# Effects of solvent extraction system on antioxidant activity of *Lamium purpureum* L.

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*Abstract*

Antioxidant and free radical scavenging activity of methanol, ethanol, ethyl acetate and chloroform extracts of aerial parts of *Lamium purpureum* L. was determined by DPPH, ABTS, FRAP and TRP assays. Contents of flavonoids and phenols were also investigated. The total phenolic content in the extracts, determined using Folin–Ciocalteu assay, ranged between 8.57 to 128.00 mg GAE/g d.e. while concentrations of flavonoids in the extracts varied from 24.20 to 39.80 mg QuE/g d.e. The highest phenolic content was found in methanol extract (128.00 mg GAE/g d.e.). The highest content of total flavonoids was identified in the methanol extract (39.80 mg QuE/g d.e.) and the lowest was in the chloroform (24.30 mg QuE/g d.e.). DPPH scavenging of the extracts was determined and obtained $IC_{50}$ values ranged from 0.12 to 3.12 mg/mL of solution. The values of ABTS radical scavenging activity ranged from 0.35 to 1.80 mg AA/g. The highest ABTS antiradical activity was registered for methanol extract. The FRAP value was found within the range 0.08 to 1.04 μmol Fe/mg. The best radical scavenger was methanol (1.04 μmol Fe/mg). In reducing power assay different extracts of *L. purpureum* showed increasing of activity with increased concentration, and all extracts possessed substantial dose dependent antioxidant activity. The best reducing capacity was obtained with methanol extract of *L. purpureum* (0.0132 mg AA/mL). The results in this study confirmed that *L. purpureum* possesses moderate antioxidant properties.

*Keywords*: *Lamium purpureum*, antioxidant activity, DPPH, ABTS, FRAP, TRP.

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*Lamium purpureum* (red deadnettle) is a species belonging to the Lamiaceae family, subfamily Lamioideae. It is a common weed of cultivated areas which grows in most of Europe [1]. Although it has not been recognized as a medicinal plant, its resemblance to other related species determined its application in form of *Lamii herba*. *Lamium* species have been used in folk medicine worldwide, as remedy in the treatment of several disorders such as trauma, fracture, paralysis, hypertension, menorrhagia and uterine hemorrhage [2,3]. Many Lamiaceae species are very important source of active natural compounds which differ widely in terms of structures, biological properties and mechanisms of action. Secondary metabolite, especially polyphenols (phenolic acids, flavonoids, phenyl propanoids and tannins) are known to be responsible for many biological activities, as well as antioxidant activity [4–8]. According to the literature data, the genus *Lamium* has been investigated from phytochemical aspects. Phenylpropanoid glycosides, iridoid glycosides, essential oils, flavonoids, anthocyanins, phytoecdysteroids, betaines have been previously studied [9–17]. It is known that many antioxidant compounds belong to various classes of secondary metabolites. Phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to oxidation of nucleic acids, proteins, lipids or DNA and can initiate many diseases [18,19]. There are several articles dealing with free radical scavenging activities of *Lamium* species [20–22] and some evidences indicating various biological aspects, such as anti-inflammatory, antioxidant, and antiproliferative properties of *Lamium* species [23–27]. The critical point in studying polyphenols in plant materials is the extraction procedure used since it dictates the nature and quantity of polyphenols that will be transferred to the extract and further characterized. Solvent extraction of polyphenolics is the initial step prior to quantification, separation, purification, and characterisation and generally involved the use of an acidified extragent.

The aim of this study was to investigate *in vitro* antioxidant capacity of various extracts of *L. purpureum* using different radical scavenge methods (DPPH, ABTS,

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FRAP and TRP assays) which were not analyzed previously. This approach should complement to their previously known therapeutic value and improve the popularization of those somehow overlooked common herb species.

## MATERIALS AND METHODS

### Plant material and reagents

Aerial parts of the analyzed species were collected at the flowering stage in June 2014 (Belgrade, Institute of Botany and Botanical Garden "Jevremovac"). A voucher specimen has been deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia (BEOU, No. 17141). The collected plants were air dried in shade place at room temperature (20 °C).

Methanol, ethanol, HCl (concentrated hydrochloric acid), EtOAc (ethyl acetate), $CH_3COOH$ (glacial acetic acid) were purchased from "Zorka Chemicals" (Sabac, Serbia). 3,4,5-Trihydroxybenzoic acid (gallic acid), 3--tert-butyl-4-hydroxyanisole (BHA) and 2,2-dyphenyl-1--picrylhydrazyl (DPPH), iron(III) chloride ($FeCl_3{\cdot}6H_2O$), iron(II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), sodium acetate ($CH_3COONa{\cdot}3H_2O$), potassium ferricyanide $K_4[Fe(CN)_6]$, trichloroacetic acid ($C_2HCl_3O_2$) were obtained from Sigma Chemicals Co., St Louis, MO, USA. Folin–Ciocalteu phenol reagent was purchased from Merck, Darmstadt, Germany. Sodium carbonate anhydrous ($Na_2CO_3$), potassium acetate ($C_2H_3KO_2$), potassium peroxidisulphate ($K_2O_8S_2$) and L(+)-ascorbic acid (vitamin C) were purchased from AnalaR Normapur, VWR, Geldenaaksebaan, Leuven, Belgium. Aluminium nitrate nona hydrate [$Al(NO_3)_3{\cdot}9H_2O$], 2,4,6-tris(2-pyridyl)-*s*--triazine (TPTZ) was purchased from Fluka Chemie AG, Buchs, Switzerland. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one (quercetin hydrate) were obtained from TCI Europe NV, Boerenveldsweg, Belgium. All chemicals were of analytical grade.

### Preparation of plant extracts

Samples of the air dried and powdered aerial parts of the *L. purpureum* (10 g) were extracted with volume of 100 mL of different solvents (methanol, ethanol, ethyl acetate and chloroform) using ultrasonic extraction. The mixture was exposed to ultrasound for first and last hour during 24 h extraction in dark and filtered through a paper filter (Whatman No. 1). The solvents were removed by evaporation under reduced pressure. The dried extracts were kept in the fridge at 4 °C, in dark glass bottles for further investigation.

### Determination of total phenolic (TPC) in the plant extracts

Phenolic compounds concentration in the different extract of *L. purpureum* was estimated by colorimetric assay, based on procedure described by Singleton and Rossi [28]. Briefly, 1mL of sample was mixed with 1 mL 10% Folin–Ciocalteu's reagent. The mixture was kept for 6 min, which was followed by addition 0.8 mL of 7.5% sodium carbonate solution. After 120 min in dark place, the absorbance of mixture was measured at 730 nm on JENWAY 6305 UV–Vis spectrophotometer. Total phenolic content was expressed as mg of gallic acid equivalent/g of dry extract (mg GAE/g d.e.). All measurements were repeated three times and expressed as mean value ± standard deviations.

### Determination of flavonoid content (TFC) in the plant extracts

The determination of the total flavonoid content was carried out as described by Park *et al*. [29]. The reaction mixture contained the sample in concentration of 1 mg/mL, $Al(NO_3)_3{\cdot}9H_2O$ and $CH_3COOK$. After 40 min in dark place, the absorbance of mixture was measured at 415 nm. The total flavonoid content in different extracts was calculated using quercetin hydrate calibration curve and expressed as mg quercetin equivalents/g of dry extract (mgQuE/g d.e.)

### Evaluation of antioxidant activity

*DPPH method*

Antioxidant activity of different extracts of *L. purpureum* was evaluated by the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging method by Blois [30]. Extracts were diluted in the appropriate solvent from which were made further dilutions in methanol and DPPH solution (0.04 mg/mL) was added. Samples were vigorously shaken and left for 30 min at room temperature in the dark. Absorbance was measured at 517 nm by JENWAY 6306 UV–Vis spectrophotometer. All measurements were carried out in triplicate. Absorbance of remaining DPPH radical in sample ($A_1$) was measured on 517 nm. Every sample and positive controls (ascorbic acid and BHA) concentration were done in triplicate and the same was done with blank probes which were prepared to contain methanol instead of investigated sample (blank absorbance $A_0$). Percent of scavenging (*RSC* / %) was calculated, as follows:

$$RSC(\%) = 100(A_o - A_1)/A_o \quad (1)$$

The extract concentration providing 50% of free radical scavenging activity ($IC_{50}$) was calculated from the graph of radical scavenging activity (RSA) percentage against extracts concentration.

*ABTS assay*

The ABTS radical scavenging method by Rice-Evans and Miller [31] and modified by Re *et al*. [32] was used. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate and its reduction in the presence of hydrogen donating antioxidants is measured spectrophotometrically at 734 nm. The reaction mixture of 19.2 mg of ABTS was dissolved in potassium persulfate before experiment. This solution was dissolved by distilled water in order to adjust working solution absorbance to 0.700 at 734 nm. The sample concentration was 1 mg/mL. Reaction mixture was prepared by mixing 100 µL of test sample and 4 mL ABTS. After 30 min incubation at 30 °C in water bath, absorbance of the mixture was measured at 734 nm. The radical scavenging activity for each extracts was determined on the basis of the linear calibration curve of ascorbic acid and was expressed as mg ascorbic acid/g of dry extract (mg Vit C/g d.e.). All previously described assays were performed on JENWAY 6305 UV–Vis spectrophotometer.

*Ferric reducing antioxidative power assay (FRAP)*

The reducing power of methanol, ethanol, ethyl acetate and chloroform extracts was determined using the ferric reducing ability of FRAP assay by Benzie and Strain [33]. This assay was based on the reducing power of a compound (antioxidant). A potential antioxidant will reduce the ferric ion ($Fe^{3+}$) to the ferrous ion ($Fe^{2+}$); the latter forms a blue complex ($Fe^{2+}$/TPTZ), which increases the absorption at 593 nm. Briefly, the FRAP reagent was prepared by mixing acetate buffer (200 µL, pH 3.6), a solution of 20 µL TPTZ and 20 µL $FeCl_3$ at 10:1:1 volume ratio. The sample solution (200 µL) and the reagent (2.95 mL $H_2O$ and 1 mL FRAP) mixed thoroughly and incubated at 37 °C. Absorbance was taken at 593 nm after 10 min on Perkin Elmer Lambda Bio UV–Vis spectrophotometer. Standard calibration curve was prepared using different concentrations of $FeSO_4 \cdot 7H_2O$. All solutions were freshly prepared. The results were expressed in µmol Fe/mg dried extract (µmol Fe/mg d.e.).

*Total reducing power assay (TRP)*

The total reducing power of different extracts of *L. purpureum* was determined according to the method of Oyaizu [34]. The total reducing power assay measures the electron donating capacity of an antioxidant. Presence of reducers causes the conversion of the $Fe^{3+}$/ferricyanide complex to the ferrous form which serves as a significant indicator of its antioxidant capacity. The reducing capacity of different extracts was compared with ascorbic acid for the reduction of $Fe^{3+}$ to $Fe^{2+}$. In this assay, the color of the test solution changes to various shades of blue, depending on the reducing power of each compound. Therefore, measuring the formation of Perls' Prussian blue at 700 nm can monitor the $Fe^{2+}$ concentration. One mL of extracts was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide $K_3[Fe(CN)_6]$ (2.5 mL, 1%). The mixtures were incubated at 50°C for 20 min. Then, trichloroacetic acid (10%, 2.5 mL) was added to the mixture and centrifuged. Finally, the upper layer (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 ml; 0.1%). The absorbance of the solution was measured at 700 nm in Perkin Elmer Lambda Bio UV–Vis spectrophotometer. Blank was prepared with all the reaction agents without extract. Higher absorbance of the reaction mixture indicated that the reducing power is increased. Ascorbic acid was used as standard. The percent increased in reduction power was calculated by using following formula:

$$\text{Increase in reduction power} = 100(A_{test} / A_{blank} - 1) \quad (2)$$

where $A_{test}$ is optical density if test solution and $A_{blank}$ is the optical density of blank solution. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations.

**Statistical analysis**

All the experiments were carried out in triplicate. The results are expressed as mean values and standard error of the mean. In all determinations, the percentage of standard deviation was accepted must be lower than 5%. The existence of significant differences among the results for total phenol and flavonoids content and the antioxidant properties of the extracts were analyzed. The $IC_{50}$ values obtained in the antioxidant assays established regression equation between the samples concentration and the scavenging effect. The data were subjected to multivariate statistical analyses, principal component analysis (PCA), and cluster analysis (CA). Correlation between various extracts of *L. purpureum* was established using regression analysis at a 95% significance level ($p \leq 0.05$). All statistical tests were used Statistica 8 software.

## RESULTS AND DISCUSSION

To explore the suitability of different extracting solvents with different polarity, we have compared total phenolic and flavonoid concentration and antioxidant properties of methanol, ethanol, ethyl acetate and chloroform extracts of aerial plant parts of *L. purpureum*. The antioxidant potential of the various extracts of *L. purpureum in vitro* was measured spectrophotometrically.

Several principles have to be considered before making a decision to choose appropriate solvent for plant extraction. Some considerations were done according to the purpose of extraction (preparation or analysis), the nature of the assayed components, the

physicochemical properties of the matrix, the availability of reagents and equipment, the cost and safety concerns. The different polarity of solvent employed in the extraction had strong association to the antioxidant activity of the natural plant extract.

Different solvents were used to accurate the extraction of the active substances with diversity in their polarity. For antioxidant and radical scavenging activity of these extracts, four different models were used: DPPH, ABTS, FRAP and TRP assay. Differences between extracts are noticed due to its polarity and results are summarized below.

**Total phenolic content**

Phenolic compounds are ubiquitous bioactive compounds and a diverse group of secondary metabolites universally present in higher plants. The Folin–Ciocalteu procedure was usually and widely used method for a rapid and useful evaluation of the phenolic content, although the assay has been shown nonspecific not only to polyphenols, but to any other substance that could be oxidized by the Folin reagent. Phenolic compounds react with Folin–Ciocalteu's reagent (FCR) only under basic conditions (adjusted by aqueous sodium carbonate). Dissociation of a phenolic proton in basic medium leads to a phenolate anion, which is capable of reducing FCR in which the molybdate in testing system is reduced forming a blue coloured molybdenum oxide with maximum absorption near 765 nm. The intensity of blue coloration produced is proportional to the total quantity of phenolic compounds present in the testing samples. The phenolic compounds in plant extract are more often associated with other molecules. The results of the total phenolic content of the examined plant extracts of *L. purpureum* are presented in Table 1.

*Table 1. Total phenolics and flavonoids contents of L. purpureum extracts*

| Type of extract (1mg/mL) | Total phenols GAE/g | Total flavonoids QE/g |
|---|---|---|
| Methanol | 128.00±0.001 | 39.8±0.000 |
| Ethanol | 89.23±0.004 | 32.8±0.001 |
| Ethyl acetate | 50.6±0.000 | 37.9±0.001 |
| Chloroform | 8.57±0.002 | 24.2±0.002 |

The highest phenolic content was found in polar solvent extracts such as methanol (128.00 mg GAE/g d.e.) and ethanol extract (89.23 mg GAE/g d.e.). The chloroform extract of *L. purpureum* contains the lowest amount of these compounds as compared to others (8.57 mg GAE/g d.e.). These results are in accordance to previous studies of different extracts of *Lamium* species. Methanol extracts of *L. album* and *L. purpureum* contain a considerable amount of phenolic metabolites [24]. Values of total polyphenols in methanol extract of *L. maculatum* were similar to our results, too [21]. Quantitative determination of phenolic compounds in butanol extract of *L. purpureum* and *L. album* showed high content of total phenols too [35]. The extractive capacity of phenolic components from *L. purpureum* material is considerably depend on the type of solvents. The total flavonoids content of extracts with different solvents from *L. purpureum* was different ($p < 0.05$). Results of the present study have shown that among all the solvent; methanol and ethanol, as more polar substances were better solvents for effective extraction of phenolic compounds as compared to solvents like ethyl acetate and chloform.

**Total flavonoids content**

The concentrations of flavonoids in *L. purpureum* extracts ranged from 24.20 to 39.80 mg QE/g d.e. The highest flavonoid content was identified in the methanol (39.8 mg QuE/g d.e.), while the lowest was in the chlorophorm (24.30 mgQuE/g d.e.) extract. The content of flavonoids was nearly equal for the methanol and ethyl acetate extracts (37.90 mg QuE/g). The results of the total flavonoids content of *L. purpureum* extracts are presented in Table 1.

Literature review showed positive correlation of flavonoid content and antioxidant capacity for some Lamiaceae species [36,37]. Flavonoids with presence of hydroxyl group in the molecule can act as proton donor and show good radical scavenging activity [38,39]. The fact, that among others, the flavonoid content depends on the used solvent for extraction was reported by Kaurinovic *et al.* [40]. Antioxidant activity of flavonoids depends of the structure, substitution pattern of hydroxyl groups, polarity of solvents and method used for extraction [41,42]. In methanol extracts of *L. amplexicaule* and *L. album* high concentration of flavonoids were obtained [22,43].

**DPPH scavenging activity**

The most common methods to determine antioxidant activity in a practical, rapid and sensitive manner are those that involve a radical chromophore, simulating the reactive oxygen species, and the free radical DPPH is one of the most widely used for *in vitro* evaluation of plant extracts and fractions. The ability of the investigated extracts of *L. purpureum* to act as free radical scavengers or hydrogen donors in transformation of $DPPH^{\bullet}$ into its reduced form DPPH–H was investigated. Free radical scavenging capacity of the tested extracts was measured by DPPH assay and results are shown in Table 2. The DPPH absorbance decreases with an increase in DPPH radical scavenging activity. Results were expressed as $IC_{50}$ concentration where 50% inhibition of the DPPH radical is obtained. Solvent used for polyphenolic extraction had significant effect on antioxidant activity, although all of the ext-

racts were able to reduce the stable, purple colored radical DPPH in to yellow-colored DPPH–H. The scavenging effect of different extracts of *L. purpureum* on the DPPH radical decreased in following order: ethanol > methanol > ethyl acetate > chloroform. The lowest antioxidant potential exhibited ethyl acetate and chloroform extracts of *L. purpureum.* $IC_{50}$ values of the synthetic antioxidants were 0.093 and 0.054 mg/mL for BHA and ascorbic acid, respectively. Free radical scavenging activities of the different *Lamium* species indicate the influence of different solvents on the DPPH radical scavenging activity. Thus, it has been found that *L. album* possess antioxidant properties obtained by DPPH assay [23]. Those data are in agreement with reported results of Matkowski and Piotrowska [24] where *L. album* possessed better scavenging potential than *L. purpureum* methanol extract. Also, significant DPPH scavenging activity was reported for butanol extracts from flowers of *L. album* and *L. purpureum* [25] as well as for butanol extracts of *L. eriocephalum, L. garganicum* and *L. purpureum* var. *purpureum* [35].

*Table 2. Antioxidant activities of L. purpureum extracts determined by DPPH, ABTS, FRAP and TRP methods*

| Extract (1mg/mL) | DPPH $IC_{50}$ mg/mL | ABTS mg of AA/g | FRAP µmol Fe/mg | TRP mg of AA/mL |
|---|---|---|---|---|
| Methanol | 0.25 | 1.80±0.138 | 1.04 | 0.0132 |
| Ethanol | 0.124 | 1.14±0.083 | 0.60 | 0.0125 |
| Ethyl acetate | 1.843 | 0.35±0.790 | 0.11 | 0.0079 |
| Chloroform | 3.12 | – | 0.08 | 0.0075 |

**ABTS scavenging activity**

Free radical scavenging capacity of the tested extracts was measured by ABTS test and results were presented in Table 2. The values of ABTS antiradical activity ranged from 0.35–1.80 mg AA/g. The activity was registered in followed order: methanol > ethanol > ethyl acetate extract. Chloroform extract of *L. purpureum* did not show any activity in ABTS assay. In the research of Yumrutas and Saygideger [22], radical scavenging activity of the different extracts of *L. amplexicaule* and *L. album* was demonstrated. Methanol extract of *L. amplexicaule* showed stronger scavenging activity in ABTS test. The scavenging potential of methanol extract of *L. album* was found higher than scavenging activity of its ethanol extract [43]. These results are in accordance with our findings.

**Ferric reducing power assay (FRAP)**

The results of total antioxidant capacity of investigated extracts, measured by the FRAP method are shown in Table 2 and they are placed within the range 0.08–1.04 µmolFe/mg. The highest value shows methanol extract (1.04 µmol Fe/mg). The lowest value expresses the extract of chloroform (0.08 µmol Fe/mg). The literature shows that FRAP method is sensitive in the measurement of total antioxidant power of the plant homogenates. Methanol extracts of Lamiaceae species (*Salvia sp.*, *Scutellaria sp.*) show the considerable antioxidant effect than ethyl acetate extracts [44].

**Total reducing power assay (TRP)**

The reducing capacity of different extracts was compared with ascorbic acid for the reduction of $Fe^{3+}$ to $Fe^{2+}$. In this assay, the color of the test solution changes to various shades of blue, depending on the reducing power of each compound. Therefore, measuring the formation of Perls' Prussian blue at 700 nm can monitor the $Fe^{2+}$ concentration. The presence of antioxidants in the sample causes the reduction of the $Fe^{3+}$/fericyanide complex to the ferrous form. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing power assay is often used to evaluate the ability of an antioxidant to donate electrons. Percentage of reduction power of *L. purpureum* extracts at various concentrations is illustrated in Fig. 1.

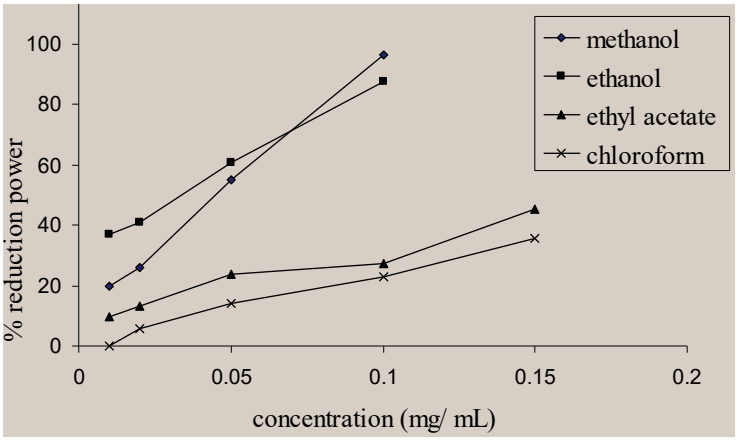


*Figure 1. Percent of reducing power of different extracts of L. purpureum.*

Our result shows the reducing power of different extracts of *L. purpureum* as a function of their concentration: higher absorbance indicated higher reducing power. The reducing power of different extracts of *L. purpureum* increased with increasing in concentration. The best reducing capacity shows methanol and ethanol extract of *L. purpureum* (0.0132 and 0.0125 mg AA/mL). The ethyl acetate (0.0079 mgAA/mL) and chloroform (0.0075 mg AA/mL) extracts possessed lower but almost equally reducing capacity.

Our results showed that *L. purpureum* could be a new potential source of natural antioxidants. As can be seen from Tables 1 and 2 the extracts with a higher content of polyphenols show the stronger antiradical activity against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ and in all applied methods. Further research is needed to identify individual components of *L. purpureum* forming antioxidative system and develop their applications for food and pharmaceutical industries. There are some difficulties to compare previously published data due to the differences in applied methods. The differences between extracts and its phenolic content were also not very striking. These results suggest a great complexity of the involved mechanisms that can vary even among related species. The apparent discrepancy between the antioxidant properties assayed by various techniques and lack of correlation to the widely distributed polyphenols have been already mentioned by Mantle *et al.* [45], but the problem still remains unresolved and will require further data accumulation and corresponding analyses of detailed chemical profile of studied plants. Also, the effects of solvent characteristics (polarity, concentration) as well as extraction procedure, time of extractions and extraction temperature had statistically significant effects on phenolic compounds and antioxidant activity [46,47].

**Correlation between antioxidant capacity and total phenolic content**

Results obtained from experimental data revealed that there might be correlation between total phenolic and antioxidant capacity of different extracts of *L. purpureum*. It has well established that the free radical scavenging activity of plant extracts is mainly due to phenolic compounds. However, some literature demonstrated that antioxidant was not solely dependent on phenolic content, but it may be due to other phytoconstituents as tannins, triterpenoid or combine effect of them. Different types of phenolic compounds have different antioxidant activity, which mainly depends on their structure as extract contains different types of phenolic compounds which have different antioxidant capacities.

The relationship between the total content of polyphenols in the studied extracts and their ability to inactivate free radicals was assessed. At the beginning of the statistical analysis we were calculating the correlation matrix, giving the correlation coefficients between each pair of variables. A data matrix contained the spectrophotometric (total phenolic content, total flavonoids content DPPH and ABTS scavenging activity, FRAP and TRP method was presented as Table 3.

Each term of the matrix is a number ranging from –1 to +1: the + or – sign indicates appositive or negative interdependence between variables (direction) and the absolute value indicates the strength of the interdependence [42]. Implementation of chemometric techniques for evaluation of antioxidant properties of *L. purpureum* extracts significant positive correlation was found ($p < 0.01$) between the total phenolic content and antioxidant activity expressed as $IC_{50}$ DPPH, ABTS, FRAP and TRP ($r$ = 0.94, 0.99, 0.95 and 0.93 respectively).

Before the chemometric analysis, the raw data were standardized by subtracting a sample mean from each variable value and dividing it by the standard deviation. This transformation procedure allowed unifying the effects of all variables on the picture of the relationships between the studied extracts. Two segmentation algorithms (hierarchical cluster analysis, and principal component analysis) were applied in order to construct the descriptive models describing the similarities and differences between the investigated extracts in terms of their antioxidant properties.

Ward's method was applied for aggregation of the samples, the squared Euclidean distance was used as the distance measure in the cluster analysis. Thus, segments with minimal internal differentiation were obtained. The dendrogram derived from a cluster analysis is shown in Figure 2.

*Table 3. Correlation matrix between total phenolic and total flavonoids content, DPPH, ABTS, FRAP andTRP assay*

| | Total phenols | Total flavonoids | DPPH $IC_{50}$ | ABTS | FRAP | TRP |
|---|---|---|---|---|---|---|
| Total phenols | 1.00 | 0.78 | –0.94 | 0.99 | 0.95 | 0.93 |
| Total flavonoids | | 1.00 | –0.68 | 0.68 | 0.58 | 0.51 |
| DPPH $IC_{50}$ | | | 1.00 | –0.91 | –0.85 | –0.94 |
| ABTS | | | | 1.00 | 0.99 | 0.96 |
| FRAP | | | | | 1.00 | 0.95 |
| TRP | | | | | | 1.00 |

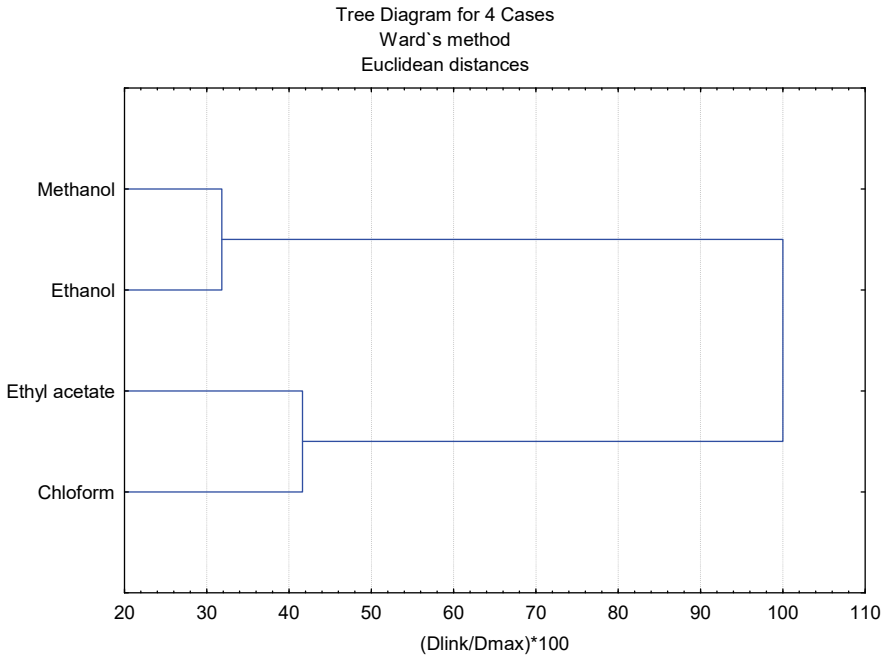


*Figure 2. The dendrogram obtained by hierarchical cluster analysis of different extracts of L. purpureum using Ward's method.*

The clusters were defined based on the values of variables describing the investigated extracts. As it can be seen, the extracts were classified into two various sub-groups according to their spectrophotometric measurements (total phenolic and total flavonoids content, DPPH, ABTS, FRAP and TRP assay). The unsupervised hierarchical cluster analysis identified two major segments. One of them consisted of methanol and ethanol extracts, whereas the second cluster was formed by less polar extracts – ethyl acetate and chloroform. Analyzed measurements (total phenolic and total flavonoids content, DPPH, ABTS, FRAP and TRP assay) were correlated with two principal components (PCs) with 97.36 percent of the total variance. The first principal component (PC1) contained 87.68 percent of the total variance. Figure 3A and B show loading plot of antioxidant markers (total phenolic and total flavonoids content, DPPH, ABTS, FRAP and TRP assay) and scores plot of different extracts of *L. purpureum*, respectively.

The high loading values of total phenolic content, ABTS, FRAP, TRP and DPPH variables on PC1 (–0.997461, –0.990907, –0.956382, –0.955621 and 0.952824, respectively) confirm, according to multivariate analysis, the major role of phenolic species in the antioxidant capacity different extracts of *L. purpureum*.

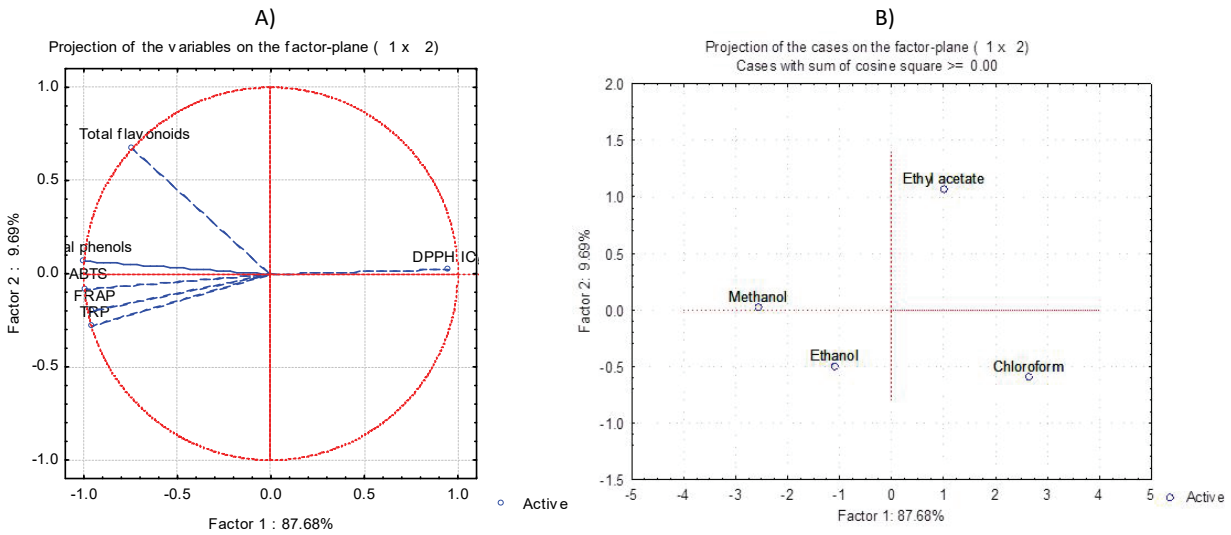


*Figure 3. A) Graph of loading plot of antioxidant markers of different extracts of L. Purpureum; B) scores plot of different extracts of L. purpureum.*

PC2 explains 9.69 percentage of the total variance, with the major loading of total flavonoids content 0.671521.

Methanol extract had the highest negative, while chloroform extract had the highest positive loadings on PC1 – 2.56287 and 2.64491, respectively (Figure 3B). Ethyl acetate extract has loadings on PC2 1.06865 (Fig 3B).

## CONCLUSION

The antioxidant properties of different *L. purpureum* extracts with different solvents (methanol, ethanol, ethyl acetate and chloroform) were demonstrated. The findings of our study revealed that extracts from *L. purpureum* possess considerable antioxidant potential. The different antioxidant activities of phenolic extracts can be attributed to different extracting solvent as the antioxidant activity depends on the type and polarity of the extracting solvent. The most efficient solvents for polyphenolic extraction were methanol and ethanol. Hence, methanol has proven to be most efficient solvent for extraction of flavonoids. The existing data is not enough in order to explain the mode of action for antioxidant, but it may enrich the strength of comprehensive data of antioxidant activity of *L. purpureum*. Ethanol and methanol extracts have proven as good scavengers, estimated by both antiradical methods – DPPH and ABTS. Obtained data confirmed the high correlation between total phenolic content and antioxidant activity. However, for better insight into the role of particular compounds in the different antioxidant mechanisms requires deeper phytochemical and pharmacological investigations.

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## IZVOD

### UTICAJ RAZLIČITIH RASTVARAČA NA ANTIOKSIDATIVNU AKTIVNOST EKSTRAKATA *Lamium purpureum* L.

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(Naučni rad)

Antioksidativna aktivnost organskih rastvarača različite polarnosti (metanol, etanol, etil acetat i hloroform) vegetativnih delova vrste *Lamium purpureum* L. određena je korišćenjem DPPH, ABTS, FRAP i TRP metoda. Određen je sadržaj fenolnih i flavonoidnih jedinjenja u ekstraktima. Dobijene vrednosti za fenole su bile između 8,57 i 128,00 mg GAE/g s.e. Izmerene koncentracije flavonoida su bile od 24,20 do 39,80 mg QuE/g s.e. Najveća količina fenola i flavonoida konstatovana je u metanolnom ekstraktu *L. purpureum* (128,00 mg GAE/g s.e. odnosno 39,80 mg QuE/g d.e.) a najmanja u hloroformskom ekstraktu. DPPH aktivnost izražena kao $IC_{50}$ ekstrakata *L. purpureum* je bila najbolja (0,12 mg/mL). Metanolni ekstrakt *L. purpureum* pokazao je najjaču antioksidativnu aktivnost u svim ostalim korišćenim testovima: ABTS (1,80 mg of AA/g), FRAP (0,08 μmol Fe/mg). U svim ispitivanim ekstraktima pokazano je da povećanjem koncentracije dolazi do povećanja ukupne redukcione moći ekstakata. Totalni redukcini potencijal metanolnog ekstrakta je bio najveći (0,0132 mg AA/mL). Antioksidativna aktivnost etil-acetatnog i hloroformskog ekstrakta *L. purpureum* u svim testovima, bila je značajno niža od antioksidativne aktivnosti polarnih rastvrača. Može se zaključiti da je metanol, dobar izbor rastvarača za ekstrakciju. Dobijeni rezultati pokazuju umereni antioksidativni potencijal *in vitro* metanolnog i etanolnog ekstrakta *L. purpureum.*

*Ključne reči*: *Lamium purpureum* • Antioksidativna aktivnost • DPPH • ABTS • FRAP • TRP