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Essential oils of *Thymus pulegioides* and *Thymus glabrescens* from Romania: chemical composition and antimicrobial activity

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Abstract: The aim of this work was to analyse the chemical composition and antimicrobial properties of essential oils isolated from two wild-growing species of thyme (*Thymus pulegioides* L. and *T. glabrescens* Willd.) originating from different locations in Romania. The yield of essential oil was determined according to European Pharmacopoeia standards. Qualitative and quantitative analysis of the oils was performed using GC and GC/MS. The antimicrobial activity was tested by the microdilution technique against Gram-negative and Gram-positive bacteria (*Escherichia coli*, *Salmonella typhimurium*, *S. enteritidis*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus faecalis*, *Bacillus subtilis*, *Micrococcus luteus*, *M. flavus* and *Listeria monocytogenes*) and human pathogen yeast *Candida albicans*. The essential oil of *Thymus pulegioides* was obtained in a yield of 0.7–1 % (v/d.w. herbal drug) and the main components were carvacrol (50.5–62.6 %), γ-terpinene (9.8–9.9 %) and *p*-cymene (5.8–7.1 %). The essential oil of *T. glabrescens* was obtained in a yield of 0.7 (v/d.w. herbal drug) and the main components were geraniol (55.5 %), neryl acetate (11.1 %) and β-bisabolene (6.7 %). The essential oils inhibited microbial growth at concentrations of 10.8–27 μl/ml.

Keywords: *Thymus pulegioides*; *Thymus glabrescens*; essential oil; antimicrobial; composition.

INTRODUCTION

Thymus species (Lamiaceae) are important aromatic plants that synthesize remarkable amount of volatile compounds referred to as essential oil.[1–4]

The essential oils of more than one hundred species of the genus *Thymus* have been chemically investigated, revealing about 360 different volatile components in total. Among these, the monoterpenes were the most prominent group while sesquiterpenes represent a lower percentage of the volatiles. Generally,

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plants of the genus *Thymus* are considered the most common source of the monoterpenoid phenols, thymol and carvacrol.[1–3,5]

Essential oils derived from plants of *Thymus* genus have been found to possess significant antifungal, insecticidal, and antimicrobial activities. These properties depend greatly on their chemical compositions and are mainly attributed to their contents of carvacrol (antifungal properties) and thymol (antiseptic).[5–10] These terpenoid phenols bind to the amine groups of the proteins of the bacterial membrane, which alters their permeability and results in the death of the bacteria. In addition, thymol and carvacrol were shown to induce a decrease in the intracellular adenosine triphosphate (ATP) pool of *E. coli* and an increase of the extracellular ATP. Antibacterial activity was also observed for the aliphatic alcohols, especially geraniol, and ester components.[5]

Previous studies reported that Gram-positive bacteria (*Staphylpcoccus aureus*, *Listeria monocytogenes* and *Bacillus cereus*) are more susceptible to essential oils than Gram-negative bacteria (*Escherichia coli* and *Salmonella enteritidis*).[5,6]

The aim of the present study was to elucidate the chemical composition of the essential oils of two of the species of *Thymus* growing wild in Romania, as well as to establish their antimicrobial and antifungal properties. The two analyzed species were *Thymus pulegioides* L. and *T. glabrescens* Willd. The essential oil of *T. pulegioides* L. has been studied extensively in the world and several chemotypes were recorded (carvacrol-type, thymol-type, linalool-type or geraniol--type).[5,8,11–15] According to different studies, *T. pulegioides* essential oil is a broad-spectrum agent that inhibits the growth of moulds and yeasts (*Candida*, *Aspergillus*) and bacteria.[5,8] *T. glabrescens* essential oil has been scantily investigated.[16–18]

EXPERIMENTAL

Plant material

Aerial parts of *Thymus pulegioides* were collected at the flowering stage from two areas of the Bucegi Mountains, Buşteni (sample A) and Sinaia area (sample B), at different altitudes (1000 and 1800 m above sea level). Aerial parts of *T. glabrescens* (sample C), were gathered at flowering stage from the district of Gorj. The plant material was dried under laboratory conditions (24–25 °C) for three weeks and stored. Dr. V. Ciocarlan from the University of Agronomy of Bucharest identified the plants and voucher specimens were stored in the herbarium of the Faculty of Pharmacy, University of Medicine and Pharmacy of Bucharest.

Isolation of the essential oils

Thirty grams of air-dried plant material (two replicates for all chemotypes) were submitted, to hydrodistillation for 3 h using a Clevenger-type apparatus, according to the standard procedure reported in the European Pharmacopoeia.[19]

Gas chromatography

Qualitative and quantitative analyses of the oils were performed using GC and GC/MS. The GC analysis was realised on a GC HP-5890 II instrument equipped with a split-splitless

injector attached to an HP-5 column (25 m×0.32 mm, 0.52 μm film thickness) and a flame-ionisation-detector (FID). The carrier gas flow rate (H_2) was 1 ml/min, the split ratio 1:30, the injector temperature 250 °C and the detector temperature was 300 °C, while the column temperature was linearly programmed from 40–260 °C (at a rate of 4 °/min) and then kept at 260 °C for an additional 20 min. The same analytical conditions were employed for the GC/MS analysis, where an HP G 1800C Series II GCD system was used together with an HP-5MS capillary column (30 m×0.25 mm, 0.25 μm film thickness). The transfer line was heated at 260 °C. The mass spectra were acquired in the EI mode (70 eV), in the *m*/*z* range 40–400. The carrier gas was helium (0.9 ml/min). Identification of the individual oil components was accomplished by comparison of the retention times of the peaks with those of standard substances and by matching the mass spectral data with those from MS libraries (Wiley 275L, NIST/NBS) using a computer search and the literature.[20] For the purpose of quantitative analysis, the area percentages obtained registered by the FID were used as the base.

Micro-organisms

The antimicrobial activity of the essential oils was evaluated against the Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028), *S. enteritidis* (ATCC 13176), *Enterobacter cloacae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853) and *Proteus mirabilis* (ATCC 14273), the Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228), *Streptococcus faecalis* (ATCC 12952), *Bacillus subtilis* (ATCC 6051), *Micrococcus luteus* (ATCC 10240), *M. flavus* (ATCC 14452), and *Listeria monocytogenes* (NCTC 7973), and the human pathogen yeast *Candida albicans* (ATCC 10231). The micro-organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia.

Antimicrobial assay

The minimal inhibitory concentrations (*MIC*) were determined by the microdilution broth method according to references of the National Committee for Clinical Laboratory Standards.[21,22]

The essential oil was diluted in dimethyl sulphoxide (DMSO) in the relation 1:1. Serial dilutions (ranging from 5 to 30 μl/ml) of the stock solutions of essential oil were tested in a microtiter plate (96 wells). The standard antibiotics streptomycin and nystatin (1 mg/ml in DMSO) were used to control the sensitivity of the tested bacteria and fungi. Two growth controls, the medium (Muller–Hinton or Sabouraud broth) and the medium with 2.0 % (v/v) DMSO, were tested for each strain.

The microplates were incubated for 24 h or 48 h at 35–37 °C. The *MIC* values were determined as the lowest concentration of oil inhibiting the visible growth of each micro-organism in the microwells.

The experiments, performed in duplicate, were repeated independently two times and essentially the same results were obtained.

RESULTS AND DISCUSSION

The essential oil was obtained from air-dried plant material in a yield of 0.99–1 % v/w for sample A – *Thymus pulegioides* from Busteni, 0.7 % v/w for sample B – *T. pulegioides* from Sinaia and 0.73 % v/w for sample C – *T. glabrescens*. These results are in conformity with the European Pharmacopoeia standard for Serpylli herba (a yield of at least 0.3 %).[19]

The chemical compositions of the examined essential oils are given in Table I.

TABLE I. The chemical composition of the tested essential oils in mass % (GC/FID (Area, %)) of *Thymus pulegioides* (samples A and B) and *T. glabrescens* (sample C) collected from different regions of Romania (*RI* = retention index; n.i = not identified)

Constituent	*RI*	*T. pulegioides* (sample A)	*T. pulegioides* (sample B)	*T. glabrescens* (sample C)
α-Thujene	922	1.0	0.7	0.1
α-Pinene	927	0.5	0.7	0.1
Camphene	942	0.2	0.2	0.1
1-Octen-3-ol	978	0.7	1.9	0.2
β-Myrcene	987	1.2	1.6	0.1
3-Octanol	996	0.2	0.7	n.i.
α-Phellandrene	1000	0.2	0.2	n.i.
α-Terpinene	1012	1.3	1.5	0.1
p-Cymene	1020	7.1	5.8	1.6
Limonene	1023	0.8	n.i.	n.i.
β-Phellandrene	1024	n.i.	0.4	n.i.
1,8-Cineole	1026	n.i.	0.4	0.3
cis-β-Ocimene	1035	0.2	0.9	n.i.
γ-Terpinene	1054	9.8	9.9	0.2
cis-Sabinene hydrate	1064	0.2	n.i.	n.i.
Linalool	1099	0.4	0.3	0.7
Borneol	1162	0.6	0.2	0.2
Terpinen-4-ol	1174	0.5	0.4	0.1
p-Cymen-8-ol	1186	0.1	n.i.	n.i.
cis-Dihydrocarvone	1197	0.1	0.1	n.i.
trans-Dihydrocarvone	1203	0.1	n.i.	n.i.
trans-Piperitol	1205	n.i.	n.i.	0.1
Nerol	1228	n.i.	n.i.	2.2
Thymol methyl ether	1230	0.5	n.i.	0.5
Carvacrol methyl ether	1240	3.4	0.2	1.4
Geraniol	1257	n.i.	n.i.	55.5
Geranial	1269	n.i.	n.i.	0.5
Thymol	1293	6.6	1.6	1.5
Carvacrol	1304	50.5	62.6	4.7
α-Cubebene	1345	n.i.	n.i.	0.1
α-Terpinyl acetate	1346	0.4	n.i.	n.i.
Neryl acetate	1362	n.i.	n.i.	11.1
Carvacrol acetate	1371	n.i.	0.1	n.i.
β-Bourbonene	1378	0.1	n.i.	1.2
trans-β-Caryophyllene	1413	5.8	5.1	3.6
β-Copaene	1422	0.1	n.i.	0.2
Aromadendrene	1434	0.1	0.1	0.1
α-Humulene	1447	0.2	0.1	0.3
allo-Aromadendrene	1453	n.i.	n.i.	0.1
cis-Muurola-4(14),5-diene	1459	n.i.	n.i.	0.1
γ-Muurolene	1470	0.2	n.i.	0.2

TABLE I. Continued

Constituent	*RI*	*T. pulegioides* (sample A)	*T. pulegioides* (sample B)	*T. glabrescens* (sample C)
Germacrene D	1475	0.1	0.1	4.0
β-Bisabolene	1503	5.2	1.9	6.7
δ-Cadinene	1517	0.4	0.2	0.5
Caryophyllene oxide	1576	0.3	0.5	0.5

The chemical composition of *T. pulegioides* essential oil did not vary depending on the harvest location. In sample A (*T. pulegioides* from Sinaia), the main components were monoterpenoid phenols (57.1 %), of which carvacrol was the most abundant (50.5 %). Monoterpenoid hydrocarbons were also important constituents of this sample, reaching up to 15.5 %, as were sesquiterpenoid hydrocarbons with 12.4 %. Monoterpenoid alcohols were present in only small percentages of 1.5 %, whilst the sesquiterpenoid ones were not identified. Other identified components were phenol methyl ethers of thymol and carvacrol (3.9 %). High percentages of phenol precursors were identified: 9.8 % γ-terpinene and 7.1 % *p*-cymene.

In sample B (*T. pulegioides* from Busteni), phenols were also the main components (64.2 %), and carvacrol was again the most important one (62.6 %), together with only 1.6 % thymol. Monoterpenoid hydrocarbons represented 22.1 % of the total peak area and sesquiterpenoid ones represented 7.7 %. Monoterpenoid alcohols and sesquiterpenoid alcohols were present in a small percentage of 1.5 %. The phenol precursors were present in almost the same quantities as in sample A: 9.9 % γ-terpinene and 5.8 % *p*-cymene.

The chemical composition of sample C (*T. glabrescens* essential oil) was essentially different from those of samples A and B. Monoterpenoid hydrocarbons were present in a very small percentage (2.17 %), whereby *p*-cymene was the only compound in this group present in a percent of over 1 % (1.6 %). The most important constituents were monoterpenoid alcohols (58.9 %), of which geraniol was the most abundant (55.5 %), while its isomer, nerol was present in concentration of 2.2 %. Sesquiterpenoid hydrocarbons were rather abundant (17.4 %), especially β-bisabolene (6.7 %), germacrene D (4.0 %) and *trans*-β-caryophyllene (3.6 %). The only ester that was identified, nerol acetate, had a significant percentage of 11.1 %. The monoterpenoid phenols were present in very small quantities: thymol, 1.5 % and carvacrol, 4.7 %.

The present results are in concordance with previous studies; the most abundant components of tested essential oils were monoterpenes.[5] Samples A and B, representing *T. pulegioides* essential oil could be classified into phenolic group of *Thymus* essential oils, while sample C (*T. glabrescens* essential oil) is of a non-phenolic type.

Evaluation of the *MIC* values showed that the oils were active against the majority of the tested strains in concentrations of 10.8 – 27 μl/ml (Table II).

TABLE II. Antimicrobial activity of *T. pulegioides* and *T. glabrescens* essential oils and standards (*MIC* / μl ml^{-1}) for Gram-negative bacteria, Gram-positive bacteria and *Candida albicans* (h.c. = higher concentrations needed than the ones tested; n.t. = not tested)

Strain	*T. pulegioides* Sinaia	*T. pulegioides* Busteni	*T. glabrescens*	Streptomycin	Nystatin
Escherichia coli	10.8	27.0	13.5	5.2	n.t.
Salmonella typhi	27.0	21.6	10.8	38	n.t.
Salmonella enteritidis	16.2	10.8	27.0	38	n.t.
Enterobacter cloacae	10.8	10.8	13.5	38	n.t.
Pseudomonas aeruginosa	27.0	10.8	10.8	16	n.t.
Proteus mirabilis	10.8	h.c.	10.8	5.2	n.t.
Staphylococcus aureus	h.c.	10.8	h.c.	5.2	n.t.
Staphylococcus epidermidis	h.c.	h.c.	h.c.	5.2	n.t.
Streptococcus faecalis	h.c.	16.2	10.8	27	n.t.
Bacillus subtilis	10.8	h.c.	16.2	5.2	n.t.
Micrococcus luteus	h.c.	h.c.	h.c.	16	n.t.
Micrococcus flavus	10.8	27.0	10.8	5.2	n.t.
Listeria monocytogenes	27.0	27.0	16.2	16	n.t.
Candida albicans	10.8	10.8	10.8	n.t.	5.2

Escherichia coli, *Enterobacter cloacae*, *Proteus mirabilis*, *Bacillus subtilis* and *Micrococcus flavus* were the strains most susceptible to sample A, *T. pulegioides* essential oil (*MIC* = 10.8 μl/ml). Higher concentrations of sample B, same type of essential oil, were required to inhibit the growth of *E. coli* or *M. flavus* (*MIC* = 27.0 μl/ml). *T. pulegioides* essential oil (sample B) inhibited the growth of *Staphylococcus aureus* (*MIC* = 10.8 μl/ml) and *Streptococcus faecalis* (*MIC* = 16.2 μl/ml). The antimicrobial activity of *T. pulegioides* essential oil was expected considering its chemical composition (the main components being phenols, which are known antimicrobial agents).

Sample C (*T. glabrescens* essential oil) inhibited the growth of *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *P. mirabilis* (*MIC* = 10.8 μl/ml). The growth of Gram-positive bacteria was inhibited by this sample at concentrations of 10.8–16.2 μl/ml. According to the literature, this activity could be related to the presence of monoterpenoid alcohols in this sample, especially of geraniol (55.5 %), which manifests an antiseptic activity comparable to that of thymol, often against *Pseudomonas*.[3]

All the tested samples showed antifungal effects by inhibiting the growth of *Candida albicans* at an *MIC* of 10.8 μl/ml. Previous studies showed antifungal

activity at *MIC* values of 0.32–0.64 μl/ml for certain Portugal varieties of *T. pulegioides* essential oil, which contained thymol (26.0 %) and carvacrol (21.0 %).[8]

Other species of the genus *Thymus*, such as *T. longicaulis*, *T. magnus* or *T. quinquecostatus*, with high amounts of monoterpenoid phenols or alcohols also exhibited a broad spectrum of activity against a variety of pathogenic bacteria and yeasts.[6,7,10]

This study confirmed once again that species of the genus *Thymus* are common sources of essential oil containing phenols or other constituents that manifest antimicrobial activity.

CONCLUSIONS

Thymus pulegioides and *T. glabrescens* from Romania are important sources of essential oils, the yield of essential oil being 0.7–1.0 % (v/d.w. herbal drug). The main constituents of the essential oil are monoterpenoid phenols (especially carvacrol) in *T. pulegioides*, and monoterpenoid alcohols (especially geraniol) in *T. glabrescens*.

The tested essential oils have antimicrobial and antifungal activity; they inhibit in small concentrations (10.8–27 μl/ml) the growth of Gram-positive and Gram-negative bacteria, and *Candida albicans*.

ИЗВОД

ЕТАРСКА УЉА *Thymus pulegioides* И *Thymus glabrescens* ИЗ РУМУНИЈЕ: ХЕМИЈСКИ САСТАВ И АНТИМИКРОБНО ДЕЛОВАЊЕ

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Циљ рада је био испитивање хемијског састава и антимикробне активности етарских уља изолованих из две самоникле врсте тимијана (*Thymus pulegioides* L. и *T. glabrescens* Willd.) пореклом са различитих локалитета у Румунији. Садржај етарског уља одређен је према актуелној Европској фармакопеји. Квалитативна и квантитативна анализа уља урађене су гаснохроматографски (GC/FID и GC/MS), док је антимикробна активност испитана микродилуционом техником на Грам-негативне и Грам-позитивне бактерије (*Escherichia coli*, *Salmonella typhimurium*, *S. enteritidis*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus faecalis*, *Bacillus subtilis*, *Micrococcus luteus*, *M. flavus* и *Listeria monocytogenes*) и хумани патогени квасац *Candida albicans*. Сува биљна дрога *T. pulegioides* је садржавала 0,7–1 % (v/m) етарског уља чије су главне компоненте биле карвакрол (50,5–62,5 %), γ-терпинен (9,8—9,9 %) и *p*-цимен (5,7–7,1 %). *T. glabrescens* је садржавао око 0,7 % (v/m) етарског уља у сувој херби, а главни састојци тога уља били су гераниол (55,5 %), нерил-ацетат (11,1 %) и β-бисаболен (6,7 %). Оба испитивана етарска уља инхибирала су развој тест-организама са којима је рађено већ при концентрацијама од 10,8–27 μl/ml.

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