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Phytochemical Investigation and Antioxidative Capacity of Triterpenes Present in Plant Species Belonging to Lamiaceae Family

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Abstract: Triterpenes are persistently associated with observed bioactivities of extracts obtained from plant material that contains these very important natural products. Many species belonging to Lamiaceae family have been used for the presence of essential oil and very little is known about the presence of the triterpene substances in this family. Qualitative and quantitative analyses of this very important substances, in the aerial parts of eight species, all belonging to Lamiaceae family, were investigated in this study. Different extracts containing triterpene substances were tested by DPPH method to evaluate their antioxidative capacity. TLC and HPLC methods, used for the analytical determination of triterpenes, showed the presence of betulin, betulinic acid, ursolic acid and oleanolic acid. Betulin (3.2 mg/g) and betulinic acid (37.1 mg/g) were the most abundant triterpene components in the hexane extracts of *Rosmarinus officinalis* L. Ursolic acid (0.14 mg/g) was the most abundant triterpene compound in the hexane extract of *Thymus pulegioides* L. All tested samples demonstrated DPPH scavenging activity in a concentration dependant manner, with a wide range of IC_{50} values from 0.4 mg/mL to 3.3 mg/mL.

INTRODUCTION

Terpenes are derived from ordinary precursors and represent the largest known group of plant secondary metabolites. Most terpenes are characteristic of the plant kingdom, but they also occur in animals, for example, sesquiterpenoid ferrohormones in insects, or diterpenes of some marine organisms. Within this group, the most important are triterpenes, from the point of view of the possibility of their industrial and therapeutic application. Triterpenes comprise about 4000 compounds that build about 40 different skeletons, with a C30 structure. Triterpenes play an important role in ecology and agronomy, in the defense against pathogens and animals, as well as in the quality of plant crops. They also play an important role in commercial use in food, cosmetics, pharmacy and industrial biotechnology sectors (Brahmkshatriya and Brahmkshatriya, 2013; Thimmappa, Geisler, Louveau, *et al.*, 2014). In terms of biological activity, the most important triterpenes are pentacyclic oleanane, ursane, lupane and tetracyclic damaran (Jäger, Trojan, Kopp, *et al.*, 2009). These include various compounds, lupane derivatives described in over 300 species of higher plants. In scientific literature, these compounds are attributed to the term "steroid triterpenes". Basically, they differ only in the oxidation state of the group bound to C-17, but the consequences of this difference with respect to their physical-chemical characteristics and pharmacological effects are very large (Dzubak, Hajduch, Vydra, *et al.*, 2006; Muffler, Leipold, Scheller, *et al.*, 2011).

Plant species from the Lamiaceae family mostly contain pentacyclic triterpenes that have anti-inflammatory, hepatoprotective, antioxidant, anticancer, antiviral, and antimicrobial activity (Dzubak, *et al.*, 2006; Shanaida, Hudz, Korzeniowska, *et al.*, 2018). Most of the

Lamiaceae species examined in this work are used to obtain essential oils, which are required in the pharmaceutical, food and cosmetic industries. Such a large production of essential oils leads to the formation of large quantities of plant residues, which contain non-volatile fractions, which can represent a good source of useful substances that are not currently used. Our aim was to investigate the presence of triterpenes in plant residues after distillation of essential oil from eight species belonging to Lamiaceae family, collected in Bosnia and Herzegovina, and to evaluate antioxidant capacity of triterpene fractions obtained with different extraction solvents.

EXPERIMENTAL

Plant material.

The aerial parts of wild plants *Rosmarinus officinalis* L. (**a**), *Salvia officinalis* L. (**b**), *Melissa officinalis* L. (**c**), *Thymus pulegioides* L. (**d**), *Lavandula officinalis* L. (**e**), *Satureja montana* L. (**f**), *Mentha piperita* L. (**g**) and *Origanum vulgare* L. (**h**), were collected at the flowering stage from May to July in Bosnia and Hercegovina in 2017. Plant material was identified by a series of comparative macroscopic, organoleptic, and TLC analyses. Plant *voucher specimens* were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Sarajevo. The raw material was dried and processed according to pharmacognostic principles. Before it was used, the plant material was stored in a dry place, in the absence of the light at room temperature.

Extraction procedure

The plant material (45.0 g), was distilled with water vapor to extract the essential oil. After steam distillation, the herbal residue was separated from water in which it was immersed, dried in an oven, in order to obtain a vegetable matrix. In order to perform quantitative extraction of triterpene compounds from the vegetable matrix, a successive extraction using three solvents of different polarity was carried out. The extraction procedure was performed in a Soxhlet apparatus for six hours. As solvents we used hexane, chloroform and methanol (each 600 ml). The extracts were concentrated under reduced pressure in a rotary evaporator.

Thin-Layer Chromatography (TLC).

In order to monitor the presence of triterpene substances in different fractions obtained by Soxhlet extraction, a TLC method was performed using pre-coated silica gel GF_{254} plates (20x20 cm, thickness 0.25 mm, Merck, Dürmsttadt). The solvent system used as eluent was benzene (Merck, Germany): ethyl acetate (Sigma-Aldrich, US): formic acid (36:12:5) (Kemika, Zagreb). Detection of triterpene substances was achieved by observation under $UV_{354/254}$, spraying with 4-anisaldehyde (Merck, Germany) - sulphuric acid (Kemika, Zagreb) and heating with a heat gun until full color development. Since the triterpene compounds are the subject of the study, the triterpene standards were used for their identification in different extracts.

High pressure liquid chromatography (HPLC)

The HPLC was used to quantifie triterpenes, using standard of betulin, betulinic acid, ursolic acid, oleanolic acid and lupeol in the extracts obtained with different solvents. This was conducted on HPLC SHIMADZU 10Avp with autosampler and spektro monitor® 3100 optical detector (LC Analytical), using a constaMetric® 3000 system for solvent release, Hyperesil ODS (Agilent Technologies) column, 4.6 x 250 mm, 5µm, mobile phase acetonitrile/aqua (700/300) acidified with ortho-phosphoric acid.

All reagents used in the experimental work were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Triterpene standards as well as investigated extracts were dissolved in acetone and used for HPLC analysis. Distilled water was used for dilution and cleaning in all analytical procedures.

1,1-Diphenyl-2-picrylhydrazyl radical-scavenging capacity (DPPH method)

The ability of phenolic derivatives to donate a hydrogen atom or an electron and scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams (Brand-Williams, Cuvelier and Berset, 1995; Thaiponga, Boonprakoba, Crosbyb, *et al.*, 2006). The tested extracts were prepared as hexane solutions (16.7 mg/ml). For each fraction analyzed, a calibration relationship diagram between reduction and concentration of dilution was calculated. Sample solution (100 µL) was mixed with 1.0 mL DPPH in anhydrous ethanol (5.25×10^{-5} mol/L). Decrease in absorbance of tested mixtures was monitored every 1 minute for 30 minutes at 517 nm using *Perkin-Elmer Lambda* 25 UV/Vis spectrophotometer. Anhydrous ethanol was used to annul the absorbance at 517 nm, DPPH solution was used as blank sample, and Trolox® (Sigma-Aldrich, US) was used as a positive control. All samples were made in triplicate.

RESULTS AND DISCUSSION

Extraction of triterpene compounds

The yield obtained by successive extraction with hexane, chloroform and methanol solvents is shown in Table 1. The highest yield, calculated as the dry extract, was obtained with methanol, followed by hexane and finally chloroform. The qualitative composition of three extracts obtained by successive extractions, was obtained by TLC.

Table 1. The yield of extracts obtained from plant material (45 g) using different solvents

Herb	Yield of dry extract		
	Hexane	Chloroform	Methanol
Rosmarinus officinalis L.	3.2 g (7.0%)	4.6 g (10.3%)	8.4 g (18.6%)
Salvia officinalis L.	6.6 g (14.8%)	1.5 g (3.3%)	11.7 g (25.9%)
Melissa officinalis L.	1.9 g (4.3%)	0.8 g (1.8%)	9.3 g (20.6%)
Thymus pulegioides L.	1.1 g (2.4%)	0.6 g (1.2%)	9.2 g (20.5%)
Lavandula officinalis L.	4.5 g (10.2%)	1.6 g (3.4%)	5.7 g (12.7%)
Satureja montana L.	2.2 g (4.8%)	0.7 g (1.6%)	7.4 g (16.5%)
Mentha piperita L.	2.6 g (5.6%)	1.7 g (3.8%)	10.5 g (23.4%)
Origanum vulgare L.	2.4 g (5.4%)	0.9 g (2.1%)	14.0 g (31.0%)

The TLC conditions used in this experiment proved to be convenient since they allowed the investigated triterpene substances to be separated and identified in different extracts. Triterpene standards used in this investigation had different Rf values as follows: lupeol (Rf 0.86), betulinic acid (Rf 0.76), ursolic acid (Rf 0.66), betulin (Rf 0.70) and oleanolic acid (Rf 0.72). Although there is a small differences in Rf values between oleanolic and betulinic acid, it was possible to distinguish them, since oleanolic acid is coloured in dark violet and betulinic acid in light violet, after detection with 4-anisaldehyde - sulfuric acid. Betulin has the lowest Rf value and is coloured in violet, while lupeol has the highest Rf value and is coloured darker violet. An example of a qualitative composition of three extracts, obtained from plant material after distillation of essential oil, using TLC, was shown for Lavandulae flos. (Figure 1.).

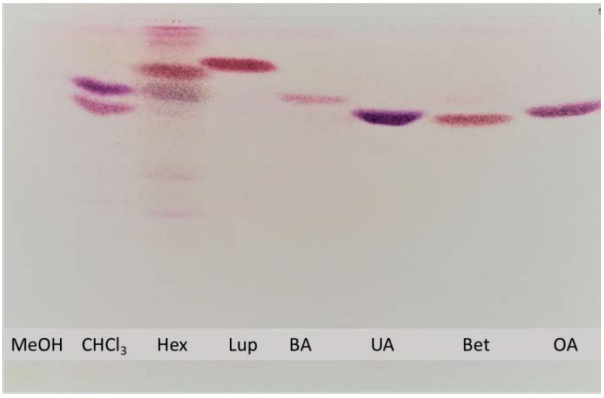

Figure 1. TLC Chromatogram of methanol (MeOH), chloroform ($CHCl_3$) and hexane (Hex) extracts of Lavandulae flos with triterpene standards: lupeol (Lup), betulinic acid (BA), ursolic acid (UA), betulin (Bet) and oleanolic acid (OA).

A fraction containing all triterpene substances could not be obtained, as different triterpenes showed different solubility in the solvents used in this investigation. The presence of triterpene substances, at any rate, in the chloroform fraction and hexane fraction were confirmed by TLC. Although TLC analysis clearly showed the absence of the investigated triterpene substances in methanol extracts, in further analysis all extracts including methanol, were subjected to HPLC in order to quantified single triterpenes by comparison with standards. The HPLC results clearly confirm the presence of triterpene substances, separated and identified by TLC method. Although the yield of methanol extract was far higher than that of hexane and chloroform extracts (Table 1), quantitative analysis showed that the latter two contained the highest amount of triterpene compounds. This indicates that the triterpene compounds are lipophilic in nature, which also determines the method of preparation of the plant materials containing them. The most common ways of preparing herbal drugs are decoct and infusion, and therefore since water is a solvent, they do not contain triterpene compounds. As a result, the use of the investigated herbs from the point of view of triterpene saponins, should involve the preparation of extracts that use lipophilic solvents.

Summarizing the results for betulin it can be concluded that the highest amount of this triterpene was found in the hexane extract of **a** (0.004 mg/ml), than hexane extract of **e** (0.002 mg/ml) followed by hexane extract of **c** (0.001 mg/ml). Chloroform extracts of **f** and **g** also contained certain amount of betulin (0.001 mg/ml) while this triterpene was not detected in the hexane extract of **h**. The highest amount of betulinic acid was detected in the hexane extract of **a** (0.05 mg/ml) and the hexane extract of **g** (0.01 mg/ml). This very important triterpene acid was detected in a very small low amount, mainly in hexane extracts of other investigated plant materials.

Interpretation of the scientific literature assign ursolic acid the role of the major bioactive principle in the plant materials with anti-inflammatory activity (Kashyap, Sharma, Tuli, *et al.*, 2016), anticancer properties (Chen, Wu, Duan, *et al.*, 2019), protective effects against cytotoxicity (Ramos-Hryb, Platt, Freitas, *et al.*, 2019) and antidiabetic activity (Wang, Zhao, Yan, *et al.*, 2019).

According to HPLC results, the highest amount of ursolic acid was detected in chloroform fractions of **e** (0.14 mg/ml) followed by **b** and **c** (009 mg/ml) and **d** (0.07 mg/ml). The content of ursolic acid was also found in hexane fractions of tested plant materials, which indicated that ursolic acid is soluble in both solvents.
After HPLC analyses of the fractions obtained from all plant materials, it has been shown that the hexane fraction of **a** was the only one containing oleanolic acid (0.001 mg/ml).

Table 2. Results of the triterpene derivatives content calculated in the hexane, chloroform and methanol extracts of individual drugs obtained by HPLC analysis.

Extract	Betulin (mg/ml) +SD	Betulinic acid (mg/ml) +SD	Ursolic acid (mg/ml) +SD	Oleanolic acid (mg/ml) +SD
a - HE	0.004 ±0	0.052 ±0.006	0.033 ±0.004	0.001 ±0
a - HL	ND	0.022 ±0.001	ND	ND
a - ME	ND	ND	ND	ND
b - HE	ND	0.002 ±0.0002	0.034 ±0.003	ND
b - HL	ND	ND	0.095 ±0.01	ND
b - ME	ND	ND	0.001 ±0	ND
c - HE	0.001 ±0.0004	0.003 ±0	0.043 ±0.005	ND
c - HL	ND	0.001 ±0.0004	0.088 ±0.008	ND
c - ME	ND	ND	ND	ND
d - HE	0.001 ±0.0005	ND	0.115 0.014	ND
d - HL	ND	ND	0.074 ±0.02	ND
d - ME	ND	0.001 ±0.0003	ND	ND
e - HE	0.002 ±0.0006	0.002 ±0	0.026 ±0.005	ND
e - HL	ND	ND	0.145 ±0.005	ND
e - ME	ND	0.001 ±0.0004	ND	ND
f - HE	ND	0.003 ±0.0003	0.062 ±0.004	ND
f - HL	0.001 ±0.0004	0.001 ±0.0005	0.035 ±0.003	ND
f - ME	ND	ND	ND	ND
g - HE	ND	0.009 ±0.001	0.020 ±0.009	ND
g - HL	0.001 ±0.0005	0.001 ±0.0003	0.013 ±0.009	ND
g - ME	ND	0.003 ±0.0005	ND	ND
h - HE	ND	0.002 ±0.0002	0.022 ±0.006	ND
h - HL	ND	ND	0.012 ±0.009	ND
h - ME	ND	ND	ND	ND

a - *Rosmarinus officinalis* L., **b** - *Salvia officinalis* L., **c** - *Melissa officinalis* L., **d** - *Thymus pulegioides* L., **e** - *Lavandula officinalis* L., **f** - *Satureja montana* L., **g** - *Mentha piperita* L., **h** - *Origanum vulgare* L., **HE** – hexane, **HL** – chroroform, **ME** – methanol, **ND**-non detected.

From the point of view of the different plant species investigated in this work, Rosmarini folium was proved to be the most important since it contains betulin, betulinic acid, ursolic acid and oleanolic acid. Figure 2 shows the HPLC chromatogram of hexane rosemary leaf extract which clearly shows the separated peaks of the tested triterpene substances, identified based on the retention times of the standard: betulinic acid (BA; Rt 15.957), oleanolic acid (OA; Rt 17.120), ursolic acid (UA; Rt 17.995) and betulin (bet; Rt 20.437). Quantification of individual triterpenes was done based on the area below the corresponding peak. The study accomplished by Andrade and coworkers corroborates our results (Andrade, Faustino, Garcia, *et al.*, 2018). Lupeol was detected neither in this material nor in the other investigated plant species.

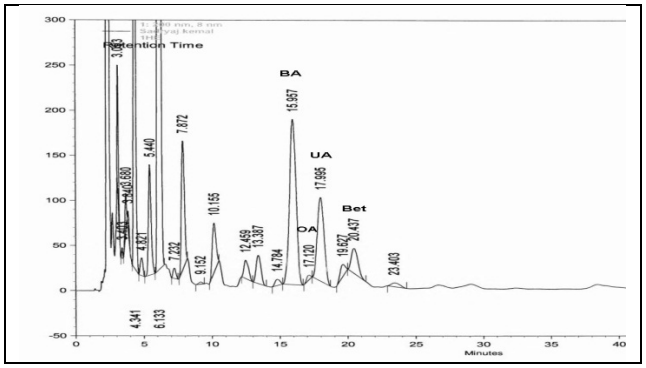

Figure 2. HPLC chromatogram of hexane extract obtained from Rosmarini folium: betulinic acid (BA), oleanolic acid (OA), ursolic acid (UA) and betulin (Bet).

From a standpoint of the presence of certain compounds, Rosmarini folium is still the plant material (drug) of choice, because it exclusively contains betulinic acid and oleanolic acid. The plant material of choice with the highest amount of determined ursolic acid was Lavandulae flos (hexane fractions: 0.14 mg/ml) (Jäger, 2009.), followed by Thymi herba (hexane fraction: 0.11 mg/ml) (Raudone, Zymone, Raudonis, *et al.*, 2017). Since these triterpenes were found in the fractions obtained from plant material after separation of essential oil by steam distillation, the possibility of using the vegetable residue after obtaining the essential oil opens up. In this way, a huge amount of plant material that is discarded in the essential oil industry can be used to extract triterpene substances. This also mean a new pleiotropic activity and possibility of new indications for the plant species of the Lamiaceae family investigated in this work. Total amount of individual triterpenes calculated per 100 g of investigated plant material is given in Table 3.

Table 3. Overview of the total content of triterpenes in the investigated plant material

Herb	**Content of single triterpenes per 100 g of investigated plant material**			
	Betulin	Betulinic acid	Ursolic acid	Oleanolic acid
Rosmarinus officinalis L.	7.0 mg	98.0 mg	42.0 mg	1.4 mg
Salvia officinalis L.	1.5 mg	4.5 mg	169.0 mg	-
Melissa officinalis L.	2.1 mg	4.5 mg	182.0 mg	-
Thymus pulegioides L.	1.1 mg	1.1 mg	265.0 mg	-
Lavandula officinalis L.	2.8 mg	4.3 mg	230.0 mg	-
Satureja montana L.	1.4 mg	5.0 mg	126.0 mg	-
Mentha piperita L.	1.4 mg	18.2 mg	42.4mg	-
Origanum vulgare L.	-	2.8 mg	42.0 mg	

Antioxidant capacity (AC) is defined as the ability of a pure substance or complex chemical mixture to slow or prevent the oxidation of other substances when both are simultaneously exposed to free radicals that cause their oxidation. Therefore, AC represents a quantitative value of resistance toward effects of free radicals that can be expressed in different ways, and it is mostly converted into the amount of standard antioxidant, usually vitamin E per unit sample (Santos-Sánchez, Salas-Coronado, Villanueva-Cañongo, *et al.*, 2019)

Triterpenes, investigated in this work, have previously been described as substances with a strong scavenging capacity against various free radicals (Parvez, Alam, Arbab, et al., 2018; Wang, Liu, Lian, *et al.*, 2019; Yin and Chan, 2007). Moreover, oleanolic acid and ursolic acid have been indicated as proton donors and consequently with reducing power (Santiago, Dayrit, Correa, *et al.*, 2014). The total triterpene fractions obtained from different plant species of the Lamiaceae family were evaluated for their antioxidant capacity using DPPH method. The total triterpene fractions for each plant material were obtained in such a way that the chloroform and hexane fractions containing most of the triterpene saponins were mixed in equal parts. This total fraction was used for antioxidant investigation.

According to the data in Table 3, it can be said that investigated triterpene fractions have promising antioxidant capacity. In modern pharmacy, "enriched extracts" are increasingly used, these are extracts concentrated in the main active substances (in our case, these are triterpenes), aware that in addition to these substances, there are concomitant substances that contribute to the final activity. Therefore, the results obtained from the values of the antioxidant activity of the conjoint fractions are useful as they give an insight into the possibility of total antioxidant activity including the accompanying substances present in the given extracts. All of the tested extracts showed antioxidant capacity in a dose-dependent manner. The IC_{50} value ranges from 0.5 mg/ml to 3.47 mg/ml (Table 4.).

The most intense antioxidant capacity was demonstrated by the triterpene fraction obtained from rosemary leaf (IC_{50} 0.5 mg/ml). Based on the literature data, the antioxidant capacity of rosemary extracts is mainly associated with total flavonoids and phenols. The obtained antioxidant effects of the rosemary leaf triterpene fraction indicate that these compounds contribute significantly to the overall antioxidant capacity of this herbal drug (Nieto, Ros, Castillo, *et al.*, 2018)

The next most significant antioxidant capacity showed the triterpene fraction obtained from lavender flower (IC_{50} 1.6 mg/ml), followed by the triterpene fraction of thyme and mint leaf with antioxidant capacity values (IC_{50} 2.5 mg/ml). The fractions obtained from the mountain savory herb (IC_{50} 3.4 mg/ml) and oregano herb (IC_{50} 3.5 mg/ml) showed the lowest value of the antioxidant capacity. The antioxidant activity data for these fractions coincide with the lowest detected values of the triterpene substances in the above-mentioned fractions.

Table 4. Results of the analysis of antioxidant capacity of different triterpene fraction

Triterpene fraction	[Sample] (mg/ml)	(%) Antioxidant activity	IC_{50} (mg/ml)
Rosmarinus officinalis	0.7	52.82	0.5±0.06
	1.4	62.12	
	2.3	72.97	
	3.2	83.43	
Salvia officinalis	1.5	15.72	3.3±0.4
	2.8	36.97	
	3.8	63.59	
Melissa officinalis	$4.7x10^{-5}$	7.31	2.9±0.3
	1.3	25.33	
	3.8	53.235	
	4.8	62.33	
Thymus pulegioides	1.5	38.27	2.5±0.5
	2.8	52.46	
	3.8	64.32	
	4.8	76.97	
Lavandula officinalis	1.5	49.42	1.6±0.1
	2.8	58.76	
	3.8	60.85	
	4.8	67.72	
Satureja montana	1.1	25.5	3.4±0.3
	2.1	39.1	
	4.3	59.0	
Mentha piperita	0.8	13.38	2.5±0.5
	1.5	29.91	
	2.8	56.46	
Origanum vulgare	1.3	29.0	3.5±0.4
	2.3	34.1	
	3.4	50.2	
Trolox	0.012	34.67	0.018±0.002
	0.018	48.67	
	0.030	77.57	

CONCLUSION

The present study shows that the aerial parts of *Mentha piperita* L., *Thymus pulegioides* L., *Rosmarinus officinalis* L., *Origanum vulgare* L., *Salvia officinalis* L., *Satureja montana* L., *Lavandula officinalis* L. and *Melissa officinalis* L., plant species from Lamiaceae family, collected in Bosnia and Hercegovina, represent an important source of triterpenes. The presence of these pharmacologically active compounds attributes new pleiotropic properties to the investigated herbal drug and opens new opportunities for their use in official pharmacy and medicine. Successive extraction with different solvents showed that chloroform was the most suitable solvent for the extraction of investigated triterpenes. TLC and HPLC experimental conditions, used in this study, proved to be sufficiently sensitive, accurate and reproducible, from the standpoint of the qualitative and quantitative analysis of the presence of triterpene substances in different extracts. Betulin (0.004 mg/ml) and betulinic acid (0.05 mg/ml) were the most abundant triterpenoid components in the hexane extracts of *Rosmarinus officinalis* L. Ursolic acid (0.14 mg/ml) was the most abundant triterpenoid component in the hexane extract of *Thymus pulegioides* L. Oleanolic acid (0.01 mg/ml) was found only in the hexane extract of *Rosmarinus officinalis* L. Due to the different solubility of individual triterpenes in the extraction solvents used, the antioxidant activity assay was performed on fractions with total triterpene content. The best antioxidative capacity was demonstrated by the triterpene fractions from the plant species *Rosmarinus officinalis* and *Lavandula angustifilia*, which were at the same time the richest in triterpenes, indicating a direct proportionality of the antioxidant activity and content of triterpenes.

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Summary/Sažetak

Triterpeni se konstantno povezuju s uočenim bioaktivnostima ekstrakata dobivenih iz biljnog materijala koji sadrže ove vrlo važne prirodne supstance. Mnoge vrste iz porodice Lamiaceae se koriste radi prisustva eteričnih ulja, a vrlo malo se zna o sadržaju triterpenskih supstanci u njima. U ovom radu provedena je kvalitativna i kvantitativna analiza ovih vrlo važnih supstanci, u nadzemnim dijelovima osam vrsta koje pripadaju porodici Lamiaceae. Dobiveni ekstrakti koji su sadržavali triterpenske supstance, testirani su DPPH metodom, kako bi se utvrdio njihov antioksidativni kapacitet**.** TLC i HPLC metode, korištene za analizu triterpena, pokazale su prisutnost betulina, betulinske kiseline, ursolne kiseline i oleanolne kiseline. Betulin (3.2 mg/g) i betulinska kiselina (37.1 mg/g) bili su najzastupljenije triterpenske komponente u heksanskom ekstraktu lista ruzmarina. Ursolna kiselina (0.14 mg/g) bila je najzastupljenija triterpenska supstanca u heksanskom ekstraktu lista timjana. Korištenjem metode DPPH, svi ispitivani uzorci pokazali su antioksidativnu aktivnost na koncentracijom ovisan način, sa širokim rasponom IC_{50} vrijednosti od 0.4 mg/ml do 3.3 mg/ml.