

THE INFLUENCE OF GEOGRAPHIC LOCATION AND ENZYME-ASSISTED EXTRACTION ON ESSENTIAL OILS COMPOSITION OF *THYMUS SERPYLLUM* GROWING WILD IN TRANSYLVANIA

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ABSTRACT. A comparative study concerning the influence of geographic location and also the influence of enzyme-assisted extraction on the essential oils isolated from Transylvanian wild *Thymus serpyllum* was achieved. The collected *Thymus serpyllum* plants, from three different regions from Transilvania, were hydrodistilled to yield essential oils. The influence of enzymatic assisted extraction on the essential oils isolated from *Thymus serpyllum* was evaluated. All analyzed plants proved to belong to carvacrol chemotype class. The major constituents of wild *Thymus serpyllum* from these three regions were carvacrol (26.58%-47.62%), p-cymene (8.61%-18.97%), methylcarvacrol (8.81%-13.35%), thymol (1.6%-11.07%), gamma-terpinene (2.09%-7.55%), beta-bysabolene (3.42%-5.18%), caryophyllene (3.17%-4.83%). Using the enzymatic treatment, the percentage in composition of some components was modified and proved to have an improvement on antioxidant and antimicrobial properties. All studied essential oils exhibited considerable antioxidant and antimicrobial activity.

Keywords: *Thymus serpyllum* essential oils, enzyme assisted extraction, antioxidant activity, antimicrobial activity, GC/MS

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INTRODUCTION

Thymus serpyllum L., also known as wild thyme, belongs to the Lamiaceae plant family. *Thymus serpyllum* is growing spontaneous in extended regions of northern and central Europe and at high altitude in Mediterranean areas¹.

Based on several studies *Thymus serpyllum* essential oil are an excellent source of bioactive compounds with powerful antioxidant, strong antibacterial and antifungal activity, anti-inflammatory, antitumor, and cytotoxic properties^{1,2,3}.

A highly variation in *Thymus serpyllum* essential oil compounds proportion was observed due to the origin, environmental condition oil also with plant stage of development and metabolism⁴. The essential oils isolated from *Thymus* genus plants, are characterized by a chemical polymorphism. Depending on source and geographical location several chemotypes (geraniol, germacrene D, citral, linalool, (E) caryophyllene, α -terpinyl acetate, carvacrol, and thymol) has been considered¹. Growing spontaneous, *Thymus serpyllum* could also been characterized by a variety of chemotypes, even within on growing area⁵.

Due to great variability in *Thymus serpyllum* essential oil composition, the evaluation of *Thymus serpyllum* grown wild in different regions is of high interest. Climatic conditions and the applied extraction technique, can influence both the qualitative composition and contents of the individual components of the isolated essential oils and consequently the bioactive properties like antioxidant and antimicrobial activity. Enzyme-assisted extraction technique is emerging as an alternative and eco-friendly approach to extract volatile oils. The biotechnological application of enzymes is not currently exploited to its maximum potential. Hydrolytic enzymes can interact with cell walls, break down their structural integrity and facilitate the release of intracellular contents from plant materials⁶. In this way, enzymes will ease the extraction and higher yields in essential oils will be obtained. Also, using this enzymatic treatment, the flavours profile can be easily modified, in sense that the percentage in composition of some components can be enhanced and also as a result of these different bioactive properties can appear. Enzymatic treatment in essential oil obtained from some spices as fenugreek, garlic, cumin, celery, pepper, mustard, chilli and citrus peel has been reported^{7,8,9,10,11,12,13}. Also results about the enzymatic influence on *Thymus capitatus* and *Rosmarinus officinalis* leaves were reported¹⁴.

In order to establish the impact of geographic location on the composition of the volatile oils, we propose a comparison study on isolated essential oils from *Thymus serpyllum* plants, collected from three different

geographic areas of Transylvania, Romania. Our purpose is to investigate the isolated essential oils chemical components and to compare them in terms of antioxidant and antimicrobial activities. Also, the influence of an enzymatic the effects of enzymatic pre-treatment on the yields, chemical composition and biological properties of *Thymus serpyllum* essential oil will be evaluated.

RESULTS AND DISCUSSION

The goal of this work was to obtain a comparative study concerning the influence of the growth regions and also the influence of enzymatic assistant extraction on the essential oils isolated from *Thymus serpyllum*. The collected *Thymus serpyllum* plants were from three different regions from Transilvania. Chemical composition, the antioxidant and antimicrobial activities of isolated essential oils were determined and compared. The influence of enzymatic assistant extraction on the essential oils isolated from *Thymus serpyllum* was investigated. The essential oil from plants collected from Nasaud area was extracted using classic Clevenger method and comparative the plant was treated with an enzymatic prepartate with a cellulosic and pectolytic activity followed by Clevenger hydrodistillation.

The *Thymus serpyllum* volatile oils obtained were light-yellow in color with herbaceous odor. Identification of chemical components in isolated essential oils by GC/MS was investigated.

The essential oils yields and the chemical composition of the essential oil analyzed by GC–MS

The yields of the obtained essential oils are provided in Table 1. Comparing the obtained essential oils from selected *Thymus serpyllum* plants, the highest yield was obtained for essential oil obtained from Nasaud area (0.50 ± 0.1 (%v/w)), followed by Tamasesti area (0.41 ± 0.1 (%v/w)). The lowest essential oils yield was obtained in case of Mociu area (0.34 ± 0.1 (%v/w)). The obtained essential oil yields are comparable, not such a big difference was observed. In case of the plant enzymatic treatment a yield improvement was observed with 12 % (0.56 ± 0.1 (%v/w)). The effect of enzymes treatment on the essential oil yield was not so high, but differences in composition were observed.

There are some reports on chemical composition and yields of the essential oils from *Thymus serpyllum* and from these studies we can conclude that chemical composition and yields are consider to be highly affected of

factors like climatic conditions, geographic growth region, stage of plant maturity and even the altitude of the growth region^{1,4,5}. Our results were similar with literature reported yields. The reported *Thymus serpyllum* essential oils yields, originated from different countries is between 0.07% to 0.6%⁵ and even higher 0.1 to 1%¹⁵. For *Thymus serpyllum* grown in Northern Kazakhstan, the essential oils yields were between 0.4- 1.4%¹⁶.

Table 1. Essential oils yields.

Yield(%v/w)	<i>Thymus serpyllum</i>
0.38±0.05	Mociu area
0.41±0.05	Tamasesti area
0.50±0.05	Nasaud area
0.56±0.05	Nasaud area enzymatically treated

The main components of the essential oils obtained from *Thymus serpyllum* plants are presented in Table 2. Identification of the constituents was based on comparison with mass fragmentation pattern and spectral comparison using NIST and Wiley mass spectra libraries of standards.

Totally 70 components were identified in the analysed plants. The composition of the studied essential oils showed that the plants are related, but differences appear in compounds relative concentration. The major constituents of *Thymus serpyllum* growing in these three regions from Transylvania were carvacrol (26.58% - 47.62%), p-cymene (8.61%-18.97%), methylcarvacrol (8.81%-13.35%), thymol (1.6%-11.07%), gamma-terpinene (2.09%-7.55%), beta-bisabolene (3.42%-5.18%), caryophyllene (3.17%-4.83%). In all three analysed essential oils, carvacrol was the majority compound.

According to *Thymus serpyllum* research studies, there is high variability in chemical composition of studied essential oils. *Thymus serpyllum* essential oil has a variety of chemotypes. Carvacrol is considered the main component, conformable to PDR from Herbal Medicine¹, but a number of studies present thymol as the major constituent in *Thymus serpyllum* essential oils^{2,17}. Thymol is the isomer of carvacrol, so differences between their ratio can exist. European Pharmacopeia agreed that a total content of carvacrol and thymol should be around 40% or more¹. Even that carvacrol and thymol are considered the principal components, some studies show other main constituents, carvacrol and thymol being just minority constituents^{1,5,18,19,20}. In some essential oil obtained from *Thymus serpyllum* growing wild in Lithuania, carvacrol and thymol weren't identified, the major components were 1,8-cineole (16.3-19.0 %), myrcene (9.7-10.7 %), 3-caryophyllene (9.6-11.3%), germacrene D (5.9-8.0%)²¹. The significant variety in composition is

connected with plant stage of development and metabolism and also with climatic conditions and geographic position of the growth region. An example was given by content of *Thymus serpyllum* essential oil growing wild in Altai Mountains, in which compositional differences appear depending on the altitude²². Also *Thymus serpyllum* grown in Kumaon region of Western Himalaya was investigated, and seasonal variation in essential oil composition was found⁴.

Table 2. Chemical composition of the obtained essential oils analysed by GC-MS

Component	M	RT	<i>Thymus serpyllum</i> %			
			Mociu	Tamasesti	Nasaud	Nasaud enzymatic
thujene	136	7.58	-	-	0.47	-
alpha -pinene	136	7.76	0.59	0.29	1.13	-
trans- ocimene	136	7.83	-	0.39	-	-
carene	136	8.07	-	0.19	0.15	-
camphene	136	8.37	0.31	-	0.79	-
beta-myrcene	136	9.23	1.17	0.81	-	-
beta-pinene	136	9.52	0.21	0.58	0.71	-
1-octen-3-ol	130	9.98	3.3	1.7	1.26	1.0
delta-carene	128	10.18	-	-	0.19	-
cis ocimene	136	10.48	-	-	-	<0.1
alpha- terpinene	136	10.62	1.13	-	0.91	-
p-cymene	134	10.99	10.66	8.61	18.97	2.37
limonene	136	11.04	-	0.31	-	-
eucaliptol	136	11.11	-	-	-	0.4
sabinene	136	11.61	-	<0.1	<0.1	-
gamma-terpinene	136	12.07	7.55	2.09	3.95	0.52
beta ocimene	136	12.97	-	-	-	<0.1
terpinolene	136	13.03	-	0.09	0.14	-
linalool	136	13.59	1.64	2.6	0.78	2
terpineol	154	13.82	-	<0.1	0.14	<0.1
octyl-acetate	172	14.16	-	0.32	-	-
verbenol	152	14.3	-	0.1	-	<0.1
tujanol	152	14.58	<0.1	<0.1	0.09	0.14
camphor	152	15.49	0.12	0.09	0.14	0.25
citronellol	156	15.62	-	-	-	0.14
izomentona	154	15.75	-	0.17	<0.1	-
Borneol	154	16.36	3.1	2.27	1.8	0.8
4-terpineol	154	16.6	1.49	1.39	0.91	3.18
thujone	152	16.78	-	0.17	0.48	-

Table 2 (continued)						
Component	M	RT	Mociu	Tamasesti	Nasaud	Nasaud enzymatic
cuminol	150	16.94	-	-	0.25	<0.1
beta-fenchol	150	17.32	0.5	0.1	0.31	0.64
cis-carveol	152	17.35	-	<0.1	-	-
p-cymen-8-ol	150	17.5	0.6	-	-	0.29
carvomenthol	156	17.96	0.15	<0.1	-	-
trans-carveol	152	18.15	-	0.1	-	-
thymol methyl ether	164	18.23	4.15	3.78	3.34	3.74
methylcarvacrol	164	18.62	13.35	8.81	9.17	8.21
thymoquinone	164	19.18	0.46	<0.1	-	0.74
geraniol	154	19.24	-	0.09	1.58	1.95
piperitona	152	19.83	0.17	2.62	0.94	-
izopiperitona	152	19.9	-	0,28	-	-
cis-geraniol	154	20.08	-	-	-	<0.1
dihydroedulan	194	20.27	-	0.1	-	-
thymol	150	20.67	4.84	1.6	11.07	24.01
carvacrol	150	21.11	31.32	47.62	26.58	28.81
isoeugenol	164	21.83	-	-	-	0.11
terpinyl acetate	196	22.24	<0.1	0.11	0.39	0.52
eugenol	164	22.65	-	-	-	0.6
carvacryl acetat	192	22.87	-	<0.1	<0.1	-
copaene	204	23.08	-	1.3	0.09	0.13
geranyl acetate	198	23.18	-	-	0.15	0.27
beta-bourbonene	204	23.36	0.1	1.18	0.22	0.37
longifolene	204	24.04	-	-	-	0.15
caryophyllene	204	24.6	4.83	3.17	4.49	4.1
germacrene	204	24.87	0.09	0.12	0.15	0.23
aromadendrene	204	25.14	-	0.09	<0.1	0.12
farnesene	204	25.44	-	-	0.09	0.19
humulene	204	25.71	<0.1	<0.1	0.24	0.25
muurolene	204	26.29	<0.1	<0.1	0.26	0.53
cubebene	204	26.51	0.14	0.43	0.24	0.33
valencene	204	26.8	-	-	0.16	0.31
beta-bysabolene	204	27.33	5.18	3.42	4.31	5.62
gamma-cadinene	204	27.55	-	<0.1	0.18	0.35
delta-cadinene	204	27.65	-	-	0.28	0.52
trans-nerolidol	222	29.11	-	-	0.55	3.11
caryophyllene oxyde	220	30.28	2.49	1.98	1.63	2.21

In our study the highest concentration of carvacrol was found in Tamasesti sample (47.62%), but the concentration of thymol was little (1.6%). Similar composition was found for Mociu sample, but a little smaller carvacrol relative concentration (31.32%), and a higher thymol concentration (4.84%) also a relative high methyl-carvacrol concentration (13.35%). This kind of composition was encountered to other essential oil samples originated from Hungary²³, North-East Italy²⁴, some area in Pakistan²⁵ and also from other Romanian region²⁶. The *Thymus serpyllum* essential oil from Nasaud area, carvacrol (26.58%), thymol (11.07%), p-cymen (18.97%) is similar in composition with samples originated from central Dalmatia and Bosnia and Herzegovina²⁷, Northern Kazakhstan¹⁶ and some area in Iran²⁸.

Even if the enzyme assisted extraction didn't have a much influence on the yield of extraction, the chemical composition suffered some modifications. In the case of carvacrol, no significant quantity increase was observed, but in case of thymol the increase was really significant from 11.07% to 24.81%. An amount decreased was observed in case of p-cymen from 18.97% to 2.37%. p-Cymen, it is known as a biological precursor of carvacrol and thymol¹ so an enzymatic transformation could explain the increase with 100% the quantity of thymol. Actually, a decreasing in the amount of monoterpene hydrocarbons was observed from 28.76% to 3.96%, while the oxygenated monoterpenes grew in quantity from 58.27% to 77.01%. New compounds were identified in enzyme assisted extracted essential oil like eucalyptol (0.4%), eugenol (0,6%) and isoegenol (0.1%), while other, from monoterpene hydrocarbons class, weren't identified.

The volatile compounds classes percentages are provided in Table 3. Oxygenated monoterpenes were the predominant class in all studied oils.

Table 3. Compound classes classification in studied essential oils

Components	<i>Thymus serpyllum</i> %			
	Mociu	Tamasesti	Nasaud	Nasaud enzymatic
Monoterpene hydrocarbons	24.92	15.11	28.76	3.96
Oxygenated monoterpenes	62.01	72.44	58.27	77.01
Sesquiterpene hydrocarbons	10.49	9.88	10.77	13.2
Oxygenated sesquiterpenes	2.49	1.98	2.18	5.32

Antioxidant activity

Antioxidant activities of obtained *Thymus serpyllum* essential oils were investigated using DPPH scavenging method. DPPH percent scavenging activities of volatile oils were measured at different concentrations between 0.5 and 5 $\mu\text{l/ml}$. EC50% is a parameter used for quantification of antioxidant activity and is giving by the concentration of essential oil used for decreasing the initial DPPH concentration by 50%. A lower EC50% indicates a higher antioxidant activity. All tested essential oils presented a high antioxidant and free radical scavenging activities. The highest antioxidant activity was found for *Thymus serpyllum* essential oil originated in Nasaud (EC50% = 2.3 ± 0.2 $\mu\text{l/ml}$), but comparable activity was revealed in Tamasesti essential oil (EC50% = 2.8 ± 0.1 $\mu\text{l/ml}$). Mociu essential oil proved also a high antioxidant activity (EC50% = 3.4 ± 0.1 $\mu\text{l/ml}$). The enzymatic extraction demonstrated to have a beneficial effect on the antioxidant activity. The essential oil obtained after enzymatic treatment exhibited a really high antioxidant activity (EC50% = 0.6 ± 0.1 $\mu\text{l/ml}$). The high antioxidant activity could be explain due to the formation by enzymatic treatment of a high quantity of thymol and also of some quantity of eugenol with it is proved to have high antioxidant properties²⁹.

Also in literature the *Thymus serpyllum* essential oil was identified like a potent antioxidant. The *Thymus serpyllum* essential oil originated from Croatia proved to have a higher antioxidant activity (EC50% = 0.4 $\mu\text{g/ml}$)²⁷, while *Thymus serpyllum* essential oil originated from Greece had also a high activity (EC50% = 0.96 $\mu\text{g/ml}$)². The high antioxidant activity in *Thymus serpyllum* essential oil, should be correlated to the phenolic compounds (thymol and carvacrol)³⁰, but also to the large number of components which have a synergic effect¹.

Table 4. Antioxidant capacity parameters

<i>Thymus serpyllum</i>	EC50%($\mu\text{L}/\text{mL}$)
Mociu area	3.4 ± 0.1
Tamasesti area	2.8 ± 0.1
Nasaud area	2.3 ± 0.2
Nasaud area enzymatically treated	0.6 ± 0.1

Antimicrobial activity

The antimicrobial activity of the obtained essential oils was evaluated against a panel of six microorganisms using of agar disc diffusion method. The data expressed as diameter of growth inhibition zone (mm). The results are illustrated in Table 5. All samples of volatile oils exhibited a high antibacterial

and antifungal activity. The highest antibacterial activity was observed against gram positive bacteria *Staphylococcus aureus*, *Bacillus cereus*. Also *Thymus serpyllum* essential oils demonstrated a very high antifungal activity on *Candida albicans*. A high antimicrobial activity on gram-negative bacteria *E. Coli* and *Klebsiella pneumonia* was observed, while against other gram-negative bacteria *Pseudomonas aeruginosa*, the

Thymus serpyllum essential oils manifested a medium antibacterial activity. The standard used, amoxicillin, proved to be not effective on gram-negative bacteria *Klebsiella pneumonia* and *Pseudomonas aeruginosa*.

An improvement in antimicrobial activity of essential oil obtained using enzymatic assisted treatment was observed, which can be explained by the composition percentages modification.

The antimicrobial activity of our *Thymus serpyllum* essential oils is in agreement with other literature results^{2,31}. From literature the antimicrobial activity is assigned to the high concentration of carvacrol in essential oils^{32,33}, but also to the presence of thymol in a high proportions^{34,35}. Our study confirms the high antimicrobial activity of essential oils containing large percentages of phenolic compounds.

The high antimicrobial activity of *Thymus serpyllum* essential oils from plants growing wild in Transylvania make them an important source of new therapeutic and antimicrobial preparates.

Table 5. Antimicrobial activity of studied essential oils

Microbial species	Inhibition diameter (mm)				
	<i>Thymus serpyllum</i> essential oil				
	Mociu	Tamasesti	Nasaud	Nasaud enzymatic	Amoxicillin
<i>Staphylococcus aureus</i>	34±1	35±1	32±1	35±1	25±1
<i>Bacillus cereus</i>	24±1	27±1	25±1	30±1	12±1
<i>E. coli</i>	24±1	30±1	22±1	25±1	18±1
<i>Klebsiella pneumoniae</i>	16±1	20±1	14±1	20±1	0
<i>Pseudomonas aeruginosa</i>	7±1	11±1	7±1	8±1	0
<i>Candida albicans</i>	26±1	30±1	26±1	30±1	18±1

CONCLUSIONS

A comparative study for analysis of influence of the geographical location and also the influence of enzymatic assistant extraction on the essential oils isolated from *Thymus serpyllum* growing wild in Transylvania was achieved.

Analysing the composition of the essential oils of *Thymus serpyllum* plants collected from different geographic areas of Transylvania showed that the plants are related, belonging to carvacrol chemotype class, but differences appear in composition and also the relative concentration of major compounds. The variation in the amount of carvacrol and other components might be connected with climatic and meteorologic conditions also with plant stage of development and metabolism.

The influence of enzymatic assistant extraction on the essential oils was studied. A highly variation in essential oil compounds proportion was observed which induced a remarkable effect on the antioxidant and antimicrobial activities. Enzyme assisted extraction could be consider as an alternative procedure to improve the quality of the essential oils and also some specific enzymes treatment might be used to modulate a specific activity. However, more studies and others hydrolytic enzymes should be investigate.

All studied essential oils demonstrated very high antioxidant and, antimicrobial activity, proving that essential oils isolated from plants growing wild in Transylvania, could be a good, inexpensive, natural, indigenous sources for natural food additives, new pharmaceutical preparates and also natural additives in cosmetic industry.

EXPERIMENTAL SECTION

MATERIAL AND METHODS

The *Thymus serpyllum* plants were collected from Nasaud area (47°17'0"N 24°24'24"E) from Bistrita-Nasaud district, Tamasesti area (7°30'12"N 23°1 1'20"E) from Maramures district, and Mociu area from Cluj district, all regions in Transylvania, Romania. The collected plants were identified at Faculty of Biology and Geology from 'Babes-Bolyai' University, Cluj-Napoca. The entire plants were naturally dried and grounded. All reactive and standards were purchased from Merck (Darmstadt, Germany). For the enzymatic treatment, a mixture of pectolytic and cellulotic enzymes, commercial preparate named Rapidase(DSM Food), was used. Antioxidant activity was determined using a Varian Cary 50 Spectrophotometer in a kinetic mode.

GC–MS Apparatus

A Trace DSQ Thermo Finnigan quadrupole mass spectrometer coupled with a Trace GC was used. The Rtx-5MS capillary column, 30mx 0.25mm, 0.25µm film thickness was used in a temperature program from 50°C, 2 min, then 8°C /min to 250°C, with 30°C /min at 310°C (10min) for essential oils compounds analysis. Helium was used as carrier gas at a flow rate of 1 mL/min. 1µL of each sample was injected into the GC-MS using the split mode (10:1) using a TriPlus autosampler (Proanalysis, Bucharest, Romania). The mass spectrometer was operated in EI mode at 70 eV, emission current was 100µA and mass spectra mass range 50-500 a.m.u. Transfer line temperature was set at 250°C, injector at 250°C and ion source at 250°C.

Extraction Procedure for Essential Oils

50 g of dried plant were submitted to hydrodistillation in a Clevenger type apparatus for 4h. The collected essential oils were dried over anhydrous Na₂SO₄. For the GC-MS analysis samples were diluted 100µL/mL in absolute ethanol.

Enzymatic Assisted Treatment

For enzymatic assisted treatment, 50 g of dried plant was treated with solution of 0.2% enzymatic prepartate in water for 24h at 37°C. After the enzymatic treatment a Clevenger hydrodistillation was achieved to yield the essential oil.

Determination of Antioxidant Activity

For determination of antioxidant activity, DPPH antioxidant assay was used. Samples essential oils were used to decolorize an ethanolic solution of 40µM DPPH. The monitoring of DPPH reduction was followed at 517nm. The percentage of DPPH scavenging activity is expressed using following formula: $DPPH_{inhibition}\% = [(A_i - A_t) / A_i] \times 100$. For determination of effective concentration (EC₅₀), different concentration of essential oils 0.5, 1, 2, 3, 4, 5 µL/mL in absolute ethanol were used. The EC₅₀ was determined by plotting the DPPH_{inhibition}% against used extract concentration.

Determination of Antimicrobial Activity

In vitro antibacterial activity of the of the essential oils were tested using Agar Diffusion Test (ADT) with evaluation of inhibition zones (IZ). For testing the antibacterial activity of the obtained essential oils, two Gram-positive, three

Gram-negative bacteria and a fungus were used as follow: *Staphylococcus aureus* ATCC 6538P, *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 10536, *Klebsiella pneumoniae* (isolated from human urine), *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans*. Amoxicillin 25 µg/disc was used as standard antibiotic in order to control the sensitivity of the microorganisms. The incubation conditions used were 24 h at 37°C. 5 µl of each volatile oils of *Thymus serpyllum* were applied on the paper disc, then disc papers were placed in the inoculated plates. After 24 h of incubation at 37 degrees Celsius the diameter of growth inhibition zones were measured.

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