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Investigation of Chemical Composition, Antibacterial and Antioxidant Activity of *Thymus daenensis* and *Thymus eriocalyx* Essential Oils against Human Pathogenic Bacteria

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ABSTRACT

Introduction: Plant essential oils can be used as alternative agents for the treatment of antibiotic-resistant pathogenic bacteria. This study aimed to investigate the chemical composition, antibacterial, and antioxidant activity of *Thymus eriocalyx* and *Thymus daenensis* essential oils against Gram-positive and Gram-negative human pathogenic bacteria. **Methods:** The aerial part of *Thymus eriocalyx* and *Thymus daenensis* in the full flowering stage were collected from West Azerbaijan Province (Urmia), Iran, in 2014. We obtained the essential oils using a Clevenger device. The disc diffusion method was used to determine the antibacterial activity of the essential oils against 15 Gram-positive and Gram-negative PTCC and ATCC bacterial standards. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were measured by microdilution broth method in 96-well plate and free radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl. Chemical analysis of the essential oils performed out by gas chromatography connected to Mass spectrometry. **Results:** Eleven (94.06%) and seven (90.76%) compounds were identified in *T. eriocalyx* and *T. daenensis* essential oils, respectively. The major components of *T. eriocalyx* essential oil were thymol (37.8%) and α-terpineol (14.91%), and of *T. daenensis* were thymol (52.36%) and carvacrol (16.72%). *T. eriocalyx* essential oil showed the highest activity against *B. cereus* with MIC of 0.93 µg mL^{-} and MBC of 1.87 µg mL^{-}. The most potent radical scavenging activity was also obtained for *T. daenensis* essential oil .**Conclusion:** Essential oil components of *T. eriocalyx* and *T. daenensis* may have the potential to be used as antimicrobial agents against antibiotic-resistant pathogenic bacteria.

INTRODUCTION

Antibiotics have been effective in treating microbial infections, but resistance to these drugs has become a challenge. There is an urgent need to identify and introduce new and useful plants to produce potential natural antibiotics with high biological potentials and the least side effects [1]. The genus *Thymus* of the family Lamiaceae [2] comprises 250 woody and cylindrical plants, short, branched, or perennial herbaceous [3].

Essential oils and extracts of thymus species are widely used in the pharmaceutical, cosmetics, and perfume industries [4]. In ancient medicine, the essential oil and extract of *Thymus* species were used to relieve seizures, painful menstruation, colic, and headache [5]. The essential oils of the *Thymus* flowers and leaves have antispasmodic and antiseptic effects [6]. The essential oil ointment of thyme is used to treat some viral diseases, especially rubella [7], calluses, warts, and abscesses [8]. Essential oils of various *Thymus* species, due to high levels of thymol and carvacrol, have potent antioxidant activity [9]. Thymol is used as a disinfectant on wounds and pimples [5]. Antibacterial, antifungal, antiviral, insecticidal, antioxidant [10], and anticancer [11] properties of different species of *Thymus* have been reported. Trombetta et al. (2005) reported the antimicrobial activity of thymol against *S. aureus* and *E. coli* [12]. Carvacrol is a natural monocyclic monoterpenoid [13], with antioxidative, antiinflammatory, antibacterial, antifungal, antiprotozoal, anticarcinogenic, antidiabetic, antinociceptive, cardioprotective, and neuroprotective properties [14]. Several studies have confirmed the bactericidal effects of thymol and carvacrol against pathogens and food

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spoilage bacteria [15].

The main constituents of *Thymus eriocalyx* essential oil collected from Markazi province (Iran) in the pre-flowering and flowering stage include thymol (42.8% and 43.1%), linalool (11.1% and 4%), γ-terpinene (6% and 6.3%), 1,8-cineole (5.6% and 3.3%), borneol (3.4% and 4.9%), α-terpineol (1.8% and 7.1%), ρ-cymene (3% and 4%), β-caryophyllene (1.8% and 3.3%), and carvacrol (2.2% and 1.9%) [16]. Thymol (36.5%), carvacrol (29.8%), ρ-cymene (10%), γ-terpinene (6.3%), and borneol (6%) were reported in the leaves essential oil of *T. spathulifolius* by water distillation method [17]. The essential oil chemical compositions of *T. kotschyanus*, *T. eriocalyx*, and *T. daenensis* aerial parts collected from Lorestan province (Iran) by water distillation method included thymol (42.6%), carvacrol (32.3%) %), ρ-cymene (4.1%), and γ-terpinene (3%) [18]. Many phenolic and flavonoid compounds were shown to have antioxidant activity by inactivating reactive oxygen species [19].

Phenolic terpenes, including thymol and carvacrol, are the essential compounds in the *Thymus* genus [20]. Monoterpenes, including ρ-cymene γ-terpinene, borneol, camphor, and camphene, were also identified in this genus [15]. Various factors, including plant species and genotype, growth stage, harvest time, collection method, drying and extracting methods, affect the quality and quantity of plant's essential oils [9]. This study aimed to investigate the chemical composition, antibacterial, and antioxidant activity of *Thymus eriocalyx* and *Thymus daenensis* essential oils against Gram-positive and Gram-negative human pathogenic bacteria.

MATERIALS AND METHODS

Mueller-Hinton agar (MHA), Nutrient Broth (NB), DPPH (2,2-diphenyl-1-picrylhydrazyl), and ascorbic acid were purchased from Merck Co. (Darmstadt, Germany). Ciprofloxacin and gentamicin discs were obtained from Paten Tab Co. (Tehran, Iran) .

Preparation of plant's essential oil . The aerial part of *T. eriocalyx* and *T. daenensis* were collected from Urmia province (Maku city), Iran, in August 2014. Samples were transferred to the Biotechnology Laboratory of Bu Ali Sina University and allowed to dry in the shade. The essential oils were extracted by the Clevenger device. One hundred and fifty g of the powdered samples were heated in water for 3 h, dehydrated by anhydrous sodium sulfate, and stored at 4 ^{o}C [21].

Bacterial Strains. Antibacterial activity of essential oils was tested *in vitro* against Gram-positive bacteria including, *Bacillus subtilis* (PTCC-1156), *Bacillus cereus* (PTCC-1247), *Streptococcus pyogenes* (PTCC-1447), *Micrococcus luteus* (ATCC 10987), *Enterococcus faecalis* (PTCC-1195), and *Staphylococcus aureus* (PTCC-1189) as well as Gram-negative bacteria including *Salmonella typhi* (PTCC-1609), *Pseudomonas aeruginosa* (PTCC-1181), *Escherichia coli* (PTCC-2922), *Shigella boydii* (PTCC1744), *Enterobacter aerogenes* (PTCC-1221), *Acinetobacter baumannii* (PTCC-4413), *Proteus mirabilis* (ATCC-1287) *Neisseria meningitides* (PTCC-4578) and *Klebsiella pneumoniae* (ATCC-1129). All bacteria were obtained from the Tehran University of Medical Sciences, Iran.

Disc diffusion assay. The antibacterial activity of essential oils was determined by disc diffusion assay [18]. Various essential oil concentrations, 10, 20, and 30 µg mL^{-} were prepared in dimethyl sulfoxide (DMSO) [22]. A 20 µl volume of each essential oil concentration was placed on discs and kept at 4 ^{0}C for 2 h, followed by incubation at 37 ^{0}C for 24 h [23]. Bacteria were grown in NB to a turbidity equivalent to 0.5 McFarland standard (1.5×10^{8} CFU) [24]. A 20 µl of bacterial suspension was cultured on an MHA plate and discs containing essential oils. Gentamicin (10 µg) and ciprofloxacin (0.005 µg) were placed on bacterial lawns before incubation at 37 ^{0}C for 24 h. Discs containing 20 µl of DMSO were also used as the negative control. The inhibitory zone formed around each disc was measured (mm) [25]. All tests were performed in triplicates.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The MIC and MBC of *T. eriocalyx* and *T. daenensis* essential oils were determined by the microdilution broth method in a 96-well plate [26]. Dilutions of the essential oils (15, 7.5, 3.75, 1.87, 0.93, and 0.46 µg mL^{-}) were prepared in NB. An extra volume of 285 µl of NB was poured into each 96-well plate, followed by 300 µl of essential oil from 30 µg mL^{-} dilution was added to the first well plate. Afterward, 300 µL of it was transferred to the second well plate, and the process continued. Finally, 15 µl of bacterial suspension (0.5 Mcfarland) was added to each well plate. The well plates were incubated at 37 ^{0}C for 24h. The lowest dilution with no growth was considered as MIC. For measuring MBC, 5 µl of bacterial suspension from the plates with a lack of bacterial growth was poured on MHA medium and cultured. The plates were incubated for 24h at 37 ^{0}C. The minimum concentration with no bacterial growth was considered as MBC.

Antioxidant activity. The free radical activity assay was performed as described by others [27]. Different dilutions (2, 4, 6, 8, and 10 µg mL^{-}) of essential oils were prepared in dimethyl sulfoxide (DMSO). The ascorbic acid and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were used as standard and reagent, respectively. The absorption of samples was recorded with a spectrophotometer at 517 nm after 30 min [28]. The IC50 of *T. eriocalyx* and *T. daenensis* essential oils and ascorbic acid were measured. The experiments were done in triplicates.

Gas Chromatography-Mass Spectrometry (GC-MS). Gas chromatography connected to mass

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spectrometry (GC/MS) was used to analyze essential oils chemical compositions in Kermanshah University, Iran. The GC/MS analysis was carried out using an Agilent 6890N coupled to Agilent S973 mass detector, with HP-5, 30 m column. The instrument was set to an initial temperature of 275 °C and maintained for 2 min. The temperature was increased to 120°C, at the rate of 8°C /min, and then to 285°C, then at the rate of an increase of 3.5°C /min. Injection port temperature was set as 350°C and the helium flow rate at 0.9 ml/min. The samples were injected in split/splitless mode. Solvent delay adjusted for 5 min and 1 µl volume injected.

Statistical analysis. The experiments were performed in a completely randomized design with the factorial test. The average comparisons were analyzed by the Duncan test at ($p<0.05$) with three replications by SPSS 16.0 software.

Table1: Essential oil chemical compositions of *T. eriocalyx* and *T. daenensis* aerial part by GCMS

T. eriocalyx	Compound content (%)	*T. daenensis*	Compound content (%)
Thymol	37.8	**Thymol**	52.36
α-Terpineol	14.91	**Carvacrol**	16.72
1,8-Cineole	8.52	**ρ-Cymene**	6.7
ρ-Cymene	7.02	**α-Terpinene**	4.08
Borneol	6.5	**E-Caryophyllene**	4.51
Linalool	5.63	**Methyl carvacrol**	3.98
Terpinen-4-ol	4.30	**Borneol**	2.41
γ-Terpinene	3.58		
E-Caryophyllene	2.63		
Methyl carvacrol	1.88		
Camphor	1.29		

RESULTS

GCMS analysis. The essential oil chemical compositions of *T. eriocalyx* and *T. daenensis* aerial parts are shown in Table1. Eleven (94.06%) and seven (90.76%) compounds were identified in *T. eriocalyx* and *T. daenensis*, respectively. The dominant chemicals in the essential oil of *T. eriocalyx* were thymol (37.8%), α-terpineol (14.91%), 1,8-cineole (8.52%), and ρ-cymene (7.02%), and thymol (52.36%), carvacrol (16.72%) and ρ-cymene (6.7%) in *T. daenensis*.

Table 2. Antibacterial activity of different concentrations of *T. eriocalyx* and *T. daenensis* essential oils against human pathogenic bacteria

Bacteria	*T. eriocalyx* (µg mL^{-})			*T.daenensis* (µg mL^{-})			Gentamicin	Ciprofloxacin
	10	20	30	10	20	30		
S. boydi	9±0.33 mm	11.3±0.86 mm	12±0.33 mm	8.5±00 mm	9.4±0.66 mm	11.2±01.2 mm	29±0.57mm	29.5±0.33mm
N. meningitides	10±0.2 mm	10±0.33 mm	11.5±0.88 mm	9.6±0.33 mm	11±0.66 mm	13±00 mm	19.66±0.33 mm	28.5±0.66 mm
A. baumannii	11±1.2 mm	13.5±0.66 mm	15±0.33 mm	11±0.88 mm	12±0.33 mm	14±0.88 mm	20±1 mm	28.5±0.66 mm
K. pneumoniae	-	10.5±0.88 mm	12.5±0.33 mm	-	-	-	20±0.57 mm	31.5±0.33 mm
S. typhi	-	-	-	-	-	-	22±0.33 mm	30±1 mm
P. aeruginosa	10±0.33 mm	12±0.66 mm	14.5±0.88 mm	-	-	-	16±0.33 mm	17.5±0.57 mm
E. coli	12±0.33 mm	14.2±0.33 mm	15±00 mm	-	-	-	19±0.57 mm	37.5±0.66 mm
E. aerogenes	-	-	-	8±00 mm	11±0.88 mm	12.5±1.2 mm	20±0.33 mm	24.5±0.66 mm
P. mirabilis	-	-	-	-	-	-	19.5±1 mm	24.5±0.57 mm
S. pyogenes	12.5±0.88 mm	14±00 mm	16.5±0.66 mm	11±0.33 mm	12±1.2 mm	13.8±0.33 mm	11±0.33 mm	28±0.33 mm
M. luteus	15.2±0.33 mm	16±0.88 mm	18±0.88 mm	14±0.66 mm	15±00 mm	15±0.88 mm	15±0.33 mm	17±0.57 mm
S. aureus	16.8±0.33 mm	16.5±0.55 mm	17.5±0.33 mm	-	-	12±0.66 mm	16.5±0.88 mm	17.5±0.88 mm
B. subtillis	17±0.66 mm	18.2±0.33 mm	19±0.66 mm	-	-	10.5±0.88 mm	15±0.88 mm	16±0.33 mm
B. cereus	17.5±0.33 mm	19±0.22 mm	22±0.88 mm	10±0.33 mm	12±0.33 mm	13.6±0.88 mm	15±0.57 mm	16.5±0.57 mm
E. faecalis	14±0.33 mm	15.5±0.88 mm	15±0.33 mm	12±0.33 mm	12.2±0.88 mm	13±0.33 mm	29.5±1 mm	33±0.57 mm

Antibacterial activity. The antibacterial activity was performed by the disc diffusion assay (22). The inhibitory zone diameters of *T. eriocalyx* and *T. daenensis* essential oils against human pathogenic bacteria are shown in Table 2. Generally, the inhibition zone diameter expanded by increasing essential oil concentration, and Gram-positive bacteria showed more sensitivity against essential oils than Gram-negative test bacteria. However, the essential oil of *T. eriocalyx* had higher antibacterial activity compared to *T. daenensis*.

B. cereus was the most susceptible organism to *T. eriocalyx* essential oil (22±0.88 mm). Moreover, *S. typhi*,

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E. aerogenes, and *P. mirabilis* were resistant to *T. eriocalyx* essential oil, and *K. pneumoniae*, *S. typhi*, *P. aeruginosa*, *E. coli*, and *P. mirabilis* to *T .daenensis* essential oil. Interestingly, *T. eriocalyx* essential oil showed a greater inhibitory zone diameter against *S. pyogenes*, *M. luteus*, *S. aureus*, *B. subtillis*, and *B. cereus* than Gentamicin.

As shown in Table3, the MIC and MBC of *T. eriocalyx* essential oil against *B. cereus* was 0.93 and 1.87 µg mL^{-}, respectively. Next, *S. typhi*, *P. mirabilis*, and *E. aerogenes* showed resistance against *T. eriocalyx* essential oil. However, no MBC of *T. eriocalyx* essential oil was observed against *K. pneumoniae*, *S. boydii*, and *P. aeruginosa*. On the other hand, *T. daenensis* essential oil on *E. faecalis* demonstrated a MIC of 3.75 µg mL^{-}, and on *S. pyogenes* an MBC of 7.5 µg mL^{-}. Moreover, *S. boydii*, *S. typhi*, *P. aeruginosa*, *P. mirabilis*, and *E. coli* showed resistance against *T. daenensis* essential oil. Finally, *P. mirabilis* and *S. typhi* showed resistance against the tested essential oils. Eventually, *T. eriocalyx* essential oil showed more inhibitory activity compared to *T. daenensis* essential oil.

Table 3. MIC and MBC of *T. eriocalyx* and *T. daenensis* essential oils against human pathogenic bacteria

Bacteria	*T. eriocalyx* (µg mL^{-})		*T. daenensis* (µg mL^{-})	
	MIC	MBC	MIC	MBC
S. boydii	15	-	-	-
N. meningitides	7.5	15	15	15
A. baumannii	7.5	15	15	-
K. pneumoniae	15	-	15	-
S. typhi	-	-	-	-
P. aeruginosa	7.5	-	-	-
E. coli	15	15	-	-
E. aerogenes	-	-	15	15
P. mirabilis	-	-	-	-
S.pyogenes	3.75	15	7.5	7.5
M.luteus	3.75	7.5	7.5	15
S. aureus	7.5	7.5	15	-
B. subtillis	1.87	3.75	7.5	-
B. cereus	0.93	1.87	15	-
E. faecalis	7.5	15	3.75	15

Assessment of antioxidant activity. As seen in Table 4, inhibition of DPPH free radicals increased by increasing essential oil concentrations. The most potent radical scavenging activity was observed for the *T. daenensis* essential oil. A significant difference was observed between the IC50 values of the essential oil of *T. eriocalyx* compare to ascorbic acid as the control.

Table4. The DPPH inhibition percentage and IC50 of aerial parts essential oil of *T. eriocalyx* and *T. daenensis*

Organ	Inhibition percentage of DPPH in different dilutions (µg mL^{-})					IC50
	2	4	6	8	10	
T. eriocalyx	88.32	91.59	95.86	96.26	97.69	0.2538^{a}
T. daenensis	96.83	96.77	97.41	98.63	98.89	0.1022^{b}
Ascorbic acid	92.92	93.8	97.8	98.82	99.72	0.1076^{b}

DPPH, Determination of 2,2-diphenyl-1-picrylhydrazyl; IC50, 50% inhibitory concentration; the same letters are not significantly different at P<0.05

DISCUSSION

The resistance of pathogenic bacteria to antibiotics has forced scientists to investigate and discover natural antibacterial agents from various sources, such as herbs [29]. The antimicrobial activity of thymol (2-isopropyl-5-methylphenol), a natural monoterpene, has been reported [30] .Teimouri et al. (2012) reported the essential oil constituents of *T. daenensis* aerial part at the flowering stage to include thymol (29.8%), carvacrol (13.6%), ρ-cymene (11.3%), borneol (6.8%), 1,8-cineole (5.89%), γ-terpinene (3.89%), camphor (3.76%), e-caryophyllene (2.98%) and α-terpinene (2.90%) by water distillation method from Kerman province, Iran [31]. Their results were similar to our findings, probably due to the common species and extracting method. The main constituents in the aerial parts essential oil of *T. kotschyanus* and *T. daenensis* collected from Hamadan province, Iran, were thymol (74.7%), ρ-cymene (6.5%), β-caryophyllene (3.8%), and methyl carvacrol (3.6%) [32]. Similar to this study, the common compounds in the *T. daenensis* essential oil were thymol, methyl carvacrol, and ρ-cymene.

According to Nejad Ebrahimi et al. (2008), the chemical compositions of the essential oil from *T. caramanicus* aerial parts collected from Kerman province (Iran) in the vegetative stage included; carvacrol (58.9%), thymol (4.6%), γ-terpinene (8%), ρ-cymene (4.6%) and α-terpinene (3%). The major components in the flowering stage were carvacrol (68.9%), thymol (5.3%), borneol (4%), γ-terpinene

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(4.6%), and ρ-cymene (6%). In the seed formation stage, carvacrol (60.2%), γ-terpinene (6.7%), and ρ-cymenes (8.9%) were the highest percentage of constitutes [4]. In a study from Turkey, carvacrol (43.13%), γ-terpinene (20.86%), ρ-cymene (13.94%), β-caryophyllene (5.10%), and thymol (4.62%) were identified in the essential oil of T. revolutus aerial parts extracted by water distillation [33].

Rasooli and Mirmostafa (2002) reported the chemical composition of aerial parts of the *T. pubescens* essential oil from Damavand mountain (Iran) in the pre-flowering stage prepared by water distillation included; carvacrol (64.79%), thymol (11.94) %), and γ-terpinene (6.12%) and in the flowering stage, carvacrol (48.75%), thymol (13.88%), ρ-cymene (12.65%) and borneol (3.77%) [34]. The compounds reported from the essential oil of *T. transcaspicus* aerial parts collected in Mashhad province during the flowering stage were thymol (56.41%), γ-terpinene (7.74%), carvacrol (7.62%), ρ-cymene (6.34%), and thymol methyl ether (4.69%) [35]. Unlike our study, Sefidkon et al. (1999) identified thymol, carvacrol, γ-terpinene, and ρ-cymene from the essential oil of *T. kotschyanus* aerial parts in different stages of growth by different methods from Tehran [36]. Such differences are due to the differences in geographical regions and climate conditions, plant collection area and time, developmental stage and plant growth conditions (light, water, mineral, and organic matter), and extract methods of essential oil. Accordingly, it could be concluded that *Thymus* spp. are generally rich in thymol and carvacrol.

The antibacterial activity of *T. pubescens* and *T. serpyllum* essential oils in the pre-flowering and flowering stages on *E. coli, S. aureus, B. subtilis,* and *P. aeruginosa* was reported [34]. The antibacterial activity of *T. pubescens* essential oil in the pre-flowering stage was related to carvacrol and thymol, and in the flowering stage was related to carvacrol, thymol, and ρ-cymene. On the other hand, *T. serpyllum* essential oil antibacterial activity in the pre-and flowering stages was related to thymol and ρ-cymene. Based on the above results, *P. aeruginosa* showed less sensitivity, which contradicted the inhibitory effect of *T. eriocalyx* essential oil in the present study. Based on the GC/MS analysis, the essential oil inhibitory activity was attributed to high levels of thymol and carvacrol compounds [37]. According to Qaralleh et al. (2009) results, *P. aeruginosa* and *S. aureus* showed the highest susceptibility against *T. capitatus* essential oil, similar to our study in *T. eriocalyx* essential oil [38]. Gram-negative bacteria, including *E. coli and K. pneumoniae* showed resistance to *T. daenensis* essential oil [31], similar to our study in the mentioned species. The highest and lowest susceptibility was observed on *B. subtilis* and *P. aeruginosa* against the essential oil of *T. caramanicus*, respectively [4]. The difference in bacterial susceptibility is related to the outer membrane of Gram-negative bacteria are hydrophobic, and the ineffectiveness of the chemicals are due to other reasons and mechanisms [39].

Therefore, the antibacterial activity of essential oils against Gram-positive bacteria may be more than Gram-negative bacteria [40, 41].

In conclusion, based on our study and other reports, essential oil components of *T. eriocalyx* and *T. daenensis* have the potential to be used as antimicrobial agents against some pathogenic bacteria.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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