the Mediterranean regions (Mozaffarian, 1996; Rechinger, 1982). Many species of *Thymus* have been widely used in folk medicine in the world for their carminative, antispasmodic, emmenagogic and tonic properties (Dorman and Deans, 2004). The species of this genus are rich in essential oils and were characterized by a great variability of both morphology and chemotypes (Stahl-Biskup, 1991). Many studies on the antimicrobial activity (Dorman and Deans, 2004; Zambonellit et al., 2004; Karaman, 2001) and antioxidative activity (Dorman and Deans, 2004; Dob et al., 2006; Youdim et al., 2002) of these oils have been reported. On the other hand, several extracts of these plants were tested for their pharmacological activity (Marti et al., 2005) and other activity (Okazaki et al., 2002). Essential oil of this plant is a rich source of thymol and carvacrol which has been reported to possess the highest antioxidant activity (Dorman and Deans, 2004; Miguel et al., 2004; Sokmen, 2004; Youdim et al., 2002). In Iran, it is predominantly

found in the south and north of the country. It is used as a food ingredient, as a tea as an herbal drug for its reputed medicinal properties (Baydar et al., 2004). The objectives of this study were (i) to investigate the antimicrobial activity of the essential oil of *T. daenensis*, and (ii) to determine the chemical composition of its hydro-distilled essential oil by GC/MS.

MATERIALS AND METHODS

Plant material and isolation procedure

The aerial parts of *T. daenensis* were collected at full flowering from its wild habitat in Jiroft, Kerman Province, at an altitude of 1700 m. Voucher specimen was deposited at the Herbarium of Medicinal Plants and Drugs Research Institute, Kerman University, Kerman, Iran. The essential oil of all air-dried samples (200 g) was isolated by hydro-distillation for 4 h, using a Clevenger-type apparatus according to the method recommended in British pharmacopoeia (1988). The distillated oils were dried over anhydrous sodium sulphate and stored in tightly closed dark vials at 4°C until analysis. The oils were yellow in color and had distinct sharp odour.

Gas chromatography/mass spectrometry (GC/MS)

GC analysis was performed using a Thermoquest gas chromatograph with a flame ionization detector (FID). The analysis was carried out on fused silica capillary DB-1 column (25 m × 0.25 mm i.d.; film thickness 0.25 µm). The injector and detector temperatures were kept at 250 and 300°C, respectively. Nitrogen was used as carrier gas at a flow rate of 1.1 ml/min; oven temperature program was 60 to 250°C at the rate of 4° /min and finally held isothermally for 15 min; split ratio was 1:50. GC-MS analysis was carried out by use of Thermoquest-Finnigan gas chromatograph equipped with fused silica capillary DB-1column (60 m × 0.25 mm i.d.; film thickness 0.25 µm) coupled with a TRACE mass (Manchester, UK). Helium was used as carrier gas with ionization voltage of 70 eV. Ion source and interface temperatures were 200 and 250°C, respectively. Mass range was from 35 to 456 amu. Oven temperature program was the same given earlier for the GC.

Identification of compounds

The constituents of the essential oils were identified by calculation of their retention indices under temperature-programmed conditions for n-alkanes (C6 to C24) and the oil on a DB-1 column under the same chromatographic conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those reported in his literature (Adams 2001; Shibamoto, 1987). For quantification purpose, relative area percentages obtained by FID were used without the use of correction factors.

Preparation of oil dilutions

The solvent showing no antimicrobial activity, that is, dimethylsulfoxide (DMSO), was selected as a diluting medium for the oil. Undiluted oil was taken as dilution 1 and 1/2, 1/4, 1/8, and 1/16 dilutions of the oil were made with DMSO. For antibacterial activity 15 µl and for antifungal property 25 µl of each dilution was used.

Antimicrobial activity

The antibacterial activity of the essential oil was evaluated by disc diffusion method using Mueller Hinton agar (Dorman and Deans, 2004) and determination of inhibition zones at different oil dilutions. The microorganisms used were as follows: *Bacillus subtilis* ATCC 9372, *Staphylococcus aureus* ATCC 25923*, Escherichia coli* ATCC 25922*, Klebsiella pneumoniae* ATCC 3583, *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 5027 and *Saccharomyces cerevisiae* ATCC 9753. The antifungal property of the oil was tested by agar-well diffusion method using Sabouraud dextrose agar. Standard reference antibiotics were used in order to control the sensitivity of the tested bacteria (ampicillin and tetracycline) and fungi (nystatine). The incubation conditions used were 24 h at 37°C for bacteria and 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 AND DISCUSSION

Chemical analysis

The air-dried aerial parts of *T. daenensis* investigated here gave an average yield of oil of 1.2% (w/w) based on dry weight of sample. The essential oil isolated by hydro-distillation was described as liquid, pale yellow in colour and the odour was representative of the plant. Qualitative and quantitative analytical results were obtained by using both GC and GC–MS techniques. Table 1 showed the compounds which were identified in the oil of *T. daenensis* and the percentage of the chemical groups in order of elution on DB-1 capillary column. Thirty compounds were identified, accounting for 97.5% of the total oil. This essential oil was characterized by very high percentage of monoterpenes (89.56%), especially oxygenated ones (61.09%), in which thymol (29.8%) and carvacrol (13.6%), Borneol (6.8%), 1, 8-cineole (5.89%) were the major components. The monoterpene hydrocarbon fraction formed 28.56% of the oil, represented by *p*-cymene (11.3%) and γ-Terpinene (3.89%) as the main compound. In contrast the sesquiterpene fraction was lower (8.75%); the hydrocarbon sesquiterpenes (6.32%) were detected in a high concentration than the oxygenated sesquiterpenes (1.53%). The previous results showed that our oil was characterized by the presence of four dominating components; thymol (29.8%), carvacrol (13.6%), *p*-cymene (11.3%) and 1, 8-cineole (5.89%). The presence of thymol and carvacrol, as well as their precursor *p*-cymene in the oil was expected, although the absence of linalool was noteworthy. These four components have been previously found as constituents of most *Thymus* oils (Mc Gimpsey et al., 1994; Stahl-Biskup et al., 1991; Sefidkon et al., 1999). A comparison of the results obtained in this study with previously reported data of the *Thymus* species oils from different countries, showed that the predominance of thymol, carvacrol, *p*-cymene and 1, 8-cineole as main components (Sefidkon and Askari, 2002). Other studies report components such as linalool

Table 1. Essential oil composition of *Thymus daenensis.*

Peak	R.I.	Compound	Amount (%)
1	921	α-thujene	2.20
2	938	α-pinene	1.50
3	955	Camphene	1.20
4	987	β-pinene	0.44
5	993	Myrcene	2.44
6	1004	α-phellandrene	0.33
7	1010	α-terpinene	2.90
8	1028	*p*-cymene	11.30
9	1030	Limonene	1.89
10	1038	1,8-cineole	5.89
11	1048	E-ocimene	0.43
12	1055	γ-terpinene	3.89
13	1077	Terpinolene	0.47
14	1099	Camphor	3.76
15	1122	Borneol	6.80
16	1138	α-terpineol	0.69
17	1219	Thymol	29.80
18	1256	Carvacrol	13.60
19	1298	Eugenol	0.12
20	1366	α-copaene	0.11
21	1392	Borbonene	0.43
22	1439	E-caryophyllene	2.98
23	1441	β-gurjunene	0.16
24	1474	α-humulene	0.13
25	1494	β-bisabolene	0.87
26	1539	γ-cadinene	0.77
27	1597	Spathulenol	0.31
29	1611	Caryophyllene oxide	1.10
30	1644	α-cadinol	0.12
		Monoterpene hydrocarbons	28.56
		Oxygenated monoterpenes	61.09
		Sesquiterpene hydrocarbons	6.32
		Oxygenated sesquiterpenes	1.53
		Unknown	2.50
Total			**97.50**

[a]Compounds listed in order of elution from a non-polar DB-1 column. [b]Rétention indices (R.I) on DB-1.

(Bucar et al., 2005), β-caryophellen (Nejad Ebrahimi et al., 2008), methylcarvacrol (Abusage et al., 2002), α-pinene (Kabouche et al., 2005), α-terpineol (Koga et al., 1999), comphene (Mojab and Nickavar, 2006), (E)-nerolidol (Kulevanova et al., 1998) and α-terpinyl acetate (Mockute and Bernotiene, 2001; Nickavar et al., 2005) as the main components.

Antimicrobial activity

The essential oil was tested against two Gram-positive and Gram-negative bacteria and three fungi. The results of the bioassays (Table 2) showed that the oil exhibited moderate to strong antibacterial activity against all the tested bacteria and strong activity against the fungi. The 1 (absolute essential oil) and 1/2 oil dilutions showed inhibitory activity against all the tested bacteria, especially *B. subtilis*, and three fungi tested. The 1/4 oil dilution was active against all tested microorganisms, except two Gram-negative strains, *K. pneumoniae* and *E. coli.*

No antimicrobial activity was observed against two Gram-negative bacteria at 1/8 and 1/16 oil dilutions. The present study revealed that the essential oil of *T. daenensis* at different dilutions also showed a similar

Table 2. Antimicrobial activity of essential oil of *Thymus daenensis*.

Micro- organism	Inhibition zone (mm)[a]							
	Oil dilutions					Standards		
	1	1/2	1/4	1/8	1/16	Ampicillin[b]	Tetracycline[c]	Nystatine[c]
Staphylococcus aureus	28	21.5*	19	15*	11	15	21	**nt**
Bacillus subtilis	20*	17	13*	9.5	7.5	13	21	nt
Escherichia coli	11	7.5	-	-	-	10	-	nt
Klebsiella pneumoniae	10*	7.5	-	-	-	10	-	nt
Aspergillus niger	26	24	16*	-	-	nt	nt	18
Candida albicans	24	22	14	-	-	nt	nt	16
Saccharomyces cerevisiae	23	21	14	-	-	nt	nt	16

[a]Includes diameter of disc (6 mm). [b]Tested at 15 µl/disc. [c]Tested at 25 µl/disc. [*]A similar inhibitory type of activity of the oil to that of standard antibiotics. (-) Inactive; (7.5 to 13) moderately active; (≥ 14) highly active; nt: not tested.

inhibitory type of activity to that of standard antibiotics (Table 2). Our results support the ethno-pharmacological uses of this plant in folk medicine and could provide useful data for the utilization of this essential oil in pharmaceutical, cosmetic and food industries. The activity was more pronounced against fungi and Gram-positive organisms than Gram-negative bacteria. It has frequently been reported that Gram-negative bacteria were resistant to the inhibitory effects of essential oil and their components (Smith et al., 1998). This resistance has been attributed to the presence of cell wall lipopolysaccharides that can screen out the essential oil (Bagci et al., 2005; Baron and Finegold, 1995).

Conclusion

There is obviously a chemical polymorphism of essential oils within the plants belonging to the genus *Thymus*. In addition, the intra specific variability of the essential oils in the genus of *Thymus* was also observed (Sefidkon and Askari, 2002). The results of investigated antimicrobial activity determined by the paper disc diffusion method showed the higher resistance of Gram-negative bacteria to the oil, which was opposite to some published results (Koga et al., 1999). 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 (Burt, 2004).

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