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Original scientific paper

A study of the antioxidants in *Oxytropis pilosa* (L.) DC.

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Abstract: The objective of this study was to estimate the antioxidative potential of *Oxytropis pilosa* (L.) DC. during the active vegetative period. The activities of the antioxidant enzymes (superoxide dismutase, catalase, peroxidase), the quantities of malonyldialdehyde, superoxide and hydroxyl radicals and reduced glutathione and the content of total flavonoids, chlorophylls *a* and *b*, carotenoids and soluble proteins were determined. The results showed that extracts from all plant organs exhibited antioxidant activity. The highest antioxidant ability was observed in the leaves where all the investigated antioxidant enzymes were active in a specific way: During the spring season, peroxidase showed the maximum activity 18.54 U mg^{-1} protein, catalase peaked in summer 9.04 U mg^{-1} protein, whereas, during the autumn season, superoxide dismutase showed maximum activity, 54.28 U mg^{-1} protein. Reduced glutathione, pigments and carotenoids present in the leaves contribute to the high antioxidant activity. Furthermore, inhibition of chemiluminescence activity of Balb/c mice blood phagocytes by crude leaf extracts at concentrations of 3.5 and 7.0 μg cm^{-3} were 30.2 and 36.5 %, respectively.

Keywords: *Oxytropis pilosa*; superoxide dismutase; catalase; peroxidase; antioxidant activity; total flavonoids, chemiluminescence.

INTRODUCTION

Oxytropis pilosa (L.) DC. is a widespread but disjunct relict species that originated in the Altai, Siberia, from where it extended to the West. The species is frequently found from the Russian and Ukrainian steppes to the Alps and thins out on its ways to Scandinavia, the Baltic States and East Europe.[1] In Serbia, it can be found with scattered distribution in the southeastern parts of the country.[2]

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Oxytropis species are well known as the "King of Herbs" in Chinese Tibetan medicine.[3] However, some *Oxytropis* species contain very toxic indolle alkaloids.[4] Current knowledge about the antioxidant properties of *O. pilosa* (L.) DC. are not supported by the available reference data.

This research was designed to study the antioxidant activity of *Oxytropis pilosa* (L.) DC. The antioxidative properties of leaf, stalk and root of *O. pilosa* (L.) DC. during the vegetative period were investigated by determining the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (C-ase) and peroxidase (P-ase); the quantities of malonyldialdehyde (MDA), the reactive oxygen species (ROS) superoxide (O_2^-) and hydroxyl radicals (·OH) and reduced glutathione (GSH), and the contents of total flavonoids, chlorophylls *a* and *b*, carotenoids and soluble proteins. The paper also describes the inhibition of the chemiluminescence activity of Balb/c mice blood phagocytes by leaves extracts.

EXPERIMENTAL

Plant material

The entire plants of *Oxytropis pilosa* (L.) DC. were collected in 2008 during the active vegetative period (April to October) from Subotinac, southeastern Serbia. The plant material was collected at three stages of growth (SG) as follows:

– 1st SG – the initial vegetation stage (April 2008)
– 2nd SG – the blooming stage (June 2008)
– 3rd SG – the seed forming stage (October 2008)

Botanical identification was made by Dr. N. Randjelović of the Botany Department, Faculty of Science, University of Niš, Serbia, where a voucher specimen is deposited.

Methods

One g of plant material was ground with quartz sand in a cold mortar. The ground material was suspended in 5 cm^3 0.1 mol dm^{-3} K_2HPO_4 at pH 7. After 10-min centrifugation at 4 °C and 15000 g, aliquots of the supernatant were used for SOD activity measurements. 20 µl of Tsuchiashi solution (chloroform/ethanol 3/5) were added to the supernatant before measurement of the enzyme activity. The SOD activity was determined in aliquots by the method of Misra and Fridovich[5] based on the inhibition of the transformation of adrenaline to adrenochrome at pH 10.2. For the other antioxidant enzymes and other biochemical determinations, the plant material was treated in the same way but the medium was 0.1 mol dm^{-3} phosphate buffer (K_2HPO_4/KH_2PO_4, pH 7) with a plant material to medium ratio of 1:5, centrifuged for 10 min at 15000 *g*. After the centrifugation, the supernatant was evaluated for:

– C-ase activity spectrophotometrically at 240 nm;[6]
– P-ase activity, using guacol as substrate;[7]
– lipid peroxidation (LP) by the thiobarbituric acid (TBA) method; the values are given as equivalent amounts of malonyldialdehyde (MDA);
– the calibration curve was prepared with malonyldialdehyde bis-diacetal; [8]
– the superoxide radical was determined by the auto-oxidation of adrenaline;[9]
– the hydroxyl radical by the inhibition of deoxyribose degradation.[10]

Moreover, the amount of GSH was determined with Ellman reagent[11] and protein by Folin reagent[12]. Total flavonoids were estimated according to Marckam.[13] The pigments were extracted with acetone and determined spectrometrically using molar extinction coefficients reported by Wettstein.[14]

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The experimental results are expressed as the mean±standard deviation of three replicates.

Chemiluminescence assay

The chemiluminescence (CL) assay was performed to measure the total antioxidant potential of plant origin. The CL intensity in phagocytosis of mouse's blood leukocytes served as a measure of the oxidant activity.[15] The CL intensities were measured on a liquid scintillation counter Beckman LS 3200 in the "out of coincidence" mode. Blood samples of Balb/c mice were prepared as previously described.[16] Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) solution, 0.1 mol dm^{-3} in dimethyl sulfoxide (Sigma) was used as the chemiluminogenic probe. The CL mixture was diluted with phosphate buffer (pH 7.2) to a final concentration of 10^{-5} mol dm^{-3}. In order to estimate enzymatic and non-enzymatic compounds of plant and their antioxidant activity, two manners of extract preparation were used. Crude leaf extract was prepared simultaneously as for the determination of the enzymatic activity. Leaves collected in the blooming stage of vegetation were used. A boiled leaf extract was obtained after cooling of the crude one (95 °C, 30 min). Blood sample without a test extract served as a control. Butylated hydroxytoluene (BHT) was used as a standard common antioxidant. The measurements of CL were performed in duplicate.

RESULTS AND DISCUSSION

Significant OH accumulation in the leaves (1.74 nmol mg^{-1} protein) contributes to a high level of LP (14.31 nmol mg^{-1} protein), but does not contribute to O_2^- accumulation in the same plant organ. The highest amount of O_2^- was detected in the root during all observed SG (Table I). The different responses of LP and O_2^- accumulation between root and leaf may be associated with the intensity level of environmental stress and different protective mechanisms.[17]

TABLE I. Quantities of reduced glutathione, malonyldialdehide, O_2^-, $^{\bullet}OH$ and protein in the organs of *Oxytropis pilosa* (L.) DC.

SG	Plant organ	Content				
		GSH U mg^{-1} prot.	MDA nmol mg^{-1} prot.	O_2^- nmol mg^{-1} prot.	$^{\bullet}OH$ nmol mg^{-1} prot.	Protein mg g^{-1}
1st	Leaf	1.98±0.18	9.74±0.97	152.68±9.76	1.70±0.33	7.95±0.60
	Stalk	0.51±0.06	5.76±0.89	251.73±8.42	1.38±0.29	7.84±0.50
	Root	0.85±0.08	6.94±0.79	276.32±11.04	1.29±0.23	7.24±0.40
2nd	Leaf	2.10±0.23	13.10±1.56	168.56±9.58	1.82±0.29	10.87±0.89
	Stalk	0.62±0.08	7.29±0.91	230.78±8.69	1.61±0.31	8.60±0.60
	Root	0.94±0.19	8.92±0.79	332.79±10.21	1.24±0.21	7.96±0.84
3rd	Leaf	1.32±0.15	14.31±2.76	214.78±10.71	1.74±0.28	7.24±0.50
	Stalk	0.34±0.05	8.10±0.82	243.44±8.89	1.43±0.22	5.25±0.70
	Root	1.15±0.14	12.56±2.05	323.56±11.04	1.05±0.19	6.24±0.41

The GSH quantity in the leaves was the highest in the blooming stage (2.10 μmol mg^{-1} protein). The present results, in comparison with literature data,[18] suggest that the research plant species was not dramatically exposed to the negative influences of $^{\bullet}OH$ and O_2^-. Of course, the low quantity of GSH does not

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automatically mean low activity of the other components of the antioxidant system.

The protein contents in the plant organs were significantly higher in blooming stage than in other SGs. A different variation of protein over the seasons was observed by Bogdanović[19] in *Picea omorika* (Panč.) Purkinye. In the study of the antioxidant defence system of high mountain and steppe plants, the average protein concentration was 29 mg g^{-1} dry in steppe plants of the family Fabaceae and 38 mg g^{-1} dry in alpine plants. This may be important under the low temperature and inadequate water conditions of alpine regions.[20]

TABLE II. Contents of total flavonoids, chlorophylls *a* and *b* and carotenoids in the leaves of *Oxytropis pilosa* (L.) DC.

SG	Flavonoids mg g^{-1} dry	Chlorophyll *a* mg g^{-1}	Chlorophyll *b* mg g^{-1}	Carotenoids mg g^{-1}
1st	0.126±0.005	1.54±0.46	0.70±0.05	0.52±0.07
2nd	0.232±0.008	1.76±0.61	0.71±0.08	0.63±0.08
3rd	0.091±0.007	1.39±0.38	0.52±0.04	0.41±0.06

The quantity of total flavonoids and the contents of pigments are presented in Table II. The highest values of all the investigated parameters were found in the blooming vegetation stage. The lower quantities of O_2^- in the leaves (Table I), where the total flavonoids (0.232 mg g^{-1} dry) and carotenoids contents (0.63 mg g^{-1}) were the highest, may support the assertion of the high antioxidant ability of leaves.

The changed antioxidant enzyme activities in plant organs of *O. pilosa* (L.) DC. show a different antioxidant metabolism in response to environmental stress. The leaves exhibited the highest SOD, C-ase and P-ase activities in all SG (Table III). The vegetation stage had a significant effect on the SOD activity in *O. pilosa* (L.) DC. the activity being significantly higher in the seed forming stage than in the other two vegetation stages. The SOD activity in the leaves of *Astragalus vulneria* (Fabaceae) was 60 U g^{-1} FW.[20] The SOD activity in *Allium ursinum*[18] was found to be as low as the activity detected in leaves of *O. pilosa* (L.) DC. (54.28 U mg^{-1} protein). SOD plays a central role in the enzymatic defence system in removing O_2^-.[21] The accumulation of O_2^- occurred in the root, together with changes in the SOD activity. The increased O_2^- production in the roots under environmental stress is correlated with the decreased SOD activity. Since enhanced information of ROS under stress conditions induce both protective responses and cellular damage,[22] the obtained results indicate that O_2^- accumulation in the root involves a protective mechanism under environmental stress in *O. pilosa* (L.) DC.

The C-ase activity in the researched plant was influenced by seasonal conditions (Table III). The highest activity in the blooming stage (9.04 U mg^{-1} protein) shows that catalase may also be involved in the protection from intense light

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and high temperature. The combined action of C-ase and SOD converts O_2^- and H_2O_2 to water and molecular oxygen, thus averting cellular damage under unfavourable conditions such as water stress.[23]

TABLE III. Activities of the antioxidant enzymes in the organs of *Oxytropis pilosa* (L.) DC.

SG	Plant organs	*SOD* U mg^{-1} prot.	C-ase U mg^{-1} protein	P-ase U mg^{-1} protein
1st	Leaf	51.43±5.78	8.32±0.87	18.54±1.20
	Stalk	25.41±3.82	5.16±0.58	14.89±0.54
	Root	23.70±3.46	6.96±0.51	5.46±0.43
2nd	Leaf	48.26±6.27	9.04±0.82	16.23±0.98
	Stalk	24.12±3.56	6.28±0.56	12.33±0.49
	Root	25.21±2.36	6.26±0.34	7.54±0.36
3rd	Leaf	54.28±4.55	7.64±0.52	15.76±0.71
	Stalk	25.89±3.24	3.85±0.28	10.64±0.51
	Root	27.36±3.82	6.14±0.31	8.72±0.43

The vegetation stage had no significant influence on peroxidase activity in the examined plant organs. The very small changes in the P-ase activity in the leaves of *O. pilosa* (L.) DC. (15.76–18.54 U mg^{-1} prot.) may indicate its physiological importance in all vegetation stages. On the other hand, literature data report significant differences in P-ase activity in some plant species.[24,25] The reason for this is several isoenzymes that have separate physiological functions. For example, in *Minium affine* exposed to extreme drought conditions, two isoenzymes were found after rehydration. The first responsible for drought protection and second involved in the cell repair system.[26]

The parameters of CL inhibition of blood phagocytes by leaf extracts of *O. pilosa* (L.) DC. (collected in the blooming vegetation stage) are given in Table IV and presented in Figs. 1 and 2. The extract of *O. pilosa* (L.) DC. reduced chemiluminiscence emission at both employed concentrations in a dose-dependent manner. Compared to the common antioxidant BHT, the inhibition was lower, but compared to commercial vegetal extracts of Isoflavin Beta and red clover, the extracts of *O. pilosa* (L.) DC. demonstrated higher levels of inhibition.[27] Sin-

TABLE IV. Parameters of the inhibition of the CL activity of blood phagocytes by *Oxytropis pilosa* (L.) DC. extracts

Sample	Inhibition[a], %	Index of inhibition[b]
BHT (3.5 μg cm^{-3})	49.1	–
BHT (7.0 μg cm^{-3})	55.8	–
Crude leaves extract (3.5 μg cm^{-3})	30.2	61.6
Crude leaves extract (7.0 μg cm^{-3})	36.5	65.5
Boiled leaves extract (3.5 μg cm^{-3}	24.4	49.6
Boiled leaves extract (7.0 μg cm^{-3})	45.8	82.0

[a]100×CL sample/CL control; [b]100×CL sample/CL BHT

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ce BHT is known to be a free radical scavenger, it can be concluded that the CL inhibitory effect of the *O. pilosa* (L.) DC. leaf extract included the scavenger effect. According to the results obtained for peroxidase activity in leaves (Table III), a high level of activity in the initial and blooming vegetation stages could be seen. It is possible that peroxidase was "responsible" for high antioxidant activity of the crude leaf extract.

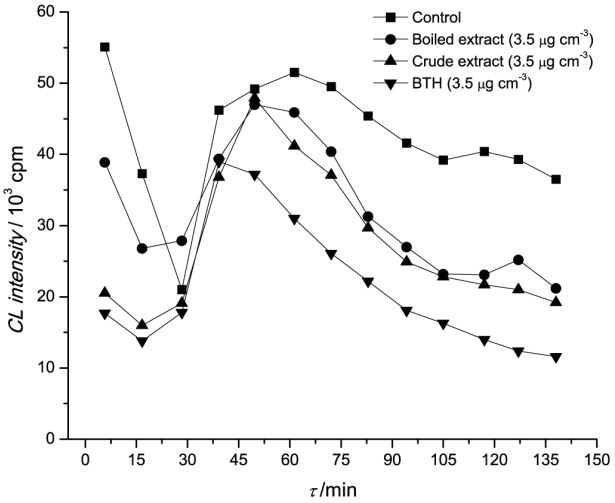

Fig. 1. Antioxidant activity of *O. pilosa* (L.) DC. extracts at lower doses.

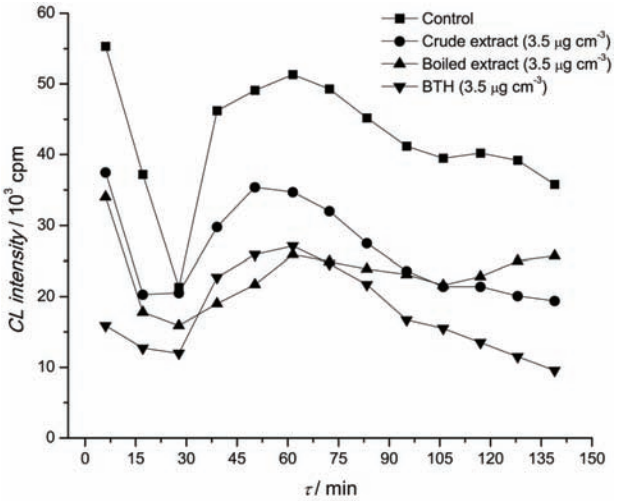

Fig. 2. Antioxidant activity of *O. pilosa* (L.) DC. extracts at higher doses.

There were some differences between the crude and boiled extracts. The crude extract showed an approximate inhibition by 33 %, while the boiled one

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inhibited by about 35 %. The lower inhibition of CL intensity in the crude leaf extract could be due to a deficit in antioxidant compounds in the reaction mixture, in contrast to the persistence of substances with pro-oxidant activity. The inhibitory activity of the boiled extract was more dose-specific than those of the crude extracts, which is in accordance with the previous explanation. The opposite effects could involve direct stimulating action on the phagocyte cell, chemical pro-oxidant action and anti-inhibitory action in different ways.[28]

CONCLUSIONS

The results reported herein can be considered as initial information on the antioxidant properties of *O. pilosa* (L.) DC. The examined antioxidant enzymes changed the levels of their activities in the vegetative period in specific way. During the spring season P-ase showed maximum activity, C-ase peaked in the summer, whereas SOD showed maximum activity during the autumn season. This suggests a complementary action of these enzymes, in response to external changes. The examined antioxidant enzymes may be used as indicators of the antioxidant ability of *O. pilosa* (L.) DC. to environmental changes. All the investigated organs possessed effective antioxidants, especially the leaves. Furthermore, the inhibition of the chemiluminiscence activity of Balb/c mice blood phagocytes by crude leaf extracts at concentrations of 3.5 and 7.0 μg cm^{-3} were 30.2 and 36.5 %, respectively. Therefore, the leaves could be used as a source of natural antioxidants and in the pharmaceutical industry for the manufacture of products with a potent oxygen radical scavenger activity.

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ИЗВОД

ПРОУЧАВАЊЕ АНТИОКСИДАНАТА У *Oxytropis pilosa* (L.) DC.

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У раду су приказани резултати проучавања антиоксидантних ензима супероксид-дисмутазе, каталазе и пероксидазе у листу, стаблу и корену самоникле популације *Oxytropis pilosa* (L.) DC. из југоисточне Србије. Такође су проучавани садржаји малонил-диалдехида, супероксид- и хидрокси-радикала, редукованог глутатиона, као и садржај укупних флавоноида, хлорофила *а* и *б*, каротеноида и растворљивих протеина. Највиша антиоксидантна активност је забележена у лишћу, у коме су антиоксидантни ензими активни на специфичан начин: у пролеће пероксидаза испољава максималну активност, 18,54 U mg^{-1} протеина; максимум активности каталазе је у лето, 9,04 U mg^{-1} протеина; док је супероксид-дизмутаза у јесен најактивнији ензимски систем, 54,28 U mg^{-1} протеина. Редуковани глутатион, пигменти и каротеноиди присутни у лишћу доприносе високој антиоксидантној активности проучаване биљне врсте. У прилог овоме је инхибиција хемилуминесцентне активности фагоцита Balb/c мишева свежим екстрактом лишћа у концентрацијама од 3,5 и 7,0 μg cm^{-3} од

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30,2, односно 36,5 %. Из овог разлога лишће *O. pilosa* (L.) DC. се може користити као извор природних антиоксиданата и бити сировинска база за производњу фармаколошких препарата значајне антиоксидантне активности.

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