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# Composition and antimicrobial studies of essential oil of *Thymus vulgaris* from Montenegro

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***Abstract*:**

**Damjanović-Vratnica, B., Caković, D., Perović, S.: *Composition and antimicrobial studies of essential oil of Thymus vulgaris from Montenegro. Biologica Nyssana, 6 (2), December 2015: 67-73.***

Chemical composition of the hydrodistilled essential oil of *Thymus vulgaris* L. (thyme) from Montenegro was analyzed by gas chromatography-mass spectrometry and its antimicrobial activity was evaluated against 10 microorganisms, including reference and clinically isolated strains. *T. vulgaris* essential oil yield was 0.42% (v/w, based on the dry leaves weight) whereas the analysis showed that major components, amongst 22 identified in the oil, were geraniol (25.66%), geranyl-acetate (20.34%), linalool (10.89%) and caryophyllene oxide (9.89%). The results of the antimicrobial activity tests revealed that the essential oil of *T .vulgaris* from Montenegro has rather strong antimicrobial activity, especially against *Staphylococcus aureus, Escherichia coli, Candida albicans* and *Klebsiella pneumoniae*. These results confirm the potential use of *T. vulgaris* essential oil in food products the as well as for therapeutic applications.

**Key words**: *Thymus vulgaris* L., essential oil, chemical composition, antimicrobial activity

***Apstrakt*:**

**Damjanović-Vratnica, B., Caković, D., Perović, S.: *Sastav i antibakterijska studija etarskog ulja Thymus vulgaris iz Crne Gore. Biologica Nyssana, 6 (2), December 2015: 67-73.***

Hemijski sastav etarskog ulja *Thymus vulgaris* L. (timijan) sa područja Crne Gore analiziran je gasno-masenom spektrometrijom i ispitano je antimikrobno dejstvo ulja na 10 mikroorganizama, uključujući referentne i klinički izolovane sojeve. Prinos etarskog ulja *T. vulgaris* bio je 0.42% (v/w, računato na težinu suvog lišća), dok su, od ukupno 22 identifikovanih komponenti, najzastupljenije: geraniol (25.66%), geranil acetat (20.34%), linalol (10.89%) i kariofilen oksid (9.89%). Dobijeni rezultati antimikrobnog ispitivanja pokazuju da etarsko ulje *T. vulgaris* sa područja Crne Gore ima prilično snažno antimikrobno dejstvo, posebno na *Staphylococcus aureus*, *Escherichia coli, Candida albicans* i *Klebsiella pneumoniae*. Ovi rezultati potvrđuju mogućnost korišćenja etarskog ulja *T. vulgaris* u prehrambenoj industriji kao i u terapeutske svrhe.

**Key words**: *Thymus vulgaris* L., etarsko ulje, hemijski sastav, antimikrobna aktivnost

## Introduction

The genus *Thymus*, member of the *Lamiaceae* family, contains about 400 species of perennial aromatic, evergreen or semi-evergreen herbaceous plants with many subspecies, varieties, subvarieties and forms (De Martino et al., 2009). *T. vulgaris*, also known as common thyme, is indigenous in the Mediterranean region and has long history as a source of the essential oil (thyme oil) and other constituents (e.g. thymol, flavonoids, caffeic acid and labiatic acid) derived from the different parts of the plant (Hudaib et al., 2002). It has been grown commercially in a number of countries for the production of the dried leaves, thyme oil, thyme extracts, and oleoresins. Thyme oil is among the world's top 10 used essential oils, which are also utilized as a preservative for food. *Thymus* species are commonly used as herbal tea, flavoring agents (condiments and spices) and for medicinal purposes (Stahl-Biskup & Saez, 2002).

Studies have found that the main chemical compositions and the concentrations of essential oil of thyme are highly variable (Jordán et al., 2006). Seven thyme chemotypes have been described whose principal volatile components are: 1,8-cineole, lanolool, α-terpineol, geraniol, *trans*-thujan-4-ol, terpinen-4-ol, thymol and carvacrol (Diaz-Maroto et al., 2006).

Many researchers investigated chemical composition and biological properties of *T. vulgaris* essential oil and extracts from various origins (Cosentino et al., 1999; Letchamo et al., 1999; Dorman & Deans, 2000; Azaz et al., 2004; De Burt, 2004; Hudaib & Aburjai, 2007; Bakkali et al., 2008; Soković et al., 2008; Imelouane et al., 2009; Lisi et al., 2011; Roby et al., 2013; Stojković et al., 2013; Borugă et al., 2014; Martins et al., 2015). It was found that, both the isolation yield and the chemical composition of the EOs can be influenced by environmental and management factors such as temperature, water stress, soil fertility, light, pest pressure, cutting date and plant maturity (Letchamo et al., 1999).

In the Mediterranean environment, there are several ecotypes of wild-growing thyme, which differ in morphological characteristics, distinguished by a strong and penetrating odor and sometimes a very evident balsamic and spicy flavor (De Lisi at al., 2011).

The genus *Thymus* is represented by 15 wild-growing species in the flora of Montenegro (Rohlena, 1942; Pulević, 2005). Essential oils composition of two species were determined (Couladis et al., 2004; Slavovska et al., 2006), but there are no studies on the chemical profile of the best known species of this genus. The aim of this study is to determine the chemical composition together with the antimicrobial properties of the essential oil from leaves of cultivated *T. vulgaris* L. from Montenegro, as natural sources of antiseptics with potential applications in the pharmaceutical and food industry.

## Material and methods

**Preparation of herb material:** Fresh leaves of *T. vulgaris* were collected manually from the collection site in the Podgorica region (Komani N 42°27'43.40", E 19°06'27.46"; central part of Montenegro) in June 2014.

The initial water inherent in the herb leaves found to be 8.9% (w/w) using a Dean and Stark apparatus with *n*-heptane as the reflux solvent. Herb material was milled in a domestic coffee mill and, after sieving in ERWEKA set of sieves, sample with a mean particle diameter size of 0.9 mm was obtained. A prepared batch was kept in an airtight resalable polypropylene bag and stored at 6 ºC for maximum 3 days before use, in order to avoid losses of volatile compounds.

**Essential oil preparation:** Herb material (70 g) was submitted to hydrodistillation in a Clevenger-type apparatus for 2 hours according to Yugoslav Pharmacopoeia IV. The obtained oil was dried over anhydrous sodium sulphate, measured, poured in hermetically sealed dark-glass containers and stored in refrigerator at 4 °C until analyzed by GC-MS.

**Gas chromatography - mass spectrometry (GC-MS):** The GC-MS analyses were carried out using a Shimadzu 2010+ gas chromatograph-mass spectrometer equipped with a ZB-5 ms (30 m x 0,25 mm x 0,25 μm) capillary column. The column temperature was programmed from 35 °C (5 min) to 300 °C at 5 °C/min. The injection port temperature was 260 °C, while the interface temperature was 305 °C. The samples of oil were injected by splitting and the split ratio was adjusted to 1:100. Helium was used as the carrier gas at a flow rate of 1.2 ml/min and 61.8 kPa inlet pressure. The MS conditions were: the ionisation voltage 70 eV, scanning interval 1.5 s, detector voltage 1.0 kV and *m/z* range 40 - 500. The components were identified by comparing their mass spectral data with those in the WILEY229 and the NIST107 mass spectra libraries, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature and whenever possible, by co-injection with authentic standards (Fluka, Great Britain).

**Microbial strains:** In order to evaluate the activity of the essential oil of *T. vulgaris*, the following microorganisms were used: reference strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231 (Torlak, Belgrade); and clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Candida albicans.*

The microorganisms were isolated from clinically treated or hospitalized patients of Medical Health Centre (Podgorica).

**Antimicrobial screening:** The agar disc diffusion method was employed to determine the antimicrobial activity of the essential oil (Erel et al., 2012). Briefly, fresh overnight cultures of the tested microorganism were adjusted with sterile saline to a concentration of approximately $1\times10^6$ CFU/ml (spectrophotometry was used to determinate optical density of cultures) and spread on the solid media plates. The above-mentioned bacteria were cultured on Nutrient broth (Torlak, Belgrade) at 37±0.1 °C while the fungi was grown on Sabouraud dextrose broth (Merck, Germany) at 20±0.1 °C. Filter paper discs (6 mm in diameter) were individually impregnated with undiluted *T. vulgaris* essential oil (4.5, 9 and 18 μg) and placed on the incubated plates.

The plates were kept at room temperature for 30 min and then incubated at 37 °C for 20 h (for bacterial strains) and 30 °C for 72 h (for fungi). Reading of the results was carried out by measuring diameters of zones of inhibition, in mm. In addition, reference antibiotic discs (provided by the Institute for Serums, Vaccines and Diagnostic Preparations - Torlak, Belgrade): ampicillin (10 μg), ceftriaxone (30 μg), erythromycin (15 μg), amykacine (30 μg), tetracycline (30 μg), for bacteria and nystatin for fungi (100 μg), were used for comparison, at the same condition as in the essential oil experiment. All tests of inhibitory activity were carried out in duplicate and the developing inhibition zones were compared with those of reference disks.

The essential oil was also subjected to the test of sterility and was found to be free of microorganisms.

## Results and discussion

Hydrodistillation of the leaves of *T. vulgaris* yielded 0.42% of essential oil (v/w, based on the dry weight of the adult leaves) with a spicy aromatic odor. The results obtained are higher than essential oil yield reported in the literature 0.09% in wild growing thyme in southern Italy (Mancini et al., 2015), and rather similar to those reported in the literature where yield was 0.15-1.2% in cultivated thyme from Italy (Hudaib et al., 2002) and 0.75% in cultivated thyme in Iran (Pirbalouti et al., 2013). Obtained essential oil yield was smaller compared to the results reported in the literature where yield was 0.9% in wild-growing thyme in Romania (Grigore et al., 2010), 1.0% in wild-growing thyme in Morocco (Imelouane et al., 2009), and significantly smaller compared to yield of 2.0% in wild-growing thyme in Spain (Arraiza et al., 2009) and 3.7-5.6% and 1.1-2. 0% in wild-growing and cultivated thyme, respectively, in Jordan (Hudaib & Aburjai, 2007).

**Table1.** Chemical composition of *T. vulgaris* essential oil (MI - Method of identification)

| No | Compound | % | MI* |
|---|---|---|---|
| 1 | α-pinene | 0.15 | 2,3 |
| 2 | Camphene | 0.23 | 2,3 |
| 3 | Sabinene | 0.9 | 2,3 |
| 4 | β-pinene | tr | 2,3 |
| 5 | α-terpenine | 0.43 | 2 |
| 6 | p-cymene | 1.67 | 2,3 |
| 7 | cis-ocimene | 0.22 | 2 |
| 8 | γ-terpenene | 0.69 | 2,3 |
| 9 | Linalool | 10.89 | 1,2,3 |
| 10 | Camphor | 0.28 | 2,3 |
| 11 | Borneol | 1.21 | 2,3 |
| 12 | Terpene-4-ol | 5.21 | 1,2,3 |
| 13 | α-terpineol | 0.34 | 2,3 |
| 14 | Nerol | 2.75 | 2,3 |
| 15 | Neral | 0.48 | 2 |
| 16 | Geraniol | 25.66 | 1,2,3 |
| 17 | Geranial | 2.82 | 2,3 |
| 18 | Carvacrol | 0.78 | 2 |
| 19 | Thymol | 0.08 | 2,3 |
| 19 | Neryl-acetate | 1.12 | 2 |
| 20 | Geranyl-acetate | 20.34 | 1,2,3 |
| 21 | β-caryophyllene | 1.05 | 2,3 |
| 22 | Bicyclogermacrene | tr | 2 |
| 23 | Elemol | 5.36 | 2,3 |
| 24 | Caryophyllene oxide | 9.89 | 1,2,3 |
| 25 | Viridiflorol | 0.52 | 2,3 |
| 26 | β-eudesmol | 2.87 | 2,3 |
| | Total | 95.94 | |
| | Monoterpene hydrocarbons | 4.29 | |
| | Oxygenated monoterpenes | 71.96 | |
| | Sesquiterpene hydrocarbons | 1.05 | |
| | Oxygenated sesquiterpenes | 18.64 | |

* 1 – co-injection with authentic compounds; 2 – MS; 3 – literature comparison; tr < 0.1

It is acknowledged that yield and chemical composition of herbal extracts are determined by a series of factors including herb genetic, climate, elevation, and topography as well as by interaction of various factors (Pirbaoiliti et al., 2013). Thus, yield and composition of aromatic plants essential oil are influenced by harvesting time, ecological and climatic conditions. It was previously found that many species of the *Thymus* genus present different intraspecific chemotypes and that chemical composition of the essential oils is variable in relation to the harvesting time, to the stage of development of the plant, and to the field environmental conditions (De Lisi et al., 2011).

The chemical composition of the hydrodistilled thyme essential oil is shown in **Tab. 1.** GC-MS analyses revealed the presence of 26 compounds representing 95.94% of the total oil.

The major components were geraniol (25.66%), geranyl-acetate (20.34%), linalool (10.89%) and caryophyllene oxide (9.89%). Other major compounds (<1.0% of the identified portion) in the gained oil were geranial (2.82%), elemol (5.36%), terpinen-4-ol (5.21%), β-eudesmol (2.87%), nerol (2.75%), *p*-cymene (1.67%), β-caryophyllene (1.05%), borneol (1.21%) and neryl-acetate (1.12%).

It was previously reported than *T. vulgaris* species has seven genetically distinct chemotypes that can be distinguished on the basis of the dominant monoterpene produced in glandular trichomes on the surface of the leaves (Diaz-Maroto et al., 2006). In southern France, six chemotypes were found and named after its dominant monoterpene: geraniol (G), *α*-terpineol (A), thuyanol-4 (U), linalool (L), carvacrol (C), and thymol (T) (Thompson et al., 2003). The six monoterpenes are all produced from geranyl pyrophosphate through a series of changes in configuration and have quite similar molecular structures. A major distinction is the phenolic nature of carvacrol and thymol, and the nonphenolic nature of the four other monoterpenes. It was found that the mean values of the proportion of dominant monoterpenes in the *α*-terpineol, geraniol, and linalool chemotypes exceeded those of the thuyanol, carvacrol, and thymol chemotypes (Thompson et al., 2003). Mancini et al. (2015) found that the most abundant compounds in *T. vulgaris* from southern Italy were thymol (46.2%–67.5%), carvacrol (5.7%–7.3%) and caryophyllene oxide (1.7%–7.3%), however De Lisi et al. (2011) reported that the predominant compounds of essential oil were geraniol, thymol and linalool (De Lisi et al., 2011). Ozcan & Chalchat (2004) found that essential oil from wild-growing *T. vulgaris* in Turkey has high content of thymol (46.2%). It was previously reported (Imelouane et al., 2009) that the oil of *T. vulgaris* from Morocco contained camphor (38.54%), camphene (17.19%), *α*-pinene (9.35%) while thyme oil from Jordan was characterized by high content of phenolic monoterpenoids (mainly thymol and carvacrol) in the range 70.8-89.0% (Hudaib & Aburjai, 2007). Grosso et al. (2010) found that in thyme essential oil from Spain the most abundant compounds were thymol (35.4-41.6%) and *p*-cymene (28.9-34.8%).

Chemical composition of obtained *T. vulgaris* essential oil from Montenegro showed that the most abundant compounds were geraniol and geranyl-acetate, thus examined *T. vulgaris* belongs to chemotype geraniol. The presence of phenolic compounds thymol and carvacrol in the oil was insignificant, while content of *p*-cymene was only 1.58%, which significantly deferred from most results from literature survey. The thyme oil from Montenegro consisted mostly of oxygenated monoterpenes (71.96%) and oxygenated sesquiterpenes (18.64%).

**Table 2.** Antimicrobial activity of the *T. globulus* essential oil and some standard antibiotics

| | **Inhibition zone** (mm)* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *T. vulgaris* oil (μg) | | | **Standard antibiotics** (μg)** | | | | | | |
| | | | | AMP | CTR | ER | AMYX | TE | NY | |
| Microorganism | 4.5 | 9 | 18 | 10 | 30 | 15 | 30 | 30 | 100 | |
| *Staphylococcus aureus* | 28 | 37 | 50 | 22 | 14 | 31 | 24 | 13 | | |
| *S. aureus* ATCC 25923 | 30 | 43 | 56 | 26 | 32 | 31 | 28 | 40 | | |
| *Escherichia coli* | 29 | 37 | 43 | - | 36 | - | 26 | - | | |
| *E. coli* ATCC 25922 | 34 | 43 | 56 | 27 | 34 | 22 | 30 | 29 | | |
| *Pseudomonas aeruginosa* | - | 14 | 17 | - | 16 | - | 21 | 16 | | |
| *P. aeruginosa* ATCC 27853 | - | 18 | 22 | - | 26 | 19 | 31 | - | | |
| *Candida albicans* | 31 | 35 | 41 | | | | | | 18 | |
| *C. albicans* ATCC 10231 | 33 | 37 | 43 | | | | | | 20 | |
| *Klebsiella pneumoniae* | 38 | 41 | 52 | 15 | 35 | 15 | 26 | 25 | | |

* Includes diameter of disc; (-) not active;

**AMP: ampicillin; CTR: ceftriaxone; ER: erythromycin; AMYK: amykacine; TE: tetracycline; NY: nystatin.

The antimicrobial activity of *T. vulgaris* essential oil is due to the presence of a mixture of monoterpenes and oxygenated monoterpenes and sesquiterpenes (most of the antimicrobial activity in the oils has been attributed to the oxygenated monoterpenes). Identification of such compounds with wide biological activity is critical for mankind. It helps in the search for chemical structures that should assist in designing new drugs as therapeautics against human pathogens (Damjanović-Vratnica et al., 2011).The antimicrobial plate diffusion assay for *Thymus vulgaris* essential oil, as summarized in the **Tab. 2**, showed that different microorganisms tested had different susceptibility to the same essential oil. The essential oil activities against tested microorganisms were increased in both cases (for ATCC strains and clinically isolated strains) with increased amount of investigated essential oil.

Thyme oil was very potent against all chosen microorganisms, except *Pseudomonas aeruginosa* (at the lowest oil concentrations), whether as clinically isolated strain or as ATCC strain. Since *Pseudomonas* species are known to have ability to metabolize a wide range of organic compounds and for this fact is used extensively in bioremediation, this may explain their high level of resistance. They may simply metabolize the compounds in the oils that are inhibitory to many of the other bacteria (Chao et al., 2000). Because of that fact, the obtained activity of 18 μg of thyme oil against *P. aeruginosa* for clinically isolated and ATCC strains is noteworthy (17 and 22 mm, respectively).

For tested ATCC strains, *T. vulgaris* essential oil showed the strongest antimicrobial activity against *E. coli* ATCC 25922, followed by *S. aureus* ATCC 25923 and fungi *C. albicans* ATCC 10231.

Regarding clinically isolated bacterial strains, the highest inhibition zone values were observed against medically important pathogens *Staphylococcus aureus* and *Escherichia coli*, ranged from 28 to 50 mm and from 29 to 43 mm, respectively. For comparison, used standard antibiotics diameters of growth inhibition zones ranged from 13 to 31 mm (for *S. aureus*) and 26 to 36 mm (for *E. coli*). Very high antibacterial activity thyme essential oil showed against *Klebsiella pneumoniae*, which were significantly susceptible to the essential oil at concentration of 9 and 18 μg, with significant diameters of growth inhibition zones (29 and 38 mm), respectively. Oil exhibited very strong activity against fungi *Candida albicans* at all concentrations, which could be significant since *C. albicans* invades different areas of the human body causing cutaneous, mucocutaneous and opportunistic infections. In our experiment, diameters of growth inhibition zones ranged from 29 to 40 mm, giving two times higher effect in comparison to nystatin. It is probably due to the high amount of monoterpene alcohols and aldehydes, already known as components that inhibit the growth of fungi, especially *C. albicans* (Dalleau at al., 2008; Leite et al., 2015).

Most of the antimicrobial activity of the essential oils has been attributed to the oxygenated monoterpenes (Bakkali et al., 2008). In the literature, it was reported that various chemical compounds have direct activity against many species of bacteria, such as terpenes and a variety of aliphatic hydrocarbons (alcohols, aldehydes and ketones). The lipophilic character of their hydrocarbon skeleton and the hydrophilic character of their functional groups are of the main importance in the antimicrobial action of essential oils components (Mancini et al., 2015).

In addition, the components present in lower amount in thyme essential oil, such as *p*-cymene, carvacrol, camphor and terpinen-4-ol, could also contribute to the antimicrobial activity of the oil. It was previously found that terpinen-4-ol exhibits very high antimicrobial effect (Barel et al., 1991, Carson & Riley, 1995; Carson et al., 2006; Pazyar et al., 2013). The antimicrobial activity of the thyme essential oil could also be associated with presence of borneol and linalool, well-known chemicals with their pronounced antimicrobial properties (Viljoen et al., 2003). In fact, it is also possible that the components which are present in lower amount might be involved in some type of synergism with the other active compounds.

Antibacterial activity of essential oils from many herb species has been extensively surveyed in last decades (Rios & Recio, 2005), but their antimicrobial mechanism has not been reported in excessive details. It was found that most active antimicrobial compounds of essential oils are terpenes and phenolics and, thus, their mode of action might be similar to that of other phenolic compounds (Shunying et al., 2005). Individual essential oil contains complex mixtures of such compounds however, a little is known about the effect of interaction between individual constituents on antimicrobial activity. Interactions between constituents may lead to additive, synergistic or antagonistic effects (Delaquis et al., 2002).

## Conclusion

The obtained results revealed that examined *Thymus vulgaris* from Montenegro belongs to chemotype geraniol. Hydrodistillation of the leaves of *T. vulgaris* yielded 0.42% of essential oil (v/w, based on the dry weight of the adult leaves) with a spicy aromatic odor. The major components, identified by GC-MS, were geraniol (25.66%), geranyl-acetate (20.34%), linalool (10.89%) and caryophyllene oxide (9.89%). This study has shown that *T. vulgaris* essential oil possesses significant activity against different microorganisms, including human pathogens, food poisoning and spoilage bacteria and blastomycete opportunistic fungi *C. albicans*. These results confirm the potential use of *T. vulgaris* essential oil in food products as well as for therapeutic applications.

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