**ORIGINAL ARTICLE**

# ANTIMICROBIAL ACTIVITY AND CHEMICAL COMPOSITION OF THYME ESSENTIAL OILS AND THE POLYPHENOLIC CONTENT OF DIFFERENT *THYMUS* EXTRACTS

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**Abstract**

*Thymus* species belonging to the *Lamiaceae* family are popular spices and contain volatile oils as main chemical constituent. Different *Thymus* species (*Thymus vulgaris, Thymus serpyllum, Thymus pulegioides,* and *Thymus glabrescens*) were collected from the Medicinal Plant Garden of the University of Medicine and Pharmacy in Târgu. Mureş (Romania). Volatile oils from these species were separated by distillation and their antimicrobial activity was determined by agar-diffusion method. SPME-GC-MS was used to determine the chemical composition of the oils. The polyphenol content of the hydrophilic extracts prepared from *Thymus serpyllum*, *Thymus pulegioides* and *Thymus glabrescens* was determined using the LC-MS technique. Thymol (32.2% and 27.4%) was the main compound in *Thymus vulgaris* and *Thymus pulegioides* oils, respectively, while carvacrol (25.8%) in *Thymus serpyllum* oil, and terpinyl acetate (47.6%) in *Thymus glabrescens* oil. Complete inhibition of the growth of *Pseudomonas aeruginosa*, *Listeria innocua* and *Streptococcus pyogenes* has been achieved by volatile oils of the four *Thymus* spp. Quantitative differences in flavonoid content of *Thymus* extracts were found using the LC-MS measurement. The detected polyphenolic and volatile oil compounds can be considered as chemically and biologically relevant agents of the examined aromatic plants.

**Rezumat**

Speciile de *Thymus* aparţinând familiei *Lamiaceae* bogate în uleiuri volatile sunt specii larg cunoscute. Diferitele specii de Thymus (*Thymus vulgaris*, *Thymus serpyllum*, *Thymus pulegioides*, *Thymus glabrescens*) au fost recoltate din Grădina de Plante Medicinale de la Universitatea de Medicină şi Farmacie din Târgu. Mureş (România). Uleiurile volatile s-au obţinut prin distilare cu vapori de apă, iar activitatea lor antimicrobiană s-a urmărit prin metoda difuziei de agar. Prin SPME-GC-MS s-a studiat conţinutul de ulei volatil. Conţinutul de polifenoli s-a determinat prin tehnica LC-MS la speciile de *Thymus serpyllum*, *Thymus pulegioides* şi *Thymus glabrescens.* Constituenţii principali au fost timolul din uleiurile volatile de *Thymus vulgaris* şi *Thymus pulegioides* (32,2% şi 27,4%), carvacrolul din uleiul volatil de *Thymus serpyllum* (25,8%) şi acetatul de terpinen din uleiul volatil de *Thymus glabrescens* (47,6%). Uleiurile volatile pot inhiba dezvoltarea microorganismelor de *Pseudomonas aeruginosa* şi *Streptococcus pyogenes*. S-au găsit diferenţe cantitative în conţinutul de flavonoide prin analizarea prin LC-MS a probelor de *Thymus*. Constituenţii determinaţi de polifenoli şi uleiuri volatile pot fi consideraţi agenţi relevanţi din punct de vedere chimic şi biologic la plantele aromatice studiate.

**Keywords**: *Thymus* spp., volatile compounds, polyphenols, antimicrobial activity

## Introduction

*Thymus* species comprise an important genus in the *Lamiaceae* family and contain volatile oils as the main chemical compounds [5]. Thymol and carvacrol are the most important constituents of volatile oils of *Thymus* species but different chemotypes can also be found in this genus [6]. Other chemical constituents of the *Thymus* species include flavonoids (e.g. thymonin, cirsilineol and 8-methoxycirsilineol), "*Labiatae* tannin" (rosmarinic acid), caffeic acid, triterpenoids, long-chain saturated hydrocarbons and aliphatic aldehydes [5]. The antimicrobial activity of the *Thymus* derived essential oils has already been demonstrated, which correlates with the thymol content of the oil [1, 2, 3]. To determine the drug quality, measurement of the main active compounds with state-of-the-art techniques is highly important. Therefore, the aim of this study was the isolation of the essential oils and water soluble compounds from different *Thymus* species

(*Thymus vulgaris, Thymus serpyllum, Thymus pulegioides* and *Thymus glabrescens*), continued by the determination of their chemical composition with solid phase microextraction - gas chromatograpy - mass spectrometry (SPME-GC-MS) and liquid chromatography - mass spectrometry (LC-MS) techniques, respectively. Moreover, our examinations focused on testing the antimicrobial activity of the essential oils by the application of agar diffusion method.

## Materials and Methods

*Plant samples and essential oil isolation*

Different *Thymus* species (*Thymus vulgaris* L., *Thymus serpyllum* L., *Thymus pulegioides* L. and *Thymus glabrescens* Willd.) were collected from the Medicinal Plant Garden of the University of Medicine and Pharmacy from Târgu Mureş in June 2009, before the flowering period. Thirty grams of dried aerial parts (herbs) were distilled for 3 h in a Neo-Clevenger apparatus [10]. The oils were dried over anhydrous sodium sulphate for 24 h, stored at 4°C in sealed brown glass vials until further examinations. The essential oil content was measured directly by a volumetric method.

*Gas chromatographic analysis of the essential oils*

*Solid phase microextraction (SPME) conditions*. Essential oil samples (100 µL) were put into vials (20 mL headspace vials, Vialab Hungary Ltd., Budapest, Hungary) sealed with a silicon/PTFE septum prior to SPME-GC/MS analysis. Sample preparation using the static headspace solid phase microextraction (sHS-SPME) technique was carried out with a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic multipurpose sampler using a 65 µM StableFlex polydimethyl siloxane/divinyl benzene (PDMS/DVB) SPME fibre (Supelco, Bellefonte, PA, USA). After an incubation period of 5 min at 100ºC, extraction was performed by exposing the fibre to the headspace of a 20 mL vial containing the plant material for 10 min at 100ºC. The fibre was then immediately transferred to the injector port of the GC/MS, and desorbed for 1 min at 250ºC. The SPME fibre was cleaned and conditioned in a CTC Combi PAL Fibre Bake-out Station (CTC Analytics AG, Zwingen, Switzerland) in pure nitrogen atmosphere at 250ºC for 15 min after desorption.

*GC-MS conditions*. The analyses were carried out with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) system equipped with an Agilent DB-5MS capillary column (30 m × 250 µm × 0.25 µm). The GC oven temperature was programmed to increase from 60ºC (3 min isothermal) to 200ºC at 8ºC/min (2 min isothermal), from 200-230ºC at 10ºC/min (5 min isothermal) and finally from 230-250ºC at 10ºC/min (1 min isothermal). High purity helium was used as carrier gas at 1.0 ml/min (37 cm/s) in constant flow mode. The injector temperature was 250ºC and the split ratio was 1:50. The mass selective detector was equipped with a quadrupole mass analyser and operated in electron ionization mode at 70 eV in full scan mode (41-500 amu at 3.2 scan/s). The data were evaluated using MSD ChemStation D.02.00.275 software (Agilent). The identification of the compounds was carried out by comparing retention times and recorded spectra with the data of authentic standards, and the NIST 05 library was also consulted.

*Determination of the antibacterial activity of the thyme essential oils*

Antimicrobial activity of the essential oils was tested by the agar diffusion method. *Thyme* essential oil samples were used in original concentrations (thereafter conc.) and 50% dilution by ethanol (thereafter diluted). Antibacterial activity was tested against the human pathogenic Gram-negative strains *Pseudomonas aeruginosa* NCAIM B.01053 (National Collection of Agricultural and Industrial Microorganisms) and *Cronobacter sakazakii* ATCC 29544$^T$ (American Type Culture Collection) as well as the Gram-positive *Listeria innocua* T1 (Hungarian Meat Research Institute) and *Streptococcus pyogenes* NCAIM B.1998. The opportunistic pathogenic yeast *Candida albicans* HA 201 (clinical isolate) and the saprophytic yeast *Saccharomyces cerevisiae* BF (baker's yeast) were also assessed in this study. Formation of inhibition zones were compared considering the different *thymus* oil samples and microorganisms [7]. Fungal and bacterial cultures of 24 h old were applied for inoculation. For the fungi YEPD (yeast extract - peptone - dextrose) and for the bacteria PDY (peptone - dextrose - yeast extract) culture media were used, respectively. Suspensions of microorganisms (100 µL) containing $10^6$ cells/mL were pipetted onto the surface of agar plates and were evenly spread. Holes of 7 mm diameter were cut in the plates, and 10 µL essential oil samples of *Thymus vulgaris*, *Thymus serpyllum*, *Thymus pulegioides*, *Thymus glabrescens* and their 50% ethanolic dilutions were pipetted separately. Fifty % ethanol solution was used as a control. Petri plates were placed in a refrigerator (4ºC) for 1 hour allowing the compounds to diffuse into the agar before microorganisms started proliferation. In the case of bacteria, the Petri plates were incubated at 37ºC for 24 h and fungi at 30ºC for 48 h, respectively.

*Determination of the phenolic compounds by LC-MS*

Air-dried samples of *Thymus glabrescens*, *Thymus pulegioides* and *Thymus serpyllum* were pulverized, and from the samples 200 mg were weighted. Extraction was ensured with ultrasonication after

addition of 1.5 mL solvent mixture [methanol:water = 6:4 (v/v)] to each sample. Purification of extracts was done by centrifugation and filtration through a 0.45 μm pore size Syringeless filter (Mini-Uniprep, Whatman). Samples were stored in the dark at 4°C until the analysis. Standard reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and water for LC-MS (LC-MS Chromasolv) was obtained from Fluka (Buchs, Switzerland) [4, 8].

*LC-MS system and parameters of measurement*

The LC-MS system consisted of a liquid chromatograph (Prominence Liquid Chromatograph LC-20 AD, Shimadzu), a micro vacuum degasser, an auto sampler, a diode array detector, a column oven, a controller and an MS detector with electrospray ion source and quadrupole analyser (Liquid Chromatograph Mass Spectrometer LCMS-2020). The LabSsolutions (Shimadzu) software controlled the LC-MS system. A C18 column (50 mm x 2.1 mm, 2.7 μm, Supelco, USA) and a gradient of mobile phase A (2% (v/v) acetic acid in water) and mobile phase B (2% (v/v) acetic acid in methanol) was used. The gradient profile was set as follows: 0.00 min 3% B eluent, 7.00 min 20% B eluent, 7.10 min 30% B eluent, 17.00 min 40% B eluent, 25.00 min 100% B eluent, 25.10 min 3% B eluent and 30.00 min 3% B eluent. The flow rate was 0.2 mL/min; the column temperature was 50ºC. The injection volume was 2 μL. UV detections were performed at 280 nm, 320 nm, and 360 nm. The electrospray source of the MS was operated in negative mode and the interface conditions were as follows: capillary voltage of −3.5 kV, CDL voltage of 0.0 V, CDL temperature of 250 ◦C and deflector voltage of 0.0 V. The nebulizing gas flow rate was 1.5 L/min; the drying gas flow rate was 3.0 L/min and was obtained from a nitrogen generator. The detector voltage was 0.95 kV.

## Results and Discussion

*SPME-GC-MS examination of essential oils*

In Table I, composition of *Thymus* essential oils can be found. Twenty-six compounds were identified using SPME-GC-MS. In the oils of *Thumus vulgaris* and *Thymus pulegioides* thymol was the main compound (32.2% and 27.4%). *Thymus serpyllum* oil contained carvacrol (25.8%), but it was also a significant compound in the *Thymus pulegioides* oil (26.7%). *Thymus glabrescens* oil contained terpinyl acetate (47.6%) as the main compound.

**Table I**

Composition of *Thymus* essential oils determined using SPME-GC-MS

| **Compounds** | **tR (min)** | **Ratio (%) of different compounds in the essential oils** | | | |
|---|---|---|---|---|---|
| | | ***Thymus vulgaris*** | ***Thymus serpyllum*** | ***Thymus pulegioides*** | ***Thymus glabrescens*** |
| α-Thujene | 5.73 | 2.2 | 1.9 | 0.3 | 0.4 |
| α-Pinene | 5.91 | 1.3 | 1.0 | 0.2 | 0.4 |
| Camphene | 6.28 | 0.6 | 0.7 | 0.1 | 0.4 |
| Sabinene | 6.78 | 0.6 | 0.6 | 0.2 | 1.9 |
| 1-Octen-3-ol | 6.89 | 3.1 | 1.7 | 1.7 | - |
| β-Pinene | 7.08 | 2.1 | 1.8 | 0.6 | 1.0 |
| α-Terpinene | 7.73 | 2.4 | 1.5 | 0.5 | 0.5 |
| p-Cymene | 7.96 | 21.7 | 25.0 | 7.4 | 4.1 |
| Caren-3-ol | 8.02 | 0.8 | - | - | 4.2 |
| 1,8-Cineol | 8.09 | 4.4 | 9.5 | - | - |
| γ-Terpinene | 8.66 | 13.0 | 0.3 | 3.2 | 4.1 |
| Terpinolen | 8.87 | 0.8 | 0.2 | - | - |
| Linalool | 9.46 | 3.4 | 0.3 | 0.4 | 2.5 |
| Camphor | 10.54 | 0.4 | 0.1 | - | - |
| Borneol | 10.99 | 0.6 | 1.8 | 1.6 | 0.4 |
| Terpinen-4-ol | 11.13 | 1.0 | 0.5 | 0.6 | 0.1 |
| α-Terpineol | 11.45 | 0.2 | 1.0 | 1.8 | 3.0 |
| Methyl thymolether | 12.01 | 0.4 | 0.2 | 5.4 | 1.4 |
| Methyl carvacrolether | 12.18 | 1.2 | 11.1 | 7.5 | - |
| Bornyl acetate | 13.01 | 0.3 | 0.25 | - | 0.5 |
| Thymol | 13.27 | 32.2 | 2.1 | 27.4 | 19.5 |
| Carvacrol | 13.38 | 5.1 | 25.8 | 26.7 | 7.3 |
| Terpinyl acetate | 14.12 | 0.7 | 10.1 | 13.3 | 47.6 |
| β-Caryophyllene | 15.43 | 0.5 | 1.3 | 1.2 | 0.9 |
| Cadinene | 16.42 | - | 0.2 | 0.2 | - |
| β-Bisabolene | 16.75 | - | 0.2 | 0.3 | - |

$t_R$ = retention time

*Antimicrobial activity of the essential oils*

Antimicrobial spectra of the essential oils originated from different *Thymus* spp. were slightly different as shown in Table II. *Thymus vulgaris* and *Thymus serpyllum* oils were the most efficient as they inhibited all the tested bacterial and yeast strains both in the original and the half-diluted concentrations. *P. aeruginosa*, *L. innocua* and *S. pyogenes* were highly and equally sensitive to the *Thymus* oils, while *C. sakazakii* exhibited limited sensitivity. Sensitivity of the two yeast strains was similar to that of *C. sakazakii*, but *S. cerevisiae* proved to be a little more sensitive than *C. albicans*.

**Table II**

Effect of thyme essential oils on different bacterial and yeast strains
Original concentrations (conc.) and 50% ethanolic dilutions (diluted) of the oils were tested

| **Volatile oils from different *Thymus* species** | | ***Microorganisms*** | | | | | |
|---|---|---|---|---|---|---|---|
| | | *P. aeruginosa* NCAIM B01053 | *C. sakazakii* ATCC 29544$^T$ | *L. innocua* T1 | *S. pyogenes* NCAIM B.1998 | *C. albicans* HA 201 | *S. cerevisiae BF* |
| ***Thymus vulgaris*** | conc. | +++ | ++ | +++ | +++ | +++ | +++ |
| | diluted | +++ | ++ | +++ | +++ | +++ | +++ |
| ***Thymus serpyllum*** | conc. | +++ | ++ | +++ | +++ | +++ | +++ |
| | diluted | +++ | ++ | +++ | +++ | +++ | +++ |
| ***Thymus pulegioides*** | conc. | +++ | ++ | +++ | +++ | +++ | +++ |
| | diluted | +++ | - | +++ | +++ | - | ++ |
| ***Thymus glabrescens*** | conc. | +++ | - | +++ | +++ | - | +++ |
| | diluted | +++ | - | +++ | +++ | - | - |

+++ = complete inhibition; ++ = limited inhibition; - = no inhibition

*Polyphenolic compounds in the extracts of three Thymus species*

The main compound in all examined *Thymus* species was rosmarinic acid, which ranged from 1372.76 µg/g to 1426.36 µg/g. The amount of caffeic acid varied between 100.60 and 109.59 µg/g of dried material. Other identified phenolic acids were ferulic acid, *p*-coumaric acid and chlorogenic acid (Table III). From the group of flavones, both apigenin and apigenin-7-glucoside were present in all samples. Flavanones, such as naringenin, eriodictyol and dihydroquercetin were identified in each sample. Flavonols (quercetin and rutin) were also detected. From the group of flavanols, catechin was detected in all samples, but epicatechin occured only in the *Thymus glabrescens* extract (192.63 ng/g). It can be noted that the three *Thymus* species examined in this study are good sources of different phenolic acids and flavonoids, but their quantity differs between the samples.

**Table III**

Polyphenolic compounds of three different *Thymus* spp extracts as determined by LC-MS

| **Polyphenol contents** | ***Origin of extracts*** | | |
|---|---|---|---|
| | ***Thymus serpyllum*** | ***Thymus glabrescens*** | ***Thymus pulegioides*** |
| Catechin (µg/g) | 2.18 | 3.60 | 2.66 |
| Caffeic acid (µg/g) | 109.59 | 100.60 | 105.28 |
| Chlorogenic acid (µg/g) | 13.03 | 4.12 | 7.39 |
| Epicatechin (ng/g) | ND | 192.63 | ND |
| p-Coumaric acid (µg/g) | 1.60 | 1.23 | 1.48 |
| Dihydroquercetin (µg/g) | 88.57 | 41.96 | 93.73 |
| Ferulic acid (µg/g) | 3.64 | 3.58 | 3.66 |
| Rutin (µg/g) | 3.84 | 2.15 | 2.14 |
| Eriodictyol (µg/g) | 31.13 | 16.08 | 30.82 |
| Rosmarinic acid (µg/g) | 1372.76 | 1436.36 | 1412.81 |
| Apigenin-7-glucoside (µg/g) | 3.90 | 28.25 | 4.94 |
| Naringenin (µg/g) | 13.48 | 9.96 | 17.46 |
| Quercetin (µg/g) | 6.58 | 0.47 | 5.32 |
| Hesperetin (ng/g) | ND | ND | ND |
| Apigenin (µg/mg) | 124.44 | 69.17 | 121.44 |

ND = not detected

## Conclusions

Essential oils from *Thymus vulgaris* and *Thymus serpyllum* showed the highest activity against the microorganisms investigated in this study, which correlates with their thymol content. Based on our results, it can be concluded that the essential oils of *Thymus* species are very efficient disinfectants and can be applied in vaporizers against different human pathogenic Gram-positive and Gram-

negative bacteria and yeasts. The LC-MS method proved to be a useful technique for determination of phenolic acids and flavonoids in the three examined *Thymus* samples. In conclusion, the polyphenolic compounds, together with essential oil constituents, might be considered as potential active ingredients of the examined *Thymus* plants.

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