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Investigating the Sensitivity and Resistance of Human Infectious Bacteria Against the Extracts of Thymus Species and Identifying Their Bioactive Compounds

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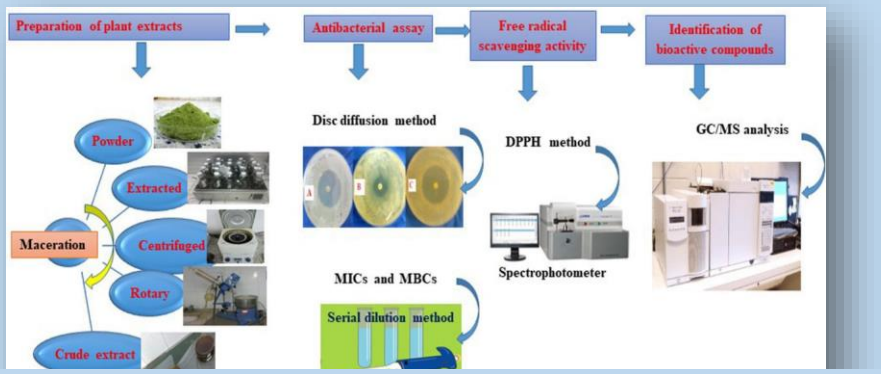
Abstract

This research was conducted with the aim of investigating the antibacterial, and antioxidant activity of *Thymus eriocalyx* and *Thymus daenensis* extracts against human pathogenic bacteria and identifies their bioactive compounds. The aerial part of *T. eriocalyx* and *T. daenensis* were collected from urmia, iran. The alcoholic extracts by maceration method and the antibacterial activity against gram-positive and gram-negative bacteria were performed by disc diffusion assay. Next, minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) by serial dilution method and free radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) were determined. In addition, the chemical compounds were analyzed by gas chromatography mass spectrometry (GC.MS). The dominant compounds in the methanol extract of *T. eriocalyx* were thymol (22.84%) and ρ-cymene (9.11%) and for *T. daenensis* as thymol (24.28%) and carvacrol (11.66%). However, sixteen (81.16%) and thirteen (76.59%) compounds were identified in the methanol extract of *T. eriocalyx* and *T. daenensis*, respectively. The most sensitive bacteria were associated to *Micrococcus luteus* and *Bacillus subtilis*, respectively, against the methanol extract of *T. Daenensis* (24±0.33 mm) and *T. eriocalyx* (21±0.88 mm) with mic of 0.78 mg ml^{-}. Finally, the lowest half maximal inhibitory concentration (IC_{50}) was 0.125 mg ml^{-} for the methanol extract of *T. daenensis*. In general, the extract of this plant can be introduced as a source for the discovery of antimicrobial drugs and antibiotics to control resistant pathogens.



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1. Introduction

The family Lamiaceae includes 236 genera with 400 species of aromatic herbaceous, perennials, and small shrubs (Ziani et al. 2019). Herbal drugs generally have fewer side effects than chemical medicines and can be used in the treatment of chronic diseases. Natural products derived from medicinal plants are the major source of drug preparation and the main basis for the development of medicinal compounds (Nalawade and Tsav 2004). The resistance of pathogenic bacteria to antibiotics has forced scientists to investigate and discover natural antibacterial agents from various sources such as herbs (Karaman et al. 2003). The key compounds of thyme include carvacrol, glycosides, flavonoids, p-cymene, borneol, linalool, alcohols, rosmarinic acid, saponins, tannins, and terpenoids have been reported (Spiewak et al. 2001). The main active ingredients in *Thymus daenensis* extracts include thymol, carvacrol, p-cymene, and gamma-terpinene (Bistgani et al. 2017). Carnosol and salvigenin were the dominant compounds in the methanol extract of *T. fontanesii* leaves, and salvianolic acid, rosmarinic acid glucoside and luteolin glucuronide were identified as the main compounds in *T. algeriensis* (Sobeh et al. 2020). Analysis of the essential oil of *T. pubescens* aerial part revealed the main components such as carvacrol, thymol, α-terpineol and p-cymene (Morteza-Semnani et al. 2006). Thymol and p-cimene-7-ol are considered as antioxidant and antimicrobial agents that provide the basis for many applications in the preservation of processed food and pharmaceutical products (Touhami et al. 2017). Rosmarinic acid has a strong anti-inflammatory effect through inhibition of NF-κB activation (Wei et al. 2018). Salvigenin has a wide range of biological effects, including antioxidant, antitumor and antibacterial (Mansourabadi et al. 2016). Antimicrobial, antioxidant and antitumor activity of thymus species has been reported (Mancini et al. 2015). The essential oil of leaves and flowers of *T. daenensis* has strong anti-rheumatic, anti-sciatic, and anti-septic properties (Mashkani et al. 2018). The antibacterial activity of *T. eriocalyx* infectious bacteria has been reported (Alamholo 2020). Alcoholic extracts with a high level of phenolic compounds including thymol and carvacrol showed the highest antioxidant and antibacterial activity (Chizzola et al. 2008). Free radicals are formed in the body by metabolizing oxygen. Active forms of oxygen are one of the most important causes of cell disorders and the occurrence of many diseases such as cardiovascular diseases, cancer, diabetes, and neurological diseases (Mazutti et al. 2008). DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Nickavar and Esbati (2012) showed the presence of the phenolic and flavonoid compounds in the hydro alcoholic extract of *T. daenensis* as well as a significant relationship between flavonoid content and free radical scavenging activity. The antioxidant activity of *T. mastichina* essential oil has been screened by DPPH free radical scavenging (Bentes et al. 2009).

The aim of this study was to investigate the antioxidant and antibacterial activity of ethanol and methanol extracts of *T. eriocalyx* and *T. daenensis* on some human pathogenic bacteria in vitro condition and also to identify chemical compounds using Gas chromatography Mass spectrometry (GC.MS).

2. Materials and methods

Culture media including Mueller-Hinton agar (MHA), and nutrient broth (NB) as well as chemical materials such as DPPH and ascorbic acid prepared from Merck Co. (Darmstadt, Germany). Also ciprofloxacin and gentamicin discs purchased from Padtan Teb Co. (Tehran, Iran).

2.1. Preparation of plant extracts

The samples of *T. eriocalyx* and *T. daenensis* were collected from Urmia, Iran (Herbarium number: AT23335 and AT23339), then, plant extracts were prepared and analyzed at Bu Ali Sina University. Methanol and ethanol solvents were used in the maceration method. The obtained solution was centrifuged at 10000 rpm for 8 minutes, and finally evaporated by rotary and transferred to an oven at 37˚C (Alamhulu and Nazeri 2015a).

2.2. Bacterial strains

Resistance and sensitivity of some human pathogenic bacteria including *Bacillus subtilis* (PTCC-1156), *Bacillus cereus* (PTCC-1247), *Streptococcus pyogenes* (PTCC-1447), *Micrococcus luteus* (ATCC 10987), *Enterococcus faecalis* (PTCC-1195),

Staphylococcus aureus (PTCC-1189), *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) were investigated on methanol and ethanol extracts of *T. eriocalyx* and *T. daenensis*.

2.3. Antibacterial assay

Antibacterial properties of *T. eriocalyx* and *T. daenensis* extracts were tested by disc diffusion assay (Tayoub et al. 2012). Ethanol and methanol extracts as 25 and 50 mg mL^{-} were prepared and then 150 µL volume of bacterial suspension as1.5×10^{8} CFU was spread on MHA medium. For incubation, a volume of 20 µL of the crude extract was poured on the discs for 24h at 37 ºC (Alamhulu and Nazeri 2016). Antibiotic discs such as gentamicin (10 µg) and ciprofloxacin (0.05 µg) as positive control and dimethyl sulfoxide (DMSO) as negative control were used (Ayoola et al. 2008). Finally, the evaluation of the inhibitory zone was performed on the used discs based on millimeters (mm).

2.4. Inhibitory and bactericidal assays

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of *T. eriocalyx* and *T. daenensis* extracts were measured by serial dilution method. Next, a volume of 385 µL of fresh NB culture medium was transferred to each tube, and 400 µL of the extract was added to the first tube and mixed. Then, 400 µL was transferred from the first tube to the second tube, and the process continued. Accordingly, a series of dilutions of 25, 12.5, 6.25, 3.125, 1.56 and, 0.78 mg mL^{-} were prepared. Next, 15 µL of bacterial suspension as 0.5 Mcfarland was added to each tube and incubated at 37 ºC for 24h. Then, the lowest dilution with no growth was considered as MIC. To measuring MBC, 5 µL of bacterial suspension was cultured and incubated from plates with no bacterial growth. Finally, the minimum concentration without bacterial growth was considered as MBC (Alamhulu and Nazeri 2015b).

2.4. Antiradical test

Different dilutions of *T. eriocalyx* and *T. daenensis* methanol extracts including 0.1, 0.2, 0.3, 0.4 and 0.5 mg mL^{-1} were prepared to calculate and evaluate free radical scavenging activity. Ascorbic acid and DPPH were used as standard and reagent, respectively. The absorbance of the samples was calculated using a spectrophotometer at a wavelength of 517 nm in triplicate (Sahin et al. 2004). Finally, the half maximal inhibitory concentration (IC_{50}) of *T. eriocalyx* and *T. daenensis* extracts and ascorbic acid was measured.

2.6. Identification of bioactive compounds

The bioactive compounds of the methanol extract were analyzed by GC.MS (Kermanshah university, Iran). GC.MS analysis was performed by an Agilent 6890N coupled to an Agilent 5973 mass detector. The instrument was set to an initial temperature of 55°C. An injection port temperature of 250 ºC and a helium flow rate of 0.9 ml/min were ensured. Finally, a volume of 0.5 µL of sample in split/splitless mode was injected.

2.7. Statistical analysis

Experiments were conducted in a factorial test in completely randomized design. Mean comparisons were analyzed with Duncan test at $P<0.05$ level by SAS software. The results were analyzed in triplicate.

3. Results

3.1. GC.MS analysis

The chemical compositions of *T. eriocalyx* and *T. daenensis* methanol extract are shown in Table 1. Sixteen (81.16%) and thirteen (76.59%) constituents were obtained in *T. eriocalyx* and *T. daenensis* extract, respectively. The chemical compounds including thymol (22.84%), ρ-cymene (9.11%), α-terpineol (6.41%), and Borneol (5.08%) were identified as the main compounds in the methanol extract of *T. eriocalyx*. The dominant compounds in *T. daenensis* included thymol (24.28%), carvacrol (11.66%) and ρ-cymene (6.33%). The chemical structures of important compounds with antimicrobial properties in *Tanacetum* spp is shown in figure 1.

Table 1. The identified compounds of *T. eriocalyx* and *T. daenensis* extract by GC. MC

T. eriocalyx	**Relative abundance (%)**	***T. daenensis***	**Relative abundance (%)**
Thymol	22.84	Thymol	24.28
Myrcene	2.08	Bisabolene	4.09
1,8-Cineole	4.87	Carvacrol	11.66
Verbenone	1.28	Citroviol	3.89
α-Terpineol	6.41	Terpinolene	4.58
ρ-Cymene	9.11	α-Terpinene	3.11
Borneol	5.28	E-Caryophyllene	5.52
Linalool	4.49	Methyl carvacrol	4.22
Eucalyptol	3.44	Eugol acetate	1.23
Terpinen-4-ol	5.08	Borneol	3.71
γ-Terpinene	2.03	ρ-Cymene	6.33
E-Caryophyllene	4.21	Myrcene	1.88
Methyl carvacrol	4.32	Eucalyptol	2.09
Carveol	2.07		
Camphor	2.77		
α-cubebene	0.88		
Total	81.16		76.59

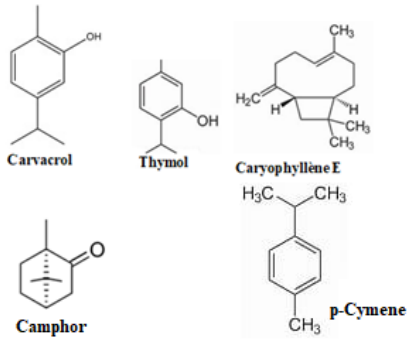

Figure 1. The chemical structures of some compounds with antimicrobial activity.

3.2. Antibacterial activity

The diameter of the inhibitory zone (mm) in the extracts of *T. eriocalyx* and *T. daenensis* on the tested bacteria is represent in Table 2. Accordingly, gram-positive bacteria showed more sensitivity to the extracts than the gram-negative bacteria. In addition, *T. daenensis* extract showed more antibacterial activity compared to *T. eriocalyx* extract.

The most susceptible bacteria were related to *M. luteus* and *B. Subtilis* on the methanol extract of *T. daenensis* (24±0.33 mm) and *T. eriocalyx* (21±0.88 mm), respectively. Moreover, *T . eriocalyx* extract on the growth of *S. typhi, A. baumannii, P. aeruginosa, E. aerogenes, P. mirabilis* and *E. faecalis*, and *T. daenensis* extract on *S. boydi S. typhi, P. aeruginosa* and *S. aureus* showed no inhibitory effect. At a concentration of 50 mg mL^{-}, the methanol extract of *T. eriocalyx* against *B. subtillis,* and *B. cereus* and *T. daenensis* ethanol and methanol extracts against *M. luteus, B. subtillis, S. pyogenes* and *B. cereus* showed the diameter of the inhibition zone more than gentamicin (fig. 2). In general, *K. pneumonia*, *S. pyogenes, M. luteus, B. subtillis* and *B. cereus* showed sensitivity to the tested extracts.

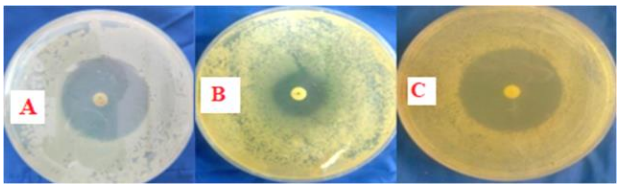

Figure 2. Inhibitory zone diameters. A: *T. daenensis* extract against *M. luteus*; B and C: *T. eriocalyx* extract against *B. cereus* and *B. subtillis.*

Table 2. Investigating the antibacterial activity of *T. eriocalyx* and *T. daenensis* extracts (Mean ± SE*).

Bacteria	*T. eriocalyx* (mg mL^{-})				*T.daenensis* (mg mL^{-})				Gentamicin	Ciprofloxacin
	Methanol		Ethanol		Methanol		Ethanol			
	25	50	25	50	25	50	25	50		
S. boydi	-	-	-	-	-	-	-	-	29±0.57	29.5±0.33
N. meningitides	-	9±0.33	-	-	8±0.33	8.5±0.55	7±0.88	8.2±0.2	19.66±0.33	28.5±0.66
A. baumannii	-	-	-	-	8±0.88	9.5±0.33	7.6±1.2	9.8±0.88	20±1	28.5±0.66
K. pneumoniae	8±0.66	9.5±0.88	7±0.55	8.3±0.66	8.5±0.55	11±00	10±0.33	11±0.66	20±0.57	31.5±0.33
S. typhi	8.5±0.33	10±0.1.2	-	-	-	-	-	-	22±0.33	30±1
P. aeruginosa	-	-	-	-	-	-	-	-	16±0.33	17.5±0.57
E. coli	-	9±0.77	-	-	9±0.33	11.5±0.66	10±0.88	12±0.55	19±0.57	37.5±0.66
E. aerogenes	-	-	-	-	9.5±0.55	10±1	12±0.33	12±0.88	20±0.33	24.5±0.66
P. mirabilis	-	-	-	-	7±0.22	7.3±0.66	7±0.66	9±0.33	19.5±1	24.5±0.57
S. pyogenes	8±0.33	11±0.66	7.5±0.88	10±0.1	9.3±0.33	13±0.55	8±0.55	12±0.66	11±0.33	28±0.33
M. luteus	12±0.55	14.3±0.33	11.5±1.2	13±0.66	17±0.66	24±0.33	15.3±0.88	18.3±0.55	15±0.33	17±0.57
S. aureus	10±0.88	13.2±0.66	-	-	-	-	-	-	16.5±0.88	17.5±0.88
B. subtillis	16±0.33	21±0.88	14±0.66	15±0.88	15±0.55	17.8±0.33	13.5±0.88	16.6±0.66	15±0.88	16±0.33
B. cereus	14±0.33	16±0.88	12±0.22	14.5±0.33	16.2±0.66	18±0.55	14±00	15.9±0.44	15±0.57	16.5±0.57
E. faecalis	-	-	-	-	14±0.33	14.5±0.88	12±0.66	13.6±0.22	29.5±1	33±0.57

The MIC and MBC of *T. eriocalyx* methanol extract were showed in 0.78 and 1.56 mg mL^{-} against *B. subtilis*. Next, some bacteria including *K. pneumonia, S. pyogenes, M. luteus, B. subtillis* and *B. cereus* showed sensitivity to *T. eriocalyx* extract. The highest inhibitory effect of *T. daenensis* extract was related to MIC at a concentration of 0.78 mg mL^{-} on *M. luteus*. However, *T. daenensis* extract had no inhibitory effect on the growth of *S. boydii*, *A. baumannii*, *S. typhi*, *P. aeruginosa, S. aureus,* and *E. coli*. In general, *T. daenensis* extract showed more inhibitory activity compared to *T. eriocalyx* extract (table 3).

Table 3. Investigation of MIC and MBC of *T. eriocalyx* and *T. daenensis* extracts against human pathogenic bacteria

Bacteria	*T. eriocalyx* (mg mL^{-})				*T.daenensis* (mg mL^{-})			
	Methanol		Ethanol		Methanol		Ethanol	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. boydi	-	-	-	-	-	-	-	-
N. meningitides	-	-	-	-	25	25	25	-
A. baumannii	-	-	-	-	-	-	-	-
K. pneumoniae	25	25	25	25	12.5	25	-	-
S. typhi	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-	-
E. coli	-	-	-	-	-	-	-	-
E. aerogenes	-	-	-	-	12.5	25	25	25
P. mirabilis	-	-	-	-	12.5	25	25	25
S. pyogenes	12.5	25	25	25	25	25	12.5	25
M. luteus	12.5	25	25	25	0.78	0.78	0.78	1.56
S. aureus	-	-	-	-	-	-	-	-
B. subtillis	0.78	1.56	1.56	3.125	6.25	12.5	1.56	3.125
B. cereus	1.56	1.56	3.125	6.25	3.125	3.125	6.25	12.5
E. faecalis	-	-	-	-	6.25	6.25	6.25	25

3.3. Assessment of antioxidant activity

The values of IC_{50} and DPPH inhibition percentage of *T. eriocalyx* and *T. daenensis* methanol extract are shown in table 4. The inhibition of DPPH free radicals showed a direct relationship with the extract concentration. *T. daenensis* extract showed the lowest IC_{50} of 0.125 mg mL^{-}. There was no significant difference between the IC_{50} value of *T. daenensis* extract compared to ascorbic acid as a control.

Table 4. DPPH inhibition percentage and IC_{50} of *T. eriocalyx* and *T. daenensis* methanol extract

Organ	Inhibition percentage of DPPH (mg mL^{-})					
	0.1	0.2	0.3	0.4	0.5	IC_{50}
T. eriocalyx	87.11	89.63	92.77	94.08	95.69	0.344^{a}
T. daenensis	91.13	92.77	94.14	95.88	98.89	0.125^{b}
Ascorbic acid	92.92	93.8	97.8	98.82	99.72	0.107^{b}

Note: The means with different letters in each row show significant differences at $P < 0.05$.

4.Discussion

Infectious diseases are still the leading cause of death worldwide, especially in developing countries (York et al.2011). Nowadays, due to the development of pathogens resistance and the high cost of treatment with chemical drugs, researchers have been led to discover the antibacterial properties of plants, which are the main source of antimicrobial properties that have been used in the treatment of infectious diseases since ancient times (Rcid et al. 2005).

In this study, bioactive compounds including thymol and ρ-cymene in *T. eriocalyx* and thymol and carvacrol in *T. daenensis* were identified as dominant compounds (table 1). The dominant components in the essential oil of *T. eriocalyx* were detected as thymol and α-terpineol, and in *T. daenensis* as thymol and carvacrol (Alamholo 2020), which was similar to the present study. According to Alizadeh et al. (2013), the main compounds of *T. daenensis* were reported as thymol, p-cymene, and β-caryophyllene. Research has shown that thymol and carvacrol have anti-inflammatory and antioxidant properties (Wattanasatcha et al. 2012). The dominant compounds including carvacrol and thymol were reported in the essential oil of *T. capitatus* collected in Egypt (Hemada and El-Darier 2011). Condurso et al. (2013) identified carvacrol, and a-terpinene from leaf essential oil of *T. capitatus* in Italy. Based on the mentioned results and the present study, in most cases the chemical compositions were similar in terms of the type of compounds.

The highest sensitivity of bacteria were associated to *M. luteus* and *B. Subtilis* against *T. daenensis* and *T. eriocalyx* methanol extract in this study (table 2). The antispasmodic, anti-inflammatory, digestive, antibacterial, antioxidant and anti-cough properties of *T. daenensis* have been reported (Mashkani et al. 2018). Aqueous and ethanol extracts of *T. capitatus* inhibit the growth of bacteria and fungi due to the presence

of flavonoids (Albano et al. 2012). Accordingly, *B. subtilis* showed the highest sensitivity with an average inhibition zone of 51.7 mm, which was sensitive to the present study. Antibacterial properties of plants may vary depending on several factors, including plant species, harvesting location, extraction time, and other environmental factors (Alamholo 2020). In this research, IC_{50} values of *T. daenensis* and *T. eriocalyx* extract were calculated as 0.125 and 0.344 mg mL^{-}, respectively (Table 4). DPPH is widely used to evaluate the antioxidant capacity and ability of compounds to act as free radical scavengers or hydrogen donors (Alamholo 2020). Asensio et al. (2011) reported the IC_{50} values of *T. mastichina* dry extracts of 0.59 – 1.78 mg mL^{-}, which showed weaker antioxidant activity than the present study. The IC_{50} values of aqueous extract of *T. atlanticus*, *T. zygis* and *T. satureioides* as 16.59, 15.43 and 14.65 µg mL^{-} were reported (Hmidani et al. 2019), which did not have the same activity as the present study. Antioxidant potential of ethanol and n-hexane extracts of *T. capitatus* showed IC_{50} values of 31 and 99 µg mL^{-} (Tabti et al. 2014). Moreover, natural antioxidants, especially phenol and flavonoids, are safe and bioactive and recently has been a focused on identifying plants with high antioxidant properties. The strong antioxidant activities could be related to the high content of phenol and flavonoids (Junejo et al. 2018). In general, the antioxidant and antibacterial activities of Thymus species could be related to the presence of bioactive compounds including thymol and carvacrol.

5. Conclusion

In conclusion, the extract of Thymus species can be recommended as a source for the processing and production of antimicrobial drugs to control pathogenic prokaryotes, especially antibiotic-resistant pathogenic bacteria in pharmaceutical and medical science.

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