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Antioxidative responses to seasonal changes and chemiluminescence assay of Astragalus onobrychis leaves extract

Invited Paper

Dragoljub L. Miladinović[1*], Budimir S. Ilić[1], Stevo J. Najman[2], Olga G. Cvetković[3], Aleksandra M. Šajnović[3], Marija D. Miladinović[1], Nikola D. Nikolić[4]

[1]*Department of Pharmacy, Faculty of Medicine, University of Niš, 18000 Niš, Serbia*

[2]*Department of Biology, Faculty of Medicine, University of Niš, 18000 Niš, Serbia*

[3]*Center of Chemistry - IChTM, University of Belgrade, Njegoševa 12, 11000 Belgrade, Serbia*

[4]*Laboratory for Geochemistry, Cosmochemistry and Astrochemistry, University of Niš, 18000 Niš, Serbia*

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Abstract: The aim of this study was to research the seasonal changes of antioxidant enzyme activity and total antioxidant capacity in leaves of *Astragalus onobrychis* L. subsp. *chlorocarpus* (Griseb.) S. Kozuharov *et* D.K. Pavlova. Leaves of *A. onobrychis* were collected during the different stages of growth and analyzed for antioxidant enzyme activity: superoxide dismutase, catalase, guaiacol peroxidase, glutathione peroxidase. Quantities of malonyldialdehyde, superoxide radicals, and hydroxyl radicals were measured as well as the content of soluble proteins. Furthermore, total antioxidant capacity was determined by the inhibition of chemiluminescence activity of blood phagocytes by leaf extracts. Stages of vegetation significantly affected the accumulation of superoxide radicals, but there were no significant differences in hydroxyl radical quantity and lipid peroxidation levels during vegetation. Soluble proteins vary greatly between different stages of growth. Seasonal changes were found to have an effect on enzymatic activities. During the spring season, guaiacol peroxidase showed the highest levels. Catalase and glutathione peroxidase increased their activities in summer, while, during the autumn season, superoxide dismutase showed maximum activity. On the basis of chemiluminescence assay, it can be concluded that leaf extract of *A. onobrychis* possesses a significant antioxidant capacity thus protecting plants during environmental stress.

Keywords: *Astragalus onobrychis • Antioxidant enzymes • Reactive oxygen species • Antioxidant capacity • Chemiluminescence*

1. Introduction

Environmental stresses, as well as developmental processes, induce the production of reactive oxygen species (ROS) in a plant. ROS have long been proposed as signal molecules that regulate various processes such as growth, development, and responses to biotic and abiotic stress factors [1]. However, at high concentrations, ROS can be toxic by destroying normal metabolism through oxidative damage to lipids, proteins and nucleic acids [2]. Since they are involved in ROS metabolism, antioxidant enzymes may play an important part in the plants' strategies of tolerance allowing survival in their habitats [3]. The most important components of the antioxidant system include several enzymes: those that directly eliminate ROS (e.g. superoxide dismutase, catalase and peroxidase) and enzymes that eliminate internal lipid peroxidation products (*e.g.* glutathione

* *E-mail: dragoljubm@gmail.com*

peroxidases) [4]. To add more evidence to the ecological studies already carried out on wild plant adaptation, it is of importance to understand the molecular, biochemical and physiological means which protect plants from environmental adversities.

Formation of activated oxygen compounds occurs during respiratory burst of phagocytic cells with the generation of photons that produce a very weak light signal, which may be amplified and measured as chemiluminescence (CL). The generation of CL has been reported to involve superoxide, singlet oxygen, hydrogen peroxide and hydroxyl radicals. Therefore, CL method is commonly used for measuring antioxidative properties of plant extracts.

Astragalus L. (Fabaceae) is generally considered the largest genus of vascular plants with an estimated number of 2500−3000 species [5]. Many species of *Astragalus* are used to restore overgrazed range, control erosion, and provide useful sources for producing important drugs [6]. The flora of Serbia includes seventeen species of the *Astragalus* genus [7].

Our previous study dealt with the mineral and non-enzymatic antioxidant composition of *Astragalus onobrychis* L. subsp. *chlorocarpus* (Griseb.) S. Kozuharov *et* D.K. Pavlova [8]. This research was designed to study the changes of enzymatic antioxidants in leaves of *A. onobrychis* from Serbia during the active vegetative period. We investigated the activities of antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), glutathione peroxidase (GPOX); quantities of malonyldialdehyde (MDA), superoxide ($O_2^{\bullet -}$), hydroxyl (•OH) radicals and the content of soluble proteins were also measured. The paper also describes the total antioxidant capacity (TAC) determined by the inhibition of CL activity of mice blood phagocytes by leaf extracts. To the authors' knowledge, the changes of the enzymatic antioxidative system related to the stage of vegetation and TAC determination by *in vitro* assay in leaves of *A. onobrychis* have not been previously examined.

2. Experimental procedure

2.1. Chemicals

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

2.2. Plant material

Astragalus onobrychis L. subsp. *chlorocarpus* (Griseb.) S. Kozuharov *et* D.K. Pavlova grows among communities of sub-Mediterranean grasslands *Astragalo-Potentilletalia* and *Scabioso-Trifolion dalmatici* in phytocoenoses which is described as the *Astragalo-Calaminthetum hungaricae* [7].The leaves were collected from healthy specimen in their natural habitat at the Seličevica mountain in 2011. The plant material was collected in three stages of growth (SG) as follows:

- 1st SG – the initial vegetation stage, spring (25 April)
- 2nd SG – the blooming stage, summer (7 July)
- 3rd SG – the seed forming stage, autumn (2 October)

Astragalus onobrychis leaves have been picked between 15:00 and 16:00 h when sun insolation was highest. Fresh leaves were immediately frozen in liquid nitrogen and transported to the laboratory where they have been stored at -70°C until preparation of extracts began.

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

Characteristics of soil were investigated by standard analytical methods and techniques [9]. Observed meteorological parameters were obtained by the State Hydrometeorological Service, from the meteorological station located in Niš (Table 1).

2.3. Antioxidants analysis

2.3.1. Extraction of enzymes

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

2.3.2. Determination of quantities MDA, $O_2^{\bullet -}$ and • OH

Lipid peroxidation (LP) was determined by the thiobarbituric acid (TBA) method [10]. A 0.5 mL aliquot of supernatant was mixed with 2 mL of 20% trichloroacetic acid (TCA) containing 0.5% TBA. The mixture was heated at 95°C for 30 min, quickly cooled, and then centrifuged at 15000 × g for 10 min. The absorbance of the supernatant at 532 nm was read and the value for non-specific absorption at 600 nm was subtracted. Values were given as equivalent amounts of MDA. The calibration curve was prepared with malonyldialdehyde bis-diacetal. $O_2^{\bullet -}$ was determined by adrenaline autooxidation [11], while •OH was determined by the inhibition of deoxyribose degradation [12].

2.3.3. Determination of antioxidant enzyme activity and protein content

All the antioxidant enzyme activities were determined spectrophotometrically at 25°C using phosphate buffer

Table 1. Observed meteorological parameters over the natural habitat of *A. onobrychis*.

Season	Air temperature (°C)	Average sunlight hours/day	Rainfall (mm)
Spring	11.94±1.78	5.72±0.64	1.24±0.16
Summer	22.98±2.14	9.10±0.97	1.25±0.13
Autumn	12.07±1.06	5.98±0.49	0.84±0.08

(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 Misra and Fridovich [11] 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 1 min compared with the control. Measurements were made at 480 nm. Catalase activity (CAT, EC 1.11.1.6) was determined at 240 nm. The decomposition of H_2O_2 was followed by a decrease in absorbance [13]. Guaiacol peroxidase activity (POD, EC 1.11.1.7.) was determined using guaiacol as the substrate at 436 nm [14]. Glutathione peroxidase activity (GPOX, EC 1.11.1.9.) was determined using cumene hydroperoxide and reduced glutathione (GSH) as substrates at 412 nm [15]. The soluble protein content was determined by the method of Bradford with bovine serum albumin as standard [16].

2.4. CL assay

The CL assay was performed to measure the TAC of plant origin. CL intensity of mice blood leukocytes served as a degree of oxidant activity [17]. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) solution was used as a chemiluminegenic probe. Butyl-hydroxytoluene (BHT), a common antioxidant, was used as a standard.

2.4.1. Blood samples

Polymorphonuclear leukocytes (PMNs) were obtained from male BALB/c mice as previously reported [18]. Venous blood was centrifuged initially at 170g to remove the platelet-rich plasma and then at 1000g to eliminate platelet-poor plasma. The buffy coat of white cells were diluted with phosphate buffered saline (PBS) pH 7.2. Dextran was added and the mixture was left for 45 min at room temperature (26°C) for sedimentation. The supernatant was centrifuged by Ficoll-gradient separation and then washed twice with distilled water to remove red blood cells. A pellet of PMNs was collected from the tube base. The cells were suspended in Hanks Balance Salt Solution (HBSS) pH 7.4 with Ca^{2+} and Mg^{2+} ($HBSS^{++}$). Cell suspensions were counted using a hemocytometer and light microscope, and they were then diluted with HBSS to obtain a final cell suspension of 1×10^6 mL^{-1}.

The sample was then taken to calculate the leukocyte formula and the absolute number of PMNs. Blood sample without testing extracts served as a control. The study was performed according to the guidelines of the Ethical Committee of the Faculty of Medicine, University of Niš, Serbia, which is in accordance with international regulations.

2.4.2. Cell viability

Cell viability was determined by the standard trypan blue exclusion method. The PMNs (1×10^6 mL^{-1}) were incubated with 3.5 or 7.0 mg mL^{-1} of plant extracts each in triplicate at room temperature for 2 h. The blue dye uptake was an indication of cell death. The percentage viability was calculated from the total cell counts [19].

2.4.3. CL measurement

CL measurements were performed using a liquid scintillation counter (Beckman LS 3200) in the coincidence off-mode. The measurement was carried out in glass vials, which previously read “background” CL, to remove vials with high cpm. The samples were measured in duplicate, every 5-10 minutes (repeated circle), with a required reading of the sample without stimulators (unstimulated CL) for 2 hours.

150 mL of blood, phosphate buffered saline pH 7.2, luminol and stimulator of phagocytes for a total volume of 1500 mL were placed in measured vials. Luminol, 0.1 mol L^{-1} in dimethylsulfoxide (DMSO), was adjusted according to the number of PMNs in the blood sample to be measured. 15 mL of Zymosan was used as the stimulator. For unstimulated chemiluminescence, PBS was added to a volume, instead of zymosan, up to 1500 mL. The measurement began with the addition of luminol solution to the diluted blood with a calculated amount of PBS. CL response of phagocytes was induced by placing the zymosan solution into the measuring sample.

In order to estimate antioxidant compounds of *A. onobrychis*, two ways of extraction preparation were used. Crude leaf extract was prepared at the same time for antioxidant activity determination. Boiled leaf extract was retrieved after cooling of the crude leaves (95°C, 30 minutes). Leaves collected in the blooming stage of vegetation were used.

Table 2. Quantities of $O_2^{\bullet -}$, $^{\bullet}OH$, MDA, protein contents and activities of antioxidant enzymes in leaves of *A. onobrychis*.

Biochemical parameters	Stages of growth			F
	1st	2nd	3rd	
$O_2^{\bullet -}$ (nmol mg^{-1} protein)	123.78 ± 7.78	163.72 ± 8.19	312.48 ± 10.56	372.20*
$^{\bullet}OH$ (nmol mg^{-1} protein)	1.76 ± 0.27	1.92 ± 0.31	1.87 ± 0.26	0.25
MDA (nmol mg^{-1} protein)	9.93 ± 0.89	11.37 ± 1.94	14.02 ± 2.03	4.46
Protein (mg g^{-1})	8.21 ± 0.64	10.35 ± 0.90	5.24 ± 0.67	35.52*
SOD (U mg^{-1} protein)	42.72 ± 4.87	27.18 ± 3.28	46.73 ± 5.13	15.79*
CAT (U mg^{-1} protein)	7.52 ± 0.68	7.93 ± 0.72	5.88 ± 0.61	7.83*
POD (U mg^{-1} protein)	8.13 ± 0.42	7.86 ± 0.48	7.08 ± 0.32	5.25*
GPOX (U mg^{-1} protein)	0.32 ± 0.02	0.45 ± 0.03	0.41 ± 0.03	18.14*

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

2.5. Data analysis

The experimental results of antioxidant analyses were expressed as a mean ± standard deviation of three replicates. The comparison of biochemical parameters was analyzed by ANOVA and Tukey test using the statistical program SPSS v19.0 (SPSS Inc., Chicago, IL, USA). The statistical significance for all tests were set at the $P \leq 0.05$ confidence level. Bivariate correlations of Pearson were used to study the interaction of studied biochemical parameters.

3. Results and discussion

3.1. Environmental conditions at A. onobrychis habitat

Soil parameters pH (7.78), redox potential (122.47 mV), and humus (1.41%) were consistent with the results of our previous studies and did not differ significantly from the investigated soils of south-eastern Serbia [20]. Temperature interval for the year was rather typical, with average temperature in summer two-fold higher than that in spring and autumn. Sunlight hours followed seasonal dynamics, with a maximum in summer (9.10 average sunlight hours/day). As for average season rainfall, values for spring (1.24 mm), summer (1.25 mm) and autumn (0.84 mm) showed dryer seasons than normal (Table 1).

3.2. $O_2^{\bullet -}$, $^{\bullet}OH$, and MDA quantities

The highest values of LP, measured as MDA equivalents and $O_2^{\bullet -}$ (Table 2), were recorded in the seed forming stage, while the highest $^{\bullet}OH$ accumulation was observed in the blooming stage. Stages of vegetation affected the accumulation of $O_2^{\bullet -}$ significantly ($F = 372.20$), but there are no significant differences in $^{\bullet}OH$ quantity ($F = 0.25$) and LP levels ($F = 4.46$) during vegetation. An increase of MDA quantity during vegetation was correlated with a significant increase of $O_2^{\bullet -}$ ($r = 0.82$; Fig. 1A) and a change of $^{\bullet}OH$ ($r = 0.70$; Fig. 1B) leaf quantities, respectively.

3.3. Protein content and antioxidant enzyme activities

Soluble proteins vary greatly between different stages of growth, and the values range from 5.24 mg g^{-1} and 10.35 mg g^{-1} (Table 2). The highest level was recorded in the blooming vegetation stage. Significant differences in protein content during vegetation ($F = 35.52$) can be related to $O_2^{\bullet -}$ quantities in leaves ($r = -0.75$; Fig. 2).

Vegetation stage had a significant effect on antioxidant enzymes activity in leaves of *A. onobrychis* (SOD, $F = 15.79$; CAT, $F = 7.83$; POD, $F = 5.25$; GPOX, $F = 18.14$; Table 2). The activity of SOD was higher in the seed forming stage (46.73 U mg^{-1} protein), than in the other two vegetation stages. The highest CAT activity was recorded in the blooming stage of vegetation (7.93 U mg^{-1} protein), similar to GPOX activity (0.45 U mg^{-1} protein). The highest value of POD activity was noted in the initial vegetation stage (8.13 U mg^{-1} protein).

CAT and POD activities were related to protein concentrations ($r = 0.94$, $r = 0.76$; Fig. 3A and 3B). In contrast, the changes in CAT and POD activities, can be associated with the increase of $O_2^{\bullet -}$ quantity ($r = -0.74$, $r = -0.74$; Fig. 3C and 3D). Negative correlation coefficients indicate an active antioxidant role of CAT and POD in protection against $O_2^{\bullet -}$ radicals.

3.4. Total antioxidant capacity

The cell viability test carried out to evaluate the cytotoxicity of plant extracts on mice blood phagocytes at 3.5 and 7.0 mg mL^{-1} indicated that cells were viable (>90%) after 2 h incubation. It is known that phagocytosis and other

Table 3. Parameters of chemiluminescence inhibition by *A. onobrychis* leaf extracts.

Sample	Percentage of inhibition[a]	Index of inhibition[b]
BHT (3.5 μg mL^{-1})	49.1	-
BHT (7.0 μg mL^{-1})	55.8	-
Crude leaves extract (3.5 μg mL^{-1})	25.2	52.1
Crude leaves extract (7.0 μg mL^{-1})	29.4	53.5
Boiled leaves extract (3.5 μg mL^{-1})	30.1	62.1
Boiled leaves extract (7.0 μg mL^{-1})	34.9	63.1

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

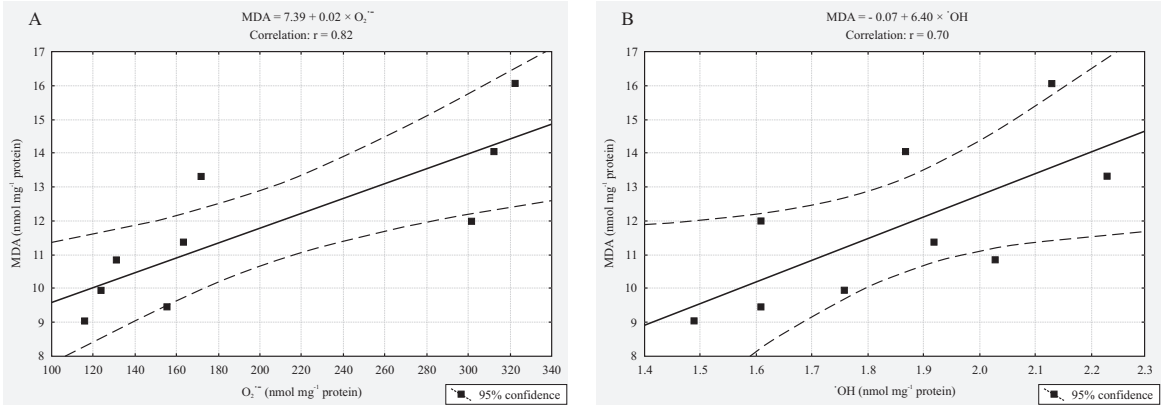

Figure 1. Evaluation of regression relationship in *A. onobrychis* leaf extract during the vegetative period considering: (A) quantities of MDA and $O_2^{\bullet -}$; (B) quantities of MDA and $^{\bullet}OH$. Solid line is the fitted linear regression line. Dashed lines represent the 95% confidence limits.

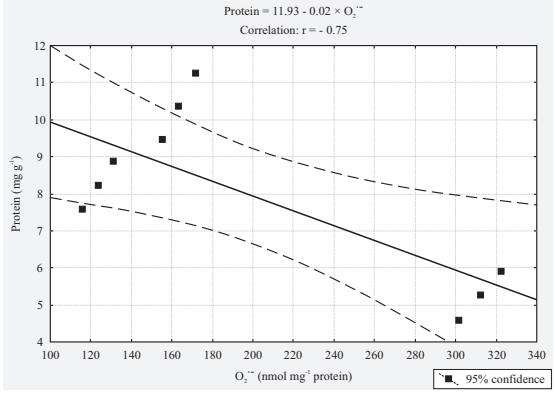

Figure 2. Evaluation of regression relationship between protein content and quantities of $O_2^{\bullet -}$ in *A. onobrychis* leaf extract during the vegetative period. Solid line is the fitted linear regression line. Dashed lines represent the 95% confidence limits.

states of phagocyte stimulation are the source of very active oxidants [21]. The parameters of the inhibitory CL activity of blood phagocytes by *A. onobrychis* extracts are given in Table 3 and Figs. 4 and 5. Based on the measurement of the area over the time course of the CL curves, we estimated the CL inhibition of plant extracts.

In relation to the CL inhibition of the same concentration of BHT, an index of inhibition was calculated. Common antioxidant BHT showed higher CL inhibition, compared with plant extracts. According to the peak values of the proportional dose dependence of the CL inhibition for BHT, we found that the concentration of 3.5 mg mL^{-1} was IC_{50}. In addition, we found that *A. onobrychis* leaf extract follows the BHT time course of CL profile. There are some differences between crude and boiled plant extracts. The crude extract shows approximately 27% of CL inhibition, but the boiled extract inhibits 32% of chemiluminescence (mean value). The inhibition index is the same for different concentrations of leaf extract.

The plants have developed adaptations to extreme climatic conditions: high temperatures, high light intensity and irradiance, drought and freezing. Antioxidant defense mechanisms can be vitally important in the survival of wild plants [22,23]. LP is an indicator of the prevalence of free radical reaction in tissues. Accumulation of $O_2^{\bullet -}$ and •OH in the leaves contribute to lipid peroxidation in *A. onobrychis* during the active vegetative period. Even the level of LP did not significantly change during the examined vegetation period, as well as the

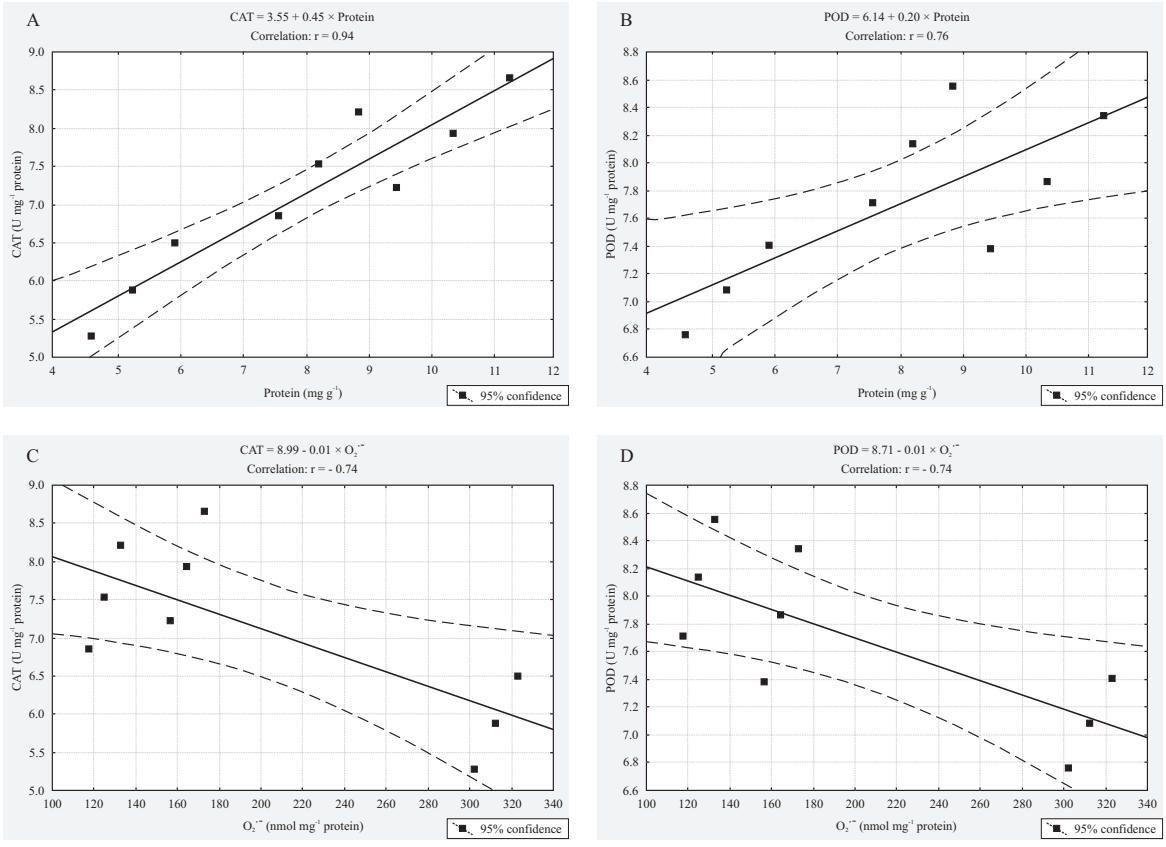

Figure 3. Evaluation of regression relationship in *A. onobrychis* leaf extract during the vegetative period considering: (A) CAT activities and protein content; (B) POD activities and protein content; (C) CAT activities and quantities of $O_2^{\bullet -}$; (D) POD activities and quantities of $O_2^{\bullet -}$. Solid line is the fitted linear regression line. Dashed lines represent the 95% confidence limits.

•OH quantities, which indicates that the researched plant species is not dramatically exposed to negative influences of ROS. As we pointed earlier, low level of rainfalls during the vegetation period (Table 1), could be the cause of drought, which is classified as an extreme abiotic condition. This factor, which is probably responsible for the significant accumulation of $O_2^{\bullet -}$, was not destructive enough to cause the occurrence of oxidative stress in the leaves of *A. onobrychis*. The results obtained for ROS came from our earlier research of Fabaceae plants [24]. The level of LP in wild populations of *Erica andevalensis*, grown in metal-enriched soils, was between 0.018 and 0.057 nmol mg^{-1}. These data suggest that *E. andevalensis* does not suffer from oxidative stress derived from metal exposure and accumulation [4]. The antioxidative protection in leaves of *Triticum aestivum* varieties, with different field drought resistance, was studied under severe recoverable soil drought at seeding stage by withholding irrigation for 7 days followed by re-watering. LP level was not changed significantly in the leaves of drought treated plants, but it rose during recovery. These findings indicate increased oxidative strain on membranes in recovery from severe drought stress but rather strict control on ROS formation in the cells. The response of these varieties was similar to conditions of drought and re-watering [25].

The soluble protein content is another indicator of oxidative damage in a plant tissue. During growth, protein content in leaves of *A. onobrychis* changed significantly and could be related to $O_2^{\bullet -}$ quantities. The lowest protein content in leaves of examined plant was in the seed forming stage. Similar results are published for *Astragalus mollissimus*, in the seed stage of vegetation, the protein content in leaves was the lowest [26]. Different protein variations over seasons had been observed in *Picea omorika*. Environmental parameters, such as maximal and minimal temperatures, insolation, wind power and frequency, peak in summer and winter, and lower protein content was established [27].

The significant accumulation of $O_2^{\bullet -}$ occurred in *A. onobrychis* leaves, in the seed forming stage, should go along with changes in SOD activity, but there is no correlation of SOD activity and the $O_2^{\bullet -}$ quantities. It can be assumed that the non-enzymatic components of

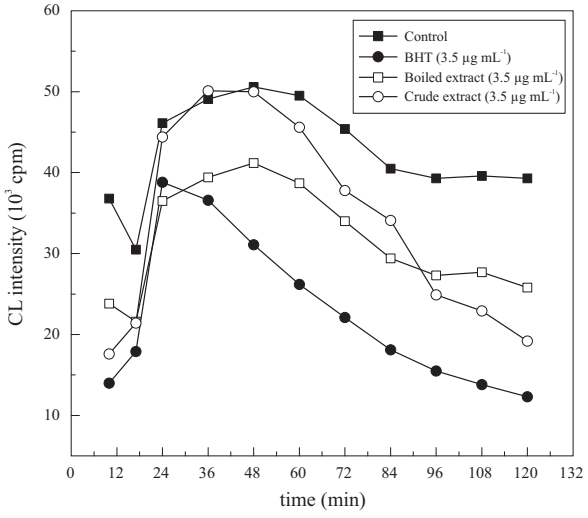

Figure 4. Inhibition of chemiluminescence by *A. onobrychis* leaf extract in lower doses.

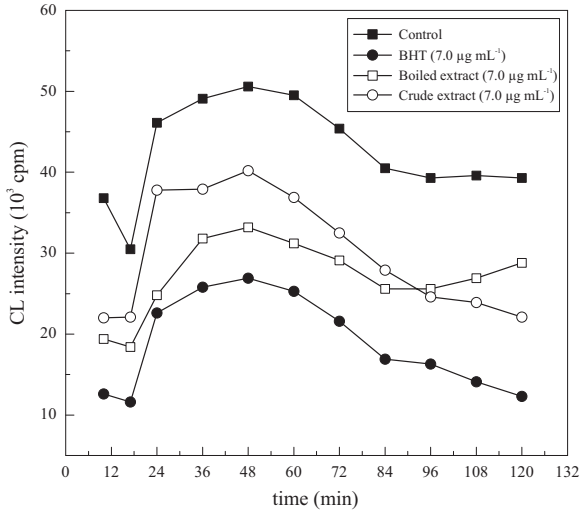

Figure 5. Inhibition of chemiluminescence by *A. onobrychis* leaf extract in higher doses.

antioxidant systems take over the role of $O_2^{\bullet -}$ scavengers. It is also possible that the amount of superoxide anion radicals was not large and that the activity of SOD was quite optimal for scavenging $O_2^{\bullet -}$ to H_2O_2. Öncel *et al*. [28] showed that both alpine and steppe plants may not need all the components of antioxidant protection. SOD activities in leaves of *Astragalus vulneria* and *Astragalus microcephalus* were 60 and 127 unit g^{-1} FW. Anderson *et al*. [29] in their study of seasonal variation in the antioxidant system in *Pinus strobus* needles, showed a minimal SOD activity in summer, and proposed that maximal activity in winter might be a response to the cold winter conditions. Salt stress treatment led to a decrease of SOD activity in the seeds of *Astragalus adsurgens*. The average values of SOD activities were 3.4 U mg^{-1} protein. The value of SOD activity of control was 5.0 U mg^{-1} protein [30]. SOD only exhibits maximum activity in the seed forming stage when determining the largest $O_2^{\bullet -}$ accumulation, which confirms its importance as an indispensable component of the functioning enzyme antioxidant system in wild plants.

The activity of SOD generates H_2O_2, while CAT and POD are the main enzymes responsible for H_2O_2 decomposition thus preventing •OH generation [3]. Statistical tests confirms this fact. Change in CAT activity during the examination vegetation period is positively correlated with protein content. Catalase, in joint action with guaiacol peroxidase, prevents •OH formation, whose amount is not significantly changed during the season. These two enzyme systems are very active and complement SOD activity. This assumption can be indirectly derived on the basis of the statistical test, namely POD and CAT activities were negatively correlated with $O_2^{\bullet -}$ quantities. In an investigation of the role of antioxidant defense system on stress tolerance of high mountain and steppe plants, no CAT activity was observed in steppe plants such as *Astragalus vulneria, Teucrium chamaedrys* and *Teucrium polium* [28]. In Kentucky bluegrass, the consistent and stable expressions of CAT activity may facilitate leaf cells in scavenging H_2O_2 in an efficient way. The combined action of CAT and SOD prevent the cellular damage under unfavourable conditions like water stress [31].

A relationship between protecting enzyme activities and osmoregulation among three genotypes of *Radix Astragali* under water deficient conditions [32] confirms that changes of SOD, CAT and POD activities are similar, which indicated that these three enzymes cooperated with each other. In the research of changes of antioxidant enzymes activity in *Astragalus membranaceus* under water stress, Kai *et al*. [33] found that POD activity initially increased slowly but rose sharply in the later stage of the stress under medium and severe drought. In the literature data, there are significant differences in POD activity in some plant species. The reason for that are several isoenzymes, which have separated physiological functions. For example, in *Triticum aestivum*, exposure to drought conditions, after rehydration, reveals three isoforms of peroxidase [25].

Glutathione peroxidases were initially shown to catalyze the glutathione dependent reduction of hydrogen peroxide and diverse alkylhydroperoxides to water or the corresponding alcohol *via* a thiol/disulfide exchange mechanism [34]. It has been found that plant GPOX protective role arises during environmental stresses and pathogen attack. However, plant GPOXs have lower activities than those of animals because they contain Cys at the putative catalytic site rather than selenocysteine, typical of animal GPOXs. This low

activity has made it difficult to clarify the physiological role of GPOX in higher plants [35]. GPOX activity recorded in *A. onobrychis* leaves extract was smaller, compared with the results of published papers [36]. Based on the results of our study, it can be assumed that GPOX from the *A. onobrychis* leaves was included in the maximum response to oxidative stress in the blooming vegetation stage, under conditions of high light intensity and the highest temperatures.

Methods of chemiluminescence are more sensitive among assays for antioxidant activity estimation because it detects the free radical trapping activity in a chain reaction that occurs both in the hydrophilic and the hydrophobic domains of biological membranes. The luminol, as a chemiluminegenic probe, is very suitable because its molecular weight is relatively small and can enter cells that then react with intracellular ROS. A kinetic profile of luminol-enhanced CL is dependent on the type of activator. Zymosan activates an oxidative burst of phagocytes by binding itself to complement and immunoglobulin receptors, which induces signal transduction that leads to the activation of protein kinase C and a consequent activation of NADPH-oxidase, the key enzyme of oxidative burst. The authors have chosen BHT as an antioxidant standard because the long lasting CL inhibition takes place, which is more suitable in experiments on phagocytes [37].

The *A. onobrychis* extracts in both concentrations were able to reduce emission of CL during the metabolic phase of phagocytosis in a dose-dependent manner. In our recent paper, CL inhibition by *O. pilosa* extracts was observed on 33% and 35% (mean value) of extracts. This can be explained by the fact that the content of flavonoids in leaves of *O. pilosa* is higher than in *A. onobrychis* [8,24]. A study by Jantan *et al*. [17] showed that most researched plant extracts did not show significant CL inhibitory effect in a similar model system, except for *Curcuma xanthorrhiza* and *Garcinia mangostana*. Compared to the common antioxidant BHT, the inhibition of CL by *A. onobrychis* extracts was lower, but compared to commercial vegetal extracts Isoflavin Beta and red clover, the extracts of *A. onobrychis* demonstrated higher level of CL inhibition [38]. Kawagoe and Nakagawa [39] have shown that, in *in vitro* conditions, quercetin is a better suppressor of CL intensity than the synthetic antioxidant BHT, in a dose-dependent manner. In the study of antioxidant activity determination of *Astragalus squarrosus* [40], it was concluded that synergistic effects of different compounds existing in the methanol extract of researched plant might be responsible for their activities against lipid peroxidation. However, the authors assume that the weak free radical scavenging activity, observed in the aerial part of the plant, might be related to the lack of polysaccharides whose concentration is highest at the root. Many studies have suggested that the presence or position of specific glycoside groups can increase or decrease the antioxidant activity of flavonoids [41].

As mentioned earlier, in order to estimate antioxidants of *A. onobrychis* by CL assay, two ways of extract preparation were used: crude leaf extract and boiled leaf extract (with denatured enzymes). The lower inhibition of CL intensity was observed in the crude leaf extract. The antioxidants present in crude leaf extract may not be implicated in the main pathway, which leads to a decrease of CL intensity. This is the case for some enzymatic antioxidants such as SOD and CAT, which were shown to be unable to affect chemiluminescence at higher concentrations. The opposite effects could involve direct stimulating action on the phagocyte cell, chemical pro-oxidant action, and anti-inhibitory action in different ways [42]. However, analyzing the results of enzymatic activity (Table 2) and results of CL inhibition of crude extracts (Table 3), it can be concluded that the enzymatic compounds in the leaves of *A. onobrychis* exhibit significant antioxidant capacity, which is 50% of CL inhibition activity of BHT. This activity protects plants under climatic conditions with highest light intensities and highest temperatures. Flavonoids, a major class of antioxidant compounds, encompass a substantial molecular weight range, which provides them with different water solubilities [8]. The cell nucleus is an aqueous domain; therefore, antioxidants which are to act there should be water soluble. Flavonoids have been shown to exhibit their antioxidant actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as ATPase and phospholipase [43]. In our study, based on the percentage and index of inhibition (Table 3), boiled extracts in which flavonoids are the most represented compounds, demonstrate a highest antioxidant activity. In addition to ecological and botanical aspects, the study of plant extracts can help in the design 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 [44].

4. Conclusions

The results presented suggest that the researched antioxidant enzymes SOD, CAT, POD and GPOX during vegetation significantly change the levels of activity in a specific way. During the spring season, POD

showed the highest level. CAT and GPOX increased their activities in summer. However, during the autumn season, SOD showed maximum activity. This suggests a complementary action of these enzymes in response to external changes. Examined antioxidant enzymes may be used as indicators of antioxidant ability of *A. onobrychis* to environmental changes. Based on CL activity, it can be concluded that the leaf extract of *A. onobrychis* possesses a significant antioxidant capacity thus protecting plants during environmental stress. Enzymatic components of *A. onobrychis* antioxidant system, during the vegetative period, work quite properly and the determined quantities of ROS are not able to damage leaf cell structures.

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