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METAL AND ANTIOXIDANT CONTENTS OF SAGE FROM SIĆEVO GORGE NATURE PARK

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In this paper we have studied seasonal changes in metal and antioxidant contents of Salvia officinalis L. (sage) from Sićevo gorge Nature Park. Collected leaves were analyzed for iron, copper, zinc and manganese concentrations, quantities of malonyldialdehyde, superoxide and hydroxyl radicals, as well as the activities of antioxidant enzymes (superoxide dismutase, catalase, guaiacol peroxidase). Also, the content of reduced glutathione, carotenoids, total flavonoids, soluble proteins, chlorophylls a and b was determined. The examined plant accumulates sufficient content of researched elements during vegetation, except for the iron in the seed-forming stage. A significant effect of growth stage on superoxide and hydroxyl radicals' accumulation was observed, but there were no significant differences in lipid peroxidation levels during vegetation. Antioxidant enzymes in the low and middle range of activities in leaves of S. officinalis were correlated with protein, chlorophyll a and b contents, except for SOD. On the other hand, contents of non-enzymatic antioxidants varied greatly between different stages of growth, as well as the contents of protected molecules, with which they were positively correlated. Results from the present study suggest that antioxidant defense system of S. officinalis functions fully and protects target molecules of reactive oxygen species. The leaves of sage from Sićevo gorge Nature Park should be considered as an important source of iron, cooper, zinc, manganese and dietary antioxidants. *Acta Medica Medianae 2015;54(1):27-33.*

***Key words**: antioxidant enzymes, non-enzymatic antioxidants, reactive oxygen species, Salvia officinalis, metal*

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Introduction

Trace metals such as iron, copper, zinc and manganese, take part in vital biochemical and physiological functions and have essential role in life maintenance. On the other hand free radicals have been implicated in aging and a number of human diseases (1).

Plants synthesize primary and secondary metabolites by simple substances such as water, carbon dioxide, nitrogen and a number of inorganic salts. The complex organic compounds, manufactured in plants leaves, include antioxidant compounds for protection against oxidative damage (2-4).

Salvia officinalis is one of the most popular medicinal and culinary herbs. Pharmacognostical handbooks describe how sage has been traditionally used to treat flatulent dyspepsia, pharyngitis, uvulitis, stomatitis, gingivitis, glos-sitis, hyperhydrosis, and galactorrhoea. Recently, it has been demonstrated that sage essential oil can improve memory, showing hope for the treatment of Alzheimer's disease (5).

Aim

The aim of this study was to estimate enzymatic and non-enzymatic antioxidants of sage, based on protection of proteins, chlorophylls *a* and *b*, target molecules of reactive oxygen species (ROS) attack. We investigated element contents: Fe, Cu, Zn, Mn, quantities of malonyldialdehyde (MDA); ROS: superoxide ($O_2^{\bullet -}$) and hydroxyl radicals ($^{\bullet}OH$); activities of antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD); contents of reduced glutathione (GSH), carotenoids, total flavonoids, soluble proteins, chlorophylls a (Chl a) and b (Chl b).

Materials and methods

Chemicals

All chemicals and reagents were of analytical reagent grade and were purchased from the Sigma–Aldrich Chemical Company.

Plant material*:* The leaves, for analysis of antioxidants and ROS, were collected from healthy Salvia officinalis L. in their natural habitat in the Sićevo gorge Nature Park, south-east Serbia, in 2011 and immediately stored in liquid nitrogen. Characteristics of soil were investigated by standard analytical methods and techniques (6). The plant material was collected in three stages of growth (SG) as follows:

-1st SG– the initial vegetation stage (spring)
-2nd SG– the blooming stage (summer)
-3rd SG– the seed-forming stage (autumn)

Element analysis

Air-dried leaves were digested using concentrated $HNO_3/HClO_4$ acid mixture in a teflon beaker, with heating to 130-140°C for 1h until a clear solution was obtained. After wet digestion, content of metals in samples was determined by atomic absorption spectrophotometry (AAS), using Perkin Elmer 1100 spectrophotometer with a graphite furnace. The accuracy of the analysis was monitored by inclusion of international refe-rence samples in the analytical program: SO-1, SO-2, SO-3 and CRM 189 (7).

Extraction of enzymes

One gram of fresh leaves was ground with quartz sand in a cold mortar. The ground material was suspended in 5ml 0.1mol l^{-1} K_2HPO_4 at pH 7.2. After 10min centrifugation at 4°C and 15000×g, the aliquots of the supernatant were used for different antioxidant activity determinations.

Determination of quantities MDA, $O_2^{\bullet-}$ and $^{\bullet}OH$*:* Lipid peroxidation (LP) was determined by the thiobarbituric acid (TBA) method (8). A 0.5ml aliquot of a supernatant was mixed with 2ml of 20% trichloroacetic acid (TCA) containing 0.5% TBA. The mixture was heated at 95°C for 30min, quickly cooled, and then centrifuged at 15000×g for 10min. The absorbance of the supernatant at 532nm was read and the value for non-specific absorption at 600nm was subtracted. Values were given as equivalent amounts of MDA. The calibration curve was prepared with malonyldialdehyde bisdiacetal. $O_2^{\bullet-}$ was determined by adrenaline autooxidation (9), while $^{\bullet}OH$ was determined by the inhibition of deoxyribose degradation (10).

Determination of antioxidant enzyme activities*:* All the antioxidant enzyme activities were determined spectrophotometrically at 25°C using phosphate buffer (pH 7.2) plant extracts. Enzymatic specific activity is expressed as μmol of the substrate transformed/min/mg protein except for superoxide dismutase activity. Superoxide dismutase activity (SOD, EC 1.15.1.1) was determined by the method of (9) based on the inhibition of transformation of adrenaline to adrenochrome at pH 10.2. One unit SOD can be regarded as that amount of enzyme which causes a 50% inhibition in the extinction change in 1min compared with the control. Measurements were made at 480nm. Catalase activity (CAT, EC 1.11.1.6) was determined at 240nm. The decomposition of H_2O_2 was followed by a decrease in absorbance (11). Guaiacol peroxidase activity (POD, EC 1.11.1.7.) was determined using guaiacol as the substrate at 436nm (12).

Determination of GSH, carotenoid, flavonoid, Chl a, Chl b and soluble protein contents*:* The amount of GSH was determined with Ellman reagent at 412nm (13). Total flavonoids were extracted with 70% methanol and estimated according to (14). Carotenoids and chlorophylls were extracted with acetone and determined spectrophotometrically using molar extinction coefficients according to Wettstein D. (15). The soluble protein content was determined by the method of Bradford M. (16) with bovine serum albumin as standard.

Data analysis

Statistical analysis was performed using the statistical software SPSS v19.0 (SPSS Inc., Chicago, IL, USA). The t-test was used to compare whether the mean of variable differs between the samples. The relationship among biochemical parameters was described as the Pearson product-moment correlation coefficient "r". Differences at $p<0.05$ were considered to be statistically significant. One-way analysis of variance (ANOVA) was applied to the data to determine differences.

Results

Element concentrations

Element concentrations found in leaves of S. officinalis are presented in Table1. The concentration levels of all elements differed significantly in vegetation stages. The Fe content in examined species increased slightly from the initial vegetation stage to the blooming stage, demonstrating maximum of accumulation (60.05 mg kg^{-1}). Copper content ranged between 6.24 mg kg^{-1} in the blooming vegetation stage and 9.18mg kg^{-1} in the seed-forming stage. Zinc, like copper, showed a similar seasonal pattern, with maximum accumulation in the seed-forming stage (60.68mg kg^{-1}). The range of Mn varied between 25.87mg kg^{-1} in the seed-forming stage and 51.35mg kg^{-1} in the initial stage of vegetation.

$O_2^{\bullet-}$, $^{\bullet}OH$, and MDA quantities*:* A signi-ficant effect of SG on ROS accumulation in S. officinalis leaves was observed (Table 1). The highest quantities of $O_2^{\bullet-}$ and $^{\bullet}OH$, in the seed-forming stage (355.52nmol mg^{-1} $protein^{-1}$ and 1.93nmol mg^{-1} $protein^{-1}$) contributed to the highest level of LP in the leaves (15.22nmol mg^{-1} $protein^{-1}$), but there were no significant differences in LP levels during vegetation. An increase of LP values, measured as MDA equivalents, during vegetation was correlated to the increase of $O_2^{\bullet-}$ quantities ($r=0.69$) in leaves, respectively.

Antioxidant enzyme activities

The results of the determination of antioxidant enzyme activities of SOD, CAT and POD in leaves of S. officinalis collected in spring, summer and autumn are summarized in Table1. Vegetation stage had no significant effect on antioxidant enzymes activity in sage. The activity of SOD was higher in the seed-forming stage (72.34 U mg^{-1} $protein^{-1}$), than in the other two vegetation stages. The highest CAT activity was recorded in the blooming stage of vegetation (11.29 U mg^{-1} $protein^{-1}$), similar to POD activity (4.71 U mg^{-1} $protein^{-1}$). SOD activities were related to copper and zinc concentrations (r=0.75, r=0.74), while activities of catalase were related to iron and manganese concentrations (r=0.96, r=0.73). POD activities were related to iron concentrations (r=0.87).

Non-enzymatic antioxidants content

Content of GSH varies greatly in different stages of growth, and the highest value was observed in the blooming stage (2.68μmol mg^{-1} $protein^{-1}$, Table1). The change in GSH contents is correlated to a change of Fe leaf accumulation (r=0.94); CAT and POD activities (r=0.96, r= 0.95).

The statistical test indicated the significant influence of seasons on the carotenoid content. The highest value of carotenoid content was found in the blooming vegetation stage (0.86mg g^{-1}, Table1). The content of carotenoid was related to iron concentrations (r=0.90); CAT and POD activities (r=0.94, r=0.97).

Flavonoids are another class of phytochemicals that contribute considerably to the antioxidant activity. Content of flavonoids varies greatly among different stages of growth, and the highest value was observed in the blooming stage (6.80mg g^{-1} dray, Table 1). The changes in flavonoid contents are correlated to a change of Fe and Mn leaf accumulation (r=0.97, r=0.74), as well as the CAT and POD activities (r=0.94, r=0.91).

Protein, chlorophyll a and b contents: Soluble proteins vary greatly in different stages of growth, and the values range from 9.82mg g^{-1} and 13.46mg g^{-1}, respectively (Table 1). The lowest level was recorded in the seed-forming stage. Protein contents in leaves were significantly higher in the blooming stage than in other SG. The changes in protein contents are correlated to a change of CAT and POD activities (r=0.90, r=0.95). Also the protein contents were related to activities (r=0.99, r=0.95), as the GSH, antioxidant compounds and protected contents of GSH, carotenoids and flavonoids (r=0.97, r=0.99, r=0.93).

The lowest Chl a and Chl b contents (1.18 and 0.45mg g^{-1}) were recorded in the seed-forming stage (Table 1). Stages of vegetation af-fected the accumulation of Chl *b* significantly, but there were no significant differences in Chl *a* content during vegetation. The changes in chlorophyll *a* contents are correlated to a change of CAT and POD activities (r=0.88, r=0.91). Also the changes in chlorophyll *a* contents are correlated with the GSH, carotenoid and flavonoid contents (r=0.79, r=0.82, r=0.70). The statistical test gives similar results of correlation analysis for chlorophyll *b*. So, the changes in Chl *b* contents are correlated to a change of CAT and POD activities (r=0.99, r=0.95), as well as the GSH, carotenoid and

Table 1. Metal concentrations, reactive oxygen species, lipid peroxidation quantities, activities of antioxidant enzymes and contents of reduced glutathione, carotenoid, flavonoid, protein and chlorophyll in S. officinalis leaves

Biochemical parameters	Stages of growth			*F*
	1st	2nd	3rd	
Fe (mg kg^{-1})	59.64 ± 4.92	60.05 ± 5.81	41.53 ± 4.10	13.46*
Cu (mg kg^{-1})	6.55 ± 0.54	6.24 ± 0.52	9.18 ± 0.71	22.03*
Zn (mg kg^{-1})	38.04 ± 3.35	20.35 ± 2.41	60.68 ± 6.51	61.91*
Mn (mg kg^{-1})	51.35 ± 5.31	38.65 ± 3.94	25.87 ± 2.62	28.88*
$O_2^{\bullet -}$ (nmol mg^{-1} $protein^{-1}$)	305.68 ± 9.76	341.58 ± 9.58	355.52 ± 12.31	17.58*
$^{\bullet}OH$ (nmol mg^{-1} $protein^{-1}$)	1.85 ± 0.21	1.14 ± 0.25	1.93 ± 0.29	8.92*
MDA (nmol mg^{-1} $protein^{-1}$)	13.11 ± 2.70	14.95 ± 3.15	15.22 ± 2.80	0.47
SOD (U mg^{-1} $protein^{-1}$)	68.83 ± 6.21	62.69 ± 5.88	72.34 ± 7.13	1.73
CAT (U mg^{-1} $protein^{-1}$)	10.92 ± 0.71	11.29 ± 0.65	9.76 ± 0.54	4.71
POD (U mg^{-1} $protein^{-1}$)	4.23 ± 0.41	4.71 ± 0.38	3.78 ± 0.37	4.33
GSH (μmol mg^{-1} $protein^{-1}$)	2.18 ± 0.31	2.68 ± 0.35	1.38 ± 0.21	14.73*
Carotenoids (mg g^{-1})	0.71 ± 0.07	0.86 ± 0.09	0.56 ± 0.06	12.20*
Flavonoids (mg g^{-1} dry)	6.10 ± 0.57	6.80 ± 0.49	4.12 ± 0.40	23.98*
Proteins (mg g^{-1})	11.23 ± 0.78	13.46 ± 0.82	9.82 ± 0.64	17.93*
Chl *a* (mg g^{-1})	1.31 ± 0.31	1.54 ± 0.42	1.18 ± 0.20	0.95
Chl *b* (mg g^{-1})	0.61 ± 0.07	0.66 ± 0.06	0.45 ± 0.06	8.95*

F - ratio between groups' variance and the variance within groups;
* - results of the variance analysis, where the seasonal changes effect is significant

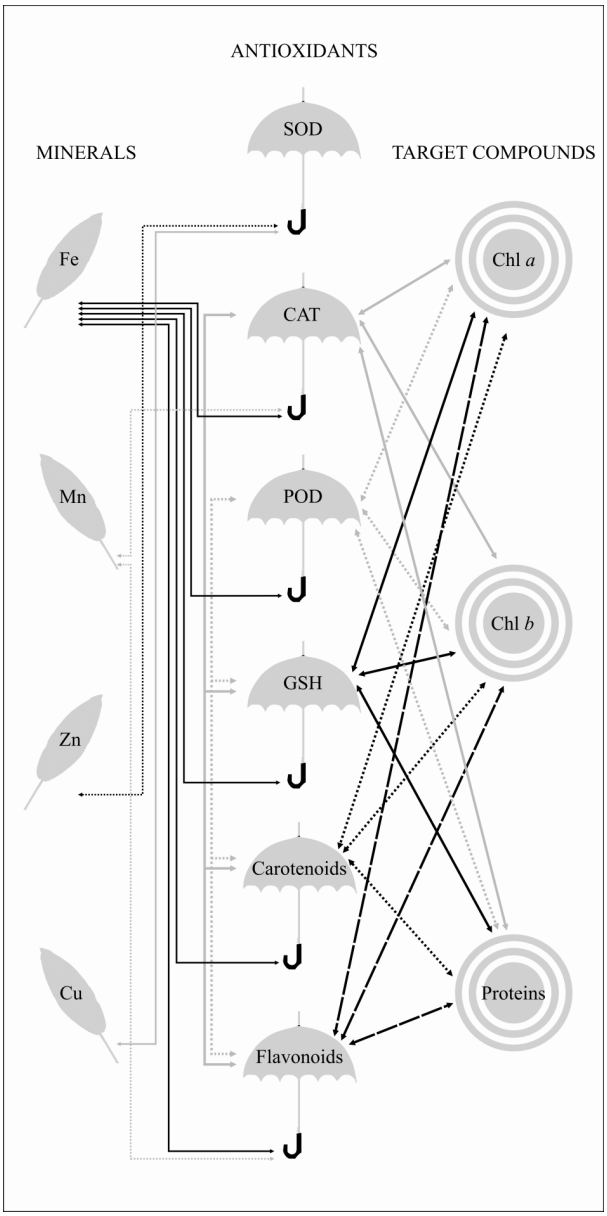

Figure 1. The interaction among the studied metals, protective antioxidant compounds and target molecules in the leaves of S. officinalis

flavonoid contents (r=0.98, r=0.96, r=0.98). Figure 1 shows the interaction of the studied elements, protective target molecules. The displayed interactions are based on the positive correlation coefficients.

Discussion

The analyzed parameters of soil: pH (7.6), redox potential (135.5mV), and humus (2.7%) were consistent with the results of our previous studies, and did not differ significantly from the investigated soils of south-eastern Serbia (4). On the base normal levels of metals in plants, it can be said that the sage leaves accumulate sufficient content of examined elements, except iron in the seed-forming stage (6). If the number of enzymatic and non-enzymatic antioxidants, with which the examined elements were in correlation, is set as criteria of their importance for the functioning of antioxidant systems of sage, then iron no doubt takes a dominant position. The variation among plants in their ability to absorb Fe is not always consistent and is affected by changing conditions of soil and climate and by stages of plant growth. In our research its content is correlated with the CAT and POD activities, as well as with the content of all researched non-enzymatic antioxidant.

Accumulation of $O_2^{\bullet-}$ in the leaves contri-bute to lipid peroxidation in S. officinalis during the active vegetative period. The level of LP did not significantly change during the examined vegetation period, which indicates that the researched plant species is not dramatically exposed to negative influence of ROS. Reactive oxygen species in Salvia reflexa was produced in larger quantities ($O_2^{\bullet-}$, 4315.90 and $^{\bullet}OH$, 529.85nmol g^{-1} f.m.) probably due to growths in maize crops which exposes it to different kinds of pesticides, used in agricultural practice.

The changes in SOD activity were correlated with the changes of Cu and Zn accumulation in a leaf of sage, which may indicate that this class of SOD is probably active in the dismutation reaction of superoxide to H_2O_2 and molecular oxygen. On the other hand there is no correlation of SOD activity and the $O_2^{\bullet-}$ quantities, although the significant accumulation of $O_2^{\bullet-}$ occurring in S. officinalis leaves, in the seed-forming stage should go along with the changes in SOD activity. It can be assumed that the non-enzymatic components of antioxidant system take over the role of $O_2^{\bullet-}$ scavengers, like glutathione and flavonoids. The activity of SOD generates H_2O_2, while CAT and POD are the main enzymes responsible for H_2O_2 decomposition, preventing $^{\bullet}OH$ generation (17). Statistical test confirms the fact that the optimum content of iron and manganese in sage leaves has contributed to the CAT activity, which was not changed significantly during the active vegetative period. In Kentucky bluegrass, the consistent and stable expressions of CAT activity may facilitate leaf cells in scavenging H_2O_2 in an efficient way (18). Our study also showed that changes in iron content in the growing season go along with the change of POD activity. The results of guaiacol peroxidase activity investigation among Salvia species indicated that S. tomentosa had the highest POD activity (19).

Based on statistical analysis joint action of glutathione, catalase and guaiacol peroxidase can be recognized in S. officinalis leaves. In a number of studies, the relative growth rate has been applied to evaluate Fe efficiency and tolerance of plants. Iron deficiency was characterized, among others, by low carotenoid content, when structural damage was well manifested (20). As we said, sage leaves accumulate sufficient content of iron, except in seed-forming stage. In this vegetation period higher carotenoids/chlorophylls ratio was determined, but oxidative damage has not occurred. The explanation of this situation may be found in the fact that the activities of CAT and POD have been sufficient to prevent a significant

drop in carotenoid concentration. If Lupinus albus was grown without Mn for 4 weeks, a loss of flavonoids content was determined (21). Based on this study an importance of Mn content for the flavonid accumulation in plants can be assumed. The relatively high content of flavonoids during the active vegetative period in S. officinalis leaves is likely to affect the low activity of guaiacol peroxidase, with which it is positively correlated. Generalić et al. (22) found significant differences in total flavonoid content in the Dalmatian sage leaf extracts depending on the season of the year they were picked in, which is consistent with the present work.

The proteins are the ones of target molecules of ROS attack and their content is an indicator of oxidative damage in a plant tissue. During the growth, protein content in leaves of S. officinalis changed significantly, and the lowest protein content was in the seed-forming stage. The changes in protein content in sage leaves are positively correlated with changes of all the studied antioxidants, except for SOD. Enhanced formation of ROS under stress conditions induces both protective responses and cellular damage. The scavenging of $O_2^{\bullet -}$ is achieved through SOD. In our case, due to optimal content of non-enzymatic antioxidants in S. officinalis leaves, especially flavonoids, probably superoxide anion radicals content was not high, and therefore the SOD response was in the middle range. In this way, the non-enzymatic antioxidants facilitate the functioning of SOD. Therefore, it was not necessary for SOD to play a primary role in the antioxidant protective system and exhibit a maximum activity, as usual. Chlorophylls have a unique and essential role in photosynthetic light-harvesting and energy transduction, and its degradation under high light intensities is considered to be the terminal symptom of oxidative stress. Results of our research indicate that stage of vegetation affected the accumulation of Chl *b* significantly, but there were no significant differences in Chl *a* content during vegetation. This fact supported the efficiency of antioxidant compounds in sage. Taking into consideration the statistical test results, we come to the conclusion that the sensitive photosynthetic pigments of sage leaves are fully protected by CAT and POD activities, as well as by the optimal content of glutathione, carotenoids and flavonoids. Correlation studies between leaf senescence rates of watercress, Petroselinum crispum and Salvia officinalis and their oxidative defense systems were conducted with detached leaves under simulated shelf-storage conditions. The relative order of leaf senescence rate, based on the rate of chlorophyll degradation and malondialdehyde accumulation, was watercress > parsley > sage. The results indicate that each herb species has developed specific defense systems, which may also prevent rapid chlorophyll loss (23).

In addition, the study of plant extracts can help in the designing of modern herbal medicinal products. It is important to note that the synergistic, additive or potentiated effects shown by the plant extract, frequently observed in the study of natural products, usually exceed the effects of single compounds, or mixtures of them at equivalent concentrations (24-26).

Conclusion

The examined plant accumulates sufficient content of researched elements during vegetation, except for the iron in the seed forming stage. A significant effect of growth stage on superoxide and hydroxyl radicals accumulation was observed but there are no significant differences in lipid peroxidation levels during vegetation. Antioxidant enzymes in the low and middle range of activities in leaves of S. officinalis are correlated with protein, chlorophyll a and b contents, except for SOD. On the other hand contents of non-enzymatic antioxidants vary greatly among different stages of growth, as well as the contents of protected molecules, with which they are positively correlated. Results from the present study suggest that the leaves of sage from Sićevo gorge Nature Park should be considered as an important source of iron, cooper, zinc, manganese and dietary antioxidants.

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SADRŽAJ METALA I ANTIOKSIDANATA ŽALFIJE IZ NACIONALNOG PARKA SIĆEVAČKA KLISURA

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U radu je proučavana sezonska promena sadržaja metala i antioksidanata u Salvia officinalis L. (žalfija) iz nacionalnog parka Sićevačka klisura. U listovima odabrane biljne vrste određena je koncentracija gvožđa, bakra, cinka i mangana; količina malonildialdehida, superoksid i hidroksil radikala, kao i aktivnost antioksidantnih enzima (superoksid dismutaze, katalaze i gvajakol peroksidaze). Takođe je određen i sadržaj redukovanog glutationa, karotenoida, ukupnih flavonoida, rastvorljivih proteina, kao i hlorofila a i b. Ispitana biljna vrsta akumulira dovoljan sadržaj metala u toku vegetacije, osim gvožđa u fazi formiranja semena. Konstatovan je značajan uticaj faze rasta na akumulaciju superoksid i hidroksil radikala, ali su zabeležene male promene na nivou lipidne peroksidacije tokom vegetacije. Antioksidantni enzimi, niske i srednje aktivnosti, u listu S. officinalis, u korelaciji su sa sadržajem proteina, hlorofila a i b (zaštićeni molekuli), ali ne i sa sadržajem SOD-a. Sa druge strane, sadržaj neenzimskih antioksidanata varira tokom vegetacionih faza, kao i sadržaj zaštićenih molekula, sa kojima su u pozitivnoj korelaciji. Rezultati ove studije sugerišu da antioksidantni odbrambeni sistem S. officinalis funkcioniše u putpunosti i štiti ciljne molekule od reaktivnih oblika kiseonika. Listovi žalfije iz nacionalnog parka Sićevačka klisura mogu se smatrati važnim izvorima gvožđa, bakra, cinka, mangana i antioksidanata. *Acta Medica Medianae 2015; 54(1) :27-33*.

***Ključne reči**: antioksidantni enzimi, neenzimski antioksidanti, reaktivni oblici kiseonika, Salvia officinalis, metali*