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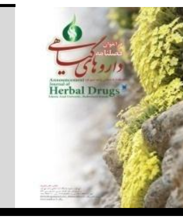

Chemical composition and antibacterial activity of essential oil from two Thymus species

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ABSTRACT

Background & Aim: *Staphylococcus aureus* is one of the bacterial pathogens and the most important factor in hospitalized infections in human community. This bacterium is becoming resistance to chemical drugs quickly. Lots of countries are trying to find new compositions as a suitable replacement for these antibiotics. The aim of this study was antibacterial activity and chemical composition of (*Thymus vulgaris* L.) and (*Thymus daenensis* Celak) cultivated in Iran.

Experimental: The aerial parts of *T. vulgaris* and *T. daenensis* were collected from stages beginning of blooming set at Estahban (Fars province) in South Iran, during 2014.The essential oil was extracted by a Clevenger approach and analyzed using GC/MS. This essential oil was examined on *S. aureus* with different sensitivity levels to vancomycin, methicilin and oxacillin antibiotics.

Results & Discussion: In *T. vulgaris* and *T. daenensis*oil, 28, 26 compounds were identified, respectively. The major constituents of the *T. vulgaris* oil were thymol (63.14%), *p*-cymene (9.45%), γ-terpinene (8.67%) and carvacrol (3.14%).The major constituents of the *T. daenensis* oil were; thymol (71.2%), γ-terpinene (8.81%), *p*-cymene (3.8%) and carvacrol (2.4%). According to these results, MIC and MBC of *T. vulgaris* and *T. Daenensis* oil on *S. aureus* were estimated 0.1 to 0.5 micro l/ml respectively.

Industrial and practical recommendations: In conclusion, the results obtained in our study indicated that *Thymus daenensis* oil had a high suppressing and fatal effects.

1. Introduction

The lives of millions of people have saved by antibiotics favor; these later have contributed the major gains in life expectancy during the last century (Bandow *et al.,* 2003). *Staphylococcus aureus* is a Gram-positive spherical bacterium approximately 1 µm in diameter. Its cells form grape-like clusters, since cell division takes place in more than one plane. It is often found as a commensal associated with skin, skin glands, and mucous membranes, particularly in the nose of healthy individuals (Crossley and Archer, 1997). *S. aureus* is a commensal and a pathogen. The anterior nares are the major site of colonization in humans. About 20–30 % of individuals are persistent carriers of S. aureus, which means they are always colonized bythis bacterium, and 30% are intermittent carriers (colonized transiently) (Wertheim and Melles, 2005). A variety of microorganisms also lead food spoilage that is encountered as one of the most important matter concerning the food industry. So far, many pathogenic microorganisms, such as S. aureus have been reported as the causal agents of foodborne diseases and/ or food spoilage (Betts *et al.,* 1999).

Thymus vulgaris L. and *Thymus daenensis* Celak from mint family (Lamiacae) traditionally being used as source of the essential oil and phenolic components derived from their different parts (Mozaffarian, 2008). The genus Thymus L. consists of about 215 species of herbaceous perennials and small shrubs in the world (Cronquist, 1988).

Essential oils extracted from fresh leaves and flowers can be used as aroma additives in food, pharmaceuticals, and cosmetics. Studies indicating the antiseptic, carminative, antimicrobial, and antioxidative properties of thyme have also been published (Zargari, 1989; Al-Bayati, 2008).

Many authors have reported antimicrobial, antifungal, antioxidant and radical-scavenging properties of essential oils. Thyme essential oils were reported to have antimicrobial activities (Beera *et al.,* 2007). In another research, aqueous and ethanolic extracts (10-200 mg/ml) of *Thymus capitatus* inhibited the growth of several bacteria and fungi (Kandil and Radwan, 1994). In studies Elabed *et al*. (2014) indicated the *T. vulgaris* essential oil antibacterial activity against *S. aureus* and exhibit antibacterial effect with MIC values 16 fold. Ghasemi Pirbalouti *et al*. (2009) reported that the essential oils of *T. daenensis* and *Thymus khuzestanicum* (MIC $_{\geq 50\%}$ = 0.63 µl ml-1 and MLC $_{\geq 99.9\%}$ = 22 µl ml-1) and the ethanol extract of *Menthalongifolia* showed higher of inhibition against *Saprolegnia parasitica* than the other extracts. Therefore, the main goal of this study chemical composition and antibacterial activity of essential oil of (*Thymus vulgaris* L.) and (*Thymus daenensis* Celak).

2. Materials and Methods

2.1. Plant Material

The aerial parts of *T. vulgaris* and *T. daenensis*were collected from stages beginning of blooming set at Estahban (Fars province) in South Iran (29°, 07' N and 54°, 02' E, 1700 m above sea level), during 2014.

2.2. Essential oil extraction

The fresh aerial parts of *T. vulgaris* and *T. daenensis* were dried inside for six days at room temperature(25 ± 5 °C), and the ground to fine a powder using Moulinex food processor. The essential oil was extracted from 100 g of ground tissue in 1 L of water contained in a 2 L flask and heated by heating jacket at 100 °C for 2 h in a Clevenger–type apparatus, according to producers outlined British Pharmacopoeia. The collected essential oil was dried over anhydrous sodium sulfate and stored at 4 °C until analyzed.

2.3. Identification of the oil components

Compositions of the essential oils were determined by GC–MS. The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system. HP-5MS column (30 m x 0.25 mm, 0.25 µm film thickness) was used with helium as carrier gas with flow rate of 1.0 mL/min. The oven temperature was kept 20 °C at 50 °C for 4 min and programmed to 280 °C at a rate of 5 °C /min, and kept 20 °C constant at 280 °C for 5 min, at split mode. The injector temperature was at 20°C at 280 °C. Transfer 20 line temperatures 280 °C. MS were taken at 70 eV. Mass range was from *m/z* 35 to 450.

Identification of the essential oil components was accomplished based on comparison of retention times with those of authentic standards and by comparison of their mass spectral fragmentation patterns (Adams, 2007).

2.4. Antibacterial assay

The tested microorganisms included the following Gram-positive bacteria, *Staphylococcus aureus* were collected from Jahrom (Fars province). For the experiments, antibacterial activity of the crude extract was investigated against *Staphylococcus aureus* bacterial strains by the paper disk diffusion technique. The extract was redissolved in methanol to make a 100 mg/ml solution and then filtered. From this solution, 40-µl aliquots were transferred onto blank paper disks with a diameter of 6 mm. Dried disks were placed onto Mueller Hinton agar medium (Merck) previously inoculated with a bacterial suspension (*ca.* 108 CFU/ml) and incubated at 35±1 °C for 24 h. Plates were then examined for the presence of growth inhibition zones, and diameters were measured, if any. Oxacillin disks (1 µg) and vancomycin disks (30 µg) as well as methicilin disks (5 µg) were used as positive controls, where appropriate. A disk loaded by 40 µl methanol instead of the extract solution served as the negative control. The experiments were carried out four times and the results were presented as mean±SD.

3. Results and discussion

3.1. Composition of the essential oils

Qualitative and quantitative analysis of the essential oils volatile profile are listed in Table 1. The yellow essential oils yield of studied were 0.17% and 0.45% (v/w) were identified in *T. vulgaris* and *T. Daenensis* oil, respectively. In *T. vulgaris* oil, 28 components were identified, which represented about 99.08% of the total detected constituents. The major constituents of the *T. vulgaris* oil were thymol (63.14%), *p*-cymene (9.45%), γ-terpinene (8.67%) and carvacrol (3.14%). In *T. daenensis*oil, 26 components were identified, which represented about 99.55% of the total detected constituents. The major constituents of the *T. daenensis*oil were; thymol (71.2%), γ-terpinene (8.81%), *p*-cymene (3.8%), and carvacrol (2.4%).

3.2. Antibacterial activity

Table 2 and Table 3 shows the antibacterial activity of *T. vulgaris* oil and *T. daenensis*oil also the inhibition zones resulted from standard antibiotic disks. In this way, and relative to the oils in which thymol is the component which define the chemotype, it is interesting to remark that, for some of the microorganisms assayed, an elevated concentration in this components does not necessary lead to the best bacteriostatic and bactericidal effectiveness of the thyme essential oils. According to these results, MIC and MBC of *T. vulgaris* oil and *T. daenensis*oil on *S. aureus* were estimated 0.1 to 0.5 µl/ml, respectively.

Staphylococcus aureus is considered as a main pathogen of causing nosocomial infections. The emergence of antibiotic resistant strains of S. aureus with infection outbreaks among hospitalized patients is a serious problem worldwide (Mastoraki *et al.*, 2008). Plant extracts and essential oils have always had choice of use for different purposes (Hammer *et al.*, 1999). Essential oils have been searched for their antibacterial, antifungal, antiviral, and antioxidant properties (Burt, 2004). Previous studies (Rasooli *et al.*, 2006) on the antimicrobial activity of the essential oils of some *Thymus* spp., most of them possessing large quantities of phenolic monoterpenes, have shown activity against viruses, bacteria, food-derived microbial strains and fungi.

Pervious works (Ghasemi Pirbalouti *et al.*, 2009) showed that essential oils of *Thymus daenensis* and *Thymbraspicata* leaves and flowers exhibited antibacterial activities against *L. monocytogenes* from chicken meat.

The essential oil and extract of some aromatic plants (for example mint family, Lamiaceae) with a higher percentage of cavracrol and thymol have a higher efficacy against microbial (Rasooli *et al.*, 2006).

For example, Golparvar *et al.* (2015) reported the highest thymol content *T. daenensis*(84.1%) cultivated in Isfahan obtained at the stage of before blooming and Salehi *et al*. (2014) reported the highest thymol content *T. vulgaris* (74.8 %) was obtained at the stage of beginning of blooming. Ghasemi Pirbalouti *et al.* (2013) reported the main constituents of the oil were thymol (33.9–70.3%), carvacrol (4.0–24.8%), γ-terpinene (3.9–10.4%) and p-cymene (4.8–8.6%) in *T. daenensis*, and thymol (35.5–44.4%), carvacrol (4.4–16.1%), γ-terpinene (10.5–11.9%) and p-cymene (8.5–16.1%) in *T. vulgaris*.

Biological activity of essential oils depends on their chemical composition which is determined by the genotype and influenced by environmental and agronomic conditions. The antifungal and antibacterial activity exhibited by Thymus genus essential oil has been demonstrated by several researchers (Rasooli and Mirmostafa, 2003). The antifungal and antibacterial activity exhibited by the extracts and essential oils of medicinal plants has been demonstrated by several researchers (Ozcan and Erkmen, 2001; Delgado and Palop, 2004). Carvacrol and thymol are structural isomers and have a phenolic hydroxyl at a different location on the phenolic ring. The hydroxyl group increased their hydrophilic ability, which could help them dissolve in microbial membrane and impair them (Sikkema *et al.*, 1995). The presence of the hydroxyl group on carvacrol and thymol plays an important role to depolarize membrane potential. The structure of cymene was like carvacrol, but lacked the hydroxyl groups. It also could decrease the membrane potential; however, higher concentrations were needed to obtain the same reduction as that obtained with carvacrol (Ultee *et al.*, 2002).

Table 1. Chemical composition of essential oils of *T. vulgaris* and *T. daenensis*

No	Compound	RI	*T. vulgaris* (%)	*T. daenensis*(%)
1	α-Thujene	932	0.88	0.78
2	α-Pinene	938	0.62	0.47
3	Camphene	948	0.47	0.15
4	Sabinene	974	0.95	0.01
5	β-Pinene	980	0.26	0.18
6	Myrcene	994	1.40	1.35
7	α-Phellandrene	1004	0.20	0.16
8	α-Terpinene	1016	1.14	1.1
9	*P*-Cymene	1027	9.45	3.8
10	1,8-cineol	1031	0.89	0.84
11	γ-Terpinene	1065	8.67	8.81
12	(E)- Sabinene hydrate	1071	0.81	0.23
13	Terpinolene	1089	2.55	0.65
14	linalool	1098	0.09	0.42
15	Camphor	1143	0.08	-
16	Borneol	1167	1.11	0.22
17	Thymol methyl ether	1235	0.59	0.24
18	Carvacrol methyl ether	1244	0.26	1.1
19	Thymol	1291	63.14	71.2
20	Carvacrol	1298	3.14	2.4
21	β-Bourbonene	1391	0.02	-
22	β-Caryophyllene	1414	1.33	2.59
23	Aromadendrene	1442	0.06	0.17
24	α-Humulene	1447	0.1	0.12
25	Germacrene-D	1478	0.09	0.4
26	γ-Cadinene	1521	0.08	-
27	Δ-Cadinene	1529	0.17	-
28	E-α-Bisabolene	1545	-	0.79
29	Caryophyllene oxide	1580	0.36	0.03
	Oil yield (V/V)		0.17	0.45
	Total		99.08	99.55

RI = Retention indices in elution order from DB-5 column

Table 2. Effect *Thymus vulgaris* essential oil on *S. aureus*.

Samples	Methicilin		Vancomycin		Oxacillin		essential oils of *T. vulgaris* (M/m)							
S. aureus	Zone diameter	Condition	Zone diameter	Condition	Zone diameter	Condition	0	0.1	0.2	0.5	1	2	4	8
1	12 mm	I****	20 mm	S***	-	R**	7×10^8	4.2×10^5	1.7×10^3	0*	0	0	0	0
2	-	R	-	R	-	R	4.32×10^8	1.6×10^5	2.1×10^3	0	0	0	0	0
3	8 mm	R	17 mm	S	-	R	4×10^8	4.42×10^5	4.0×10^{٤}	0	0	0	0	0
4	11 mm	I	14 mm	S	-	R	4.44×10^8	8.1×10^5	3.4×10^{٥}	0	0	0	0	0
5	24 mm	S	17 mm	S	-	R	5.67×10^8	3.1×10^5	7×10^3	0	0	0	0	0

**** I(Semi sensitive), *** S(Sensitive), ** R(Resistant).

Table 3. Effect *Thymus daenensis* essential oil on *S. aureus*.

Samples *S. aureus*	Methicilin		Vancomycin		Oxacillin		essential oils of *T. daenensis* (M/m)							
	Zone diameter	Condition	Zone diameter	Condition	Zone diameter	Condition	0	0.1	0.2	0.5	1	2	4	8
1	12 mm	I****	20 mm	S***	-	R**	7×10^8	3.1×10^5	0*	0*	0	0	0	0
2	-	R	-	R	-	R	4.32×10^8	3.2×10^5	0	0	0	0	0	0
3	8 mm	R	17 mm	S	-	R	4×10^8	3.12×10^5	3.2×10^4	0	0	0	0	0
4	11 mm	I	14 mm	S	-	R	4.44×10^8	5×10^3	3×10^3	0	0	0	0	0
5	24 mm	S	17 mm	S	-	R	5.67×10^8	6×10^4	0	0	0	0	0	0

**** I(Semi sensitive), ***S(Sensitive), **R(Resistant).

It was shown that the presence of free hydroxyl group is essential for antimicrobial activity of carvacrol and that this compound could act as a protonophore (Arfa *et al.,* 2006). It gets inserted in cytoplasmic membrane, changes the membrane physical and chemical properties and affects both lipid ordering and stability of bilayer, resulting in an increase of proton passive flux across the membrane. The bacteriostatic properties of this oil are suspected to be associated with the carvacrol content, which has been tested previously and was found to have a significant antibiotic activity (Ultee *et al.,* 2002). Also, synergism between carvacrol and its precursor *p*-cymene has been noted. *P-cymene* is a very weak antibacterial, and swells bacterial cell membranes to a greater extent than carvacrol does. By this mechanism p-cymene probably enables carvacrol to be more easily transported into the cell so that a synergistic effect is achieved when the two are used together. Carvacrol, which is the main component of *Thymus capitatus* 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 (Ultee *et al.,* 1999). The effect of carvacrol on *Staphylococus* was investigated by (Knowles *et al.,* 2005).

Stojkovic *et al.* (2013) reported the essential oil of thyme expressed the same MICs on *S. typhimurium* and *S. aureus* bacteria tested (1.0 µl/ml). In fact, other constituents, such as γ-terpinene, have been considered to display relatively good activity due to their possible synergistic or antagonistic effects (Vardar-Unlu and Candan, 2003) which is in agreement with our results showing that low amounts of γ-terpinene during the vegetative phase may justify the low antimicrobial activity during this period. This strong antibacterial effect could be due to flavonoids, which have been shown to be active against MRSA (Sato *et al.,* 2004). Also, increasingly adverse drug reactions to the synthetic antibiotics and the increasing resistance of some pathogens to synthetic antibiotics, has been another argument against the use of these chemicals as therapeutics (Friedman *et al.,* 2002). In our study, most of the antimicrobial activity in essential oils from *T. vulgaris* appears to be associated with phenolic compounds (thymol and carvacrol), these results agree with those reported by other authors (Karaman *et al.* 2001; Rota *et al.* 2008).

Cos and Vlietinck (2006) reported that Gram negative bacteria are generally more resistant compared to the Gram-positive ones. Also, Shan *et al.* (2005) reported that Gram-positive bacteria (*L. monocytogenes*, *Staphylococcus aureus* and *B.cereus*) were generally more sensitive to the tested extracts than Gram-negative (*Escherichia coli* and *Salmonella anatum*).

4. Conclusion

In the present work, the thymol, *p*-cymene, γ-terpinene and carvacrol were the major components of *T. vulgaris* and *T. daenensis* oil. Antimicrobial effects of the extracts, although being selective in terms of pathogenic bacteria and comparatively weak bactericides can also contain some potential for practical applications as a complementary property, e.g., in designing hurdle food preservation technologies. In addition, carvacrol and thymol had almost the same antibacterial activity on *Staphylococcus aureus*. Among the plants tested, the essential oils of *T. daenensis* showed the best antimicrobial activity.

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