Content of antioxidants in extracts of mistletoe (*Viscum album* L.), yew (*Taxus baccata* L.), pine (*Pinus sylvestris* L.) and fir (*Abies alba* Mill.).

ANNA BARBASZ, BARBARA KRECZMER*, ELŻBIETA RUDOLPHI-SKÓRSKA, APOLONIA SIEPRAWSKA, DANUTA WOŹNICA

Institute of Biology
Pedagogical University
Podchorążych 2
30-084 Kraków, Poland

*corresponding author: e-mail: basiakreczmer@gmail.com

S u m m a r y

Imbalance between the intensity of oxidative processes (that induce the formation of reactive oxygen species) and counteracting antioxidant system is called oxidative stress. Most of the pathological changes in living organisms is associated with the processes of carcinogenesis induced by free radicals. State of equilibrium is maintained due to the presence of antioxidant enzymes (e.g. superoxide dismutase, peroxidase) and other biologically active substances such as glutathione, ascorbic acid and beta-carotene. These compounds enable the removal of reactive oxygen species in cells. The purpose of this study was to investigate the oxidative activity of mistletoe extracts and their potential hosts: fir and pine, and yew trees, which also have therapeutic properties. The results of performed analysis of enzymatic antioxidants (superoxide dismutase and peroxidase) lead to the conclusion that their activity in the tissues of mistletoe is much lower than in the tissues of fir, pine and yew. It was found, however, a much higher content of non-enzymatic antioxidants such as ascorbic acid, glutathione or beta-carotene in the tissues of mistletoe compared to other plants analyzed. Thus, extracts from mistletoe are a rich source of antioxidants easily assimilated to organisms receiving them.

Key words: *mistletoe, fir, pine, yew, oxidative stress, antioxidants*

INTRODUCTION

The disturbance of the balance between production and inactivation of free radicals leading to the occurrence of oxidative stress may be induced by environmental

factors such as pollution, extreme temperatures and shortages of water [1] but also by natural metabolic processes occurring in cells. Cancers, like many other human diseases are associated with the formation of reactive oxygen species (ROS) in the cells, which react with components of cells (nucleic acids, lipids, proteins) causing their serious damage [2]. To scavenge ROS and alleviate their deleterious organism in the course of the evolution effects have evolved various defense mechanisms involving enzymatic and non-enzimatic antioxidant systems [3]. Enzimatic antioxidants include superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.16), peroxidase (POD, EC 1.11.1.7) as well as non-enzimatic antioxidants including glutathione, ascorbic acid and carotenoids. Among ROS-scavenging enzymes, SOD rapidly catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2 and $O_2^{\cdot}$ H_2O_2 is scavenged by peroxidase and by the ascorbate-glutathione cycle. Glutathione (GSH) is a major antioxidant involved in the protection of cellular structures through the direct binding of free radicals, and by maintaining an appropriate redox status of cells. Glutathione is also involved in the regeneration of reduced forms of ascorbic acid and tocopherol in the cells. The reduction in the glutathione content results in simultaneous lowering of ascorbic acid level which indicates a close associatd of the effects of glutathione, ascorbic acid and tocopherol [4].

Therefore, natural sources of antioxidants which could be used for medical purposes are sought [5]. Extracts of mistletoe (*Viscum album*) are used for treatment of many diseases such as cancer, hypertension, and diabetes. They have also immunomodulatory [6] and antibacterial [7] properties. Therefore, it seemed interesting to examine the oxidative activity of extracts of mistletoe (*Viscum album*) as well as of the tissues of its potential hosts i.e. firs and pines. Due to similar therapeutic effects observed for extract from the needles of yew trees *(Taxus baccata* L.*),* this material was also tested for comparison.

MATERIALS AND METHODS

Leaves of white mistletoe (*Viscum album* L.), and the needles of fir (*Abies alba* Mill.), yew (*Taxus baccata* L. *Fastigiata*) and Scots pine (*Pinus sylvestris* L.) were used in the study. Mistletoe, fir and pine were collected in Czasław (Racichowice community near Kraków). Habitat was a mixed forest with a predominance of coniferous trees. Yew were collected within a private estate in Łapanów (Łapanów community near Kraków). Leaves of mistletoe were collected in spring (April), summer (August), autumn (October) and winter (December-January), each time from the same fir tree. Fir, yew and pine needles from spring growth were collected during the period April-May. Plant material was frozen with liquid nitrogen and kept at –80° C for biochemical analysis.

Activity of antioxidant enzymes

Approximately 0.1 g of tissue was homogenized at 4°C in a solution containing 0.1% bovine serum albumin (BSA). For removing low-molecular substances

homogenate was centrifuged for 10 minutes at 14 000 x g. The obtained supernatant was dialyzed for 8–12 hours in dialysis sacks (SIGMA) immersed in phosphate buffer (0.05 M, pH 7.0) at 4°C. After cleaning, the activity of superoxide dismutases, peroxidases, and the protein content was measured in the supernatant.

Superoxide dismutases (SOD)

SOD activity was determined spectrophotometrically using cytochrome [8] as a substance undergoing oxidation. The reaction mixture contained 0.1 mM EDTA, 1 mM cytochrome c, 1 mM xanthine, and phosphate buffer pH 7.8. The mixture was aerated by stirring on a magnetic stirrer for 2.5 hours. Then, 8–10 $\times$ 10^{-3} cm^3 oxidase (activity 50U, Grade I, Sigma) was introduced to the reaction mixture. Reaction kinetics was studied spectrophotometrically at 550 nm for 2 minutes. The amount of enzyme which causes 50% decrease of rate of cytochrome c reduction at 25°C was taken as an unit of enzyme activity. The enzyme activity was recalculated relatively to the amount of protein quantified with use of Bradford method (1976) [9].

Peroxidases (POX)

Peroxidase activity was quantified spectrophotometrically by a modified method of Lück (1962) [10] by measuring the amount of the products of oxidation of p-phenylenediamine (PPD) in the presence of H_2O_2. 0.02 cm^3 of PPD (1%) and 2 cm^3 of phosphate buffer (0.05 M) were added to 20 cm^3 of supernatant. Subsequently, 0.02 cm^3 of 0.03 M H_2O_2 was added to the reaction mixture for reaction initiation. The change in absorbance at $\lambda = 460$ nm just after H_2O_2 injection and after 2 min was read. The response time was selected in such a way that sample absorbance did not exceed 0.4 units. The POX activity was recalculated relatively to the total amount protein present in the sample.

The ascorbic acid content

0.1 g of leaves were homogenized in 0.5 cm^3 of deionized water. The homogenate was centrifuged at 14 000 x g for 10 minutes. The ascorbic acid content in the supernatant was determined with a refractometer RQflex 10 (Merck).

Determination of glutathione concentration

0.4 g of plant material was homogenized in 5 cm^3 of 0.1 M phosphate buffer pH 7.4 containing 10 mM EDTA. The homogenate was centrifuged at 15 000 x g

for 15 min. In order to remove the protein from the samples, 0.5 cm^3 10% TCA and 0.5 cm^3 of 10 mM EDTA was successively added to the supernatant. After 10 min. of incubation at 4°C. the mixture was centrifuged at 6 000 x g for 5 min. Then, solution containing 2.3 cm^3 of distilled water, 0.1 cm^3 of 10 mM EDTA and 0.3 cm^3 3,2 M Tris-HCl buffer pH 8.1 was mixed with 0.2 cm^3 of supernatant. Subsequently, 0.1 cm^3 of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) of 2.6 mM in 0.05 mM acetate buffer, pH 5.0 was added. After 10 min. the extinction at 412 nm was read relatively to the blank sample.

Determination of ferric reducing power

The ferric reducing power of studied extracts was determined by measuring the ability of samples to reduce Fe^{+3} ions as described by Oyaizu (11). Briefly, appropriate volumes of studied samples (0–1.0 cm^3) were mixed with 2.5 cm^3 of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 cm^3 of 1% potassium ferricyanide. The mixtures were incubated at 50ºC for 20 min. Next, 2.5 cm^3 of 10% trichloroacetic acid was added and, subsequently, centrifuged at 650 x g for 10 min. 5 cm^3 of the resulting supernatant was mixed with equal volume of water and 1 cm^3 of 0.1% ferric chloride. The absorbance of obtained solution was read at 700 nm against blank. Higher absorbance of the reaction mixture indicated greater reducing power.

Determination of β-carotene

Quantification of carotenoids was performed by high performance liquid chromatography (HPLC). The system consisted of Agilent 1100 HPLC analyzer with DAD detector. Carotenoids were identified on the basis of retention parameters of the relevant standards and UV-VIS spectra. The wavelength used in analysis was 450 nm. Separation of samples was performed on Waters Spherisorb ODS2 Column, 3 μm, 4.6 x 150 mm, at a flow rate of 1 ml/min and applied phase gradients: acetonitrile/water (250/33 v/v) (A) and ethyl acetate (B): 80% A (at 0 min.) to 40% A (at 6.5 min.), then 20% A (from 8.5 to 12 min.).

RESULTS AND DISCUSSION

Oxidative properties of plant extracts were tested by determining the content of essential cellular antioxidants, that is enzymes SOD, POX and low molecular weight antioxidants: ascorbic acid, glutathione, carotenoids and ferric reducing power. Tested compounds, by mutual interaction catalyze a series of coupled reactions leading to the inactivation of ROS in cells [12]. Cellular antioxidants assure a consistent mechanism scavenging free radicals. The lower activity of some steps of this mechanism is compensated by a greater mobilization of other [13].

The conducted experiments revealed that the activity of enzymes studied is much lower in extracts from mistletoe than in the tissues of its potential hosts and yew. In the case of superoxide dismutases (SOD) their activity was nearly eleven times higher in the tissues of fir and pine trees compared to the mistletoe. High activity of these enzymes was also observed in tissues of yew (seven times higher) (fig. 1).

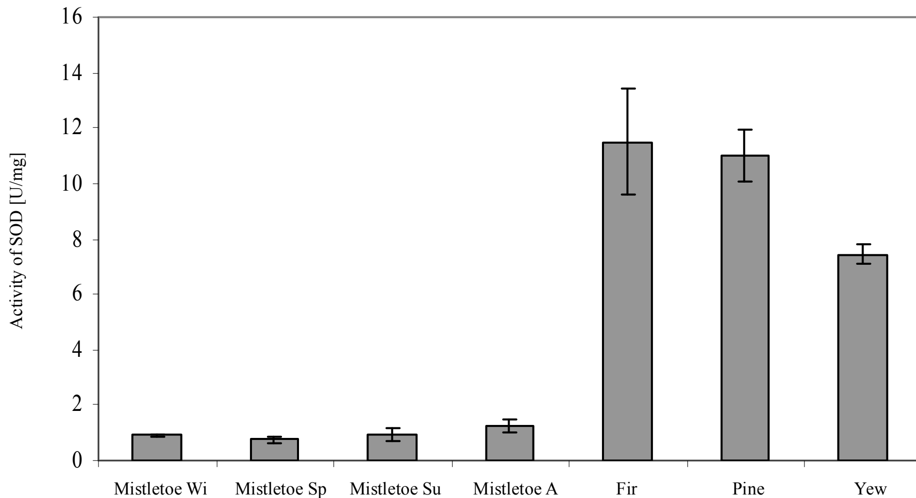

Figure 1.
Superoxide dismutase (SOD) activity in plant preparations derived from mistletoe, fir, pine and yew. The data represent mean values ± SE, n=5. Wi – Winter, Sp – Spring, Su – Summer, A – Autumn.

As in the case of SOD, a lower peroxidase activity was found in mistletoe extracts when compared to the tissues of fir and pine (fig. 2). It is noticeable that the activity of POX in the fir tree is twice as high as that in the pine tree, while the activity of SOD in both species was within the error limits the same. The time of mistletoe harvesting had no significant effect on the activities of studied enzymes. The relatively low activity of antioxidant enzymes in mistletoe extracts may be compensated by higher concentrations of ascorbic acid (fig. 3) and glutathione (fig. 4). Mistletoe extracts from plants harvested at different seasons indicated almost the same levels of ascorbic acid. Similarly to mistletoe extracts, high content of ascorbic acid was also found in extracts of fir and yew trees, while the lowest content exhibited the pine tissues (fig. 3). Another results were found for the glutathione concentration. The study showed the impact of harvest time on the content of this antioxidant in the mistletoe. The highest level of glutathione was found in mistletoe tissues collected in winter, the least in these from summer harvesting (fig. 4). It is noticeable that the fir tree, which tissues exhibit a high activity of antioxidant enzymes and high levels of ascorbic acid has the lowest level of glutathione which can confirm the compensatory activity of these compounds in the antioxidative protection system of cells. Ferric reducing power measures the ability of the extracts to donate

electron to Fe (III) (fig. 5). The higher the ferric reducing power value, the greater the antioxidant activity. In terms of antioxidant activities of mistletoe extracts, have the highest ferric reducing power values, suggesting that mistletoe is of very high primary antioxidant activity compared with the others analyzed plants.

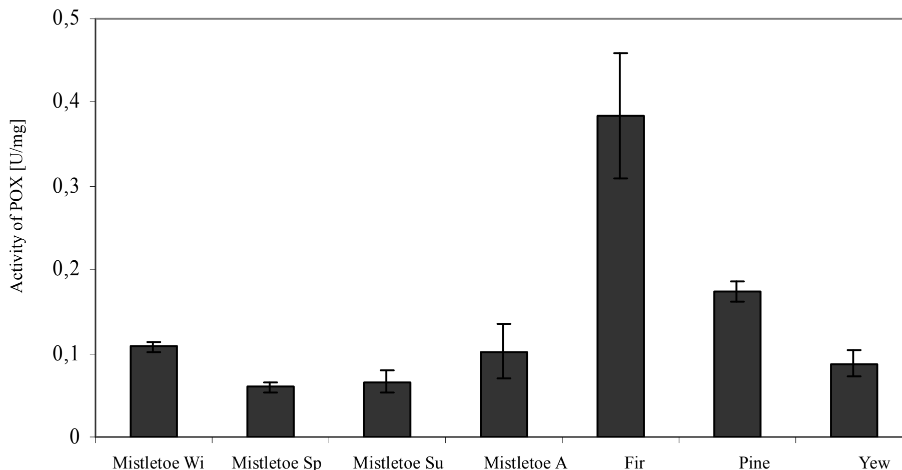

Figure 2.

Peroxidase (POX) activity in plant preparations derived from mistletoe, fir, pine and yew. The data represent mean values ± SE, n=5. Wi – Winter, Sp – Spring, Su – Summer, A – autumn.

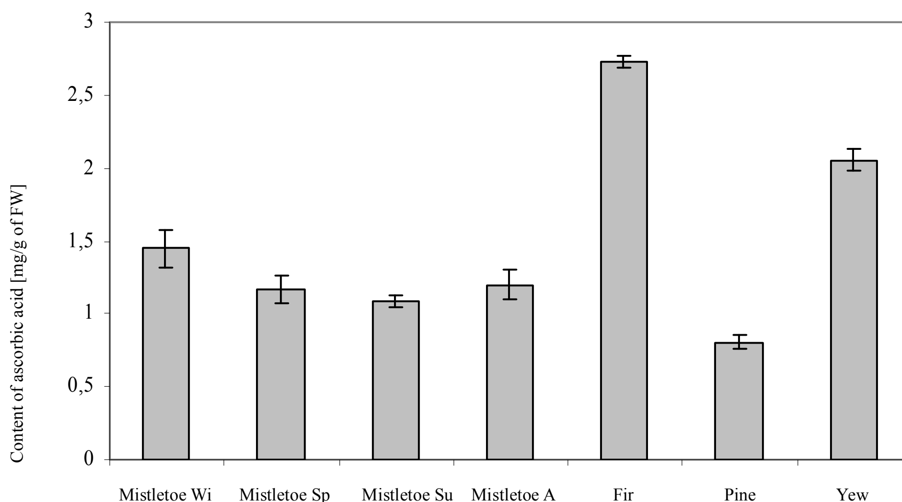

Figure 3.
The ascorbic acid content in aqueous extracts of mistletoe, fir, pine and yew. The data represent mean values ± SE, n=5.
Wi – Winter, Sp – spring, Su – summer, A – autumn.

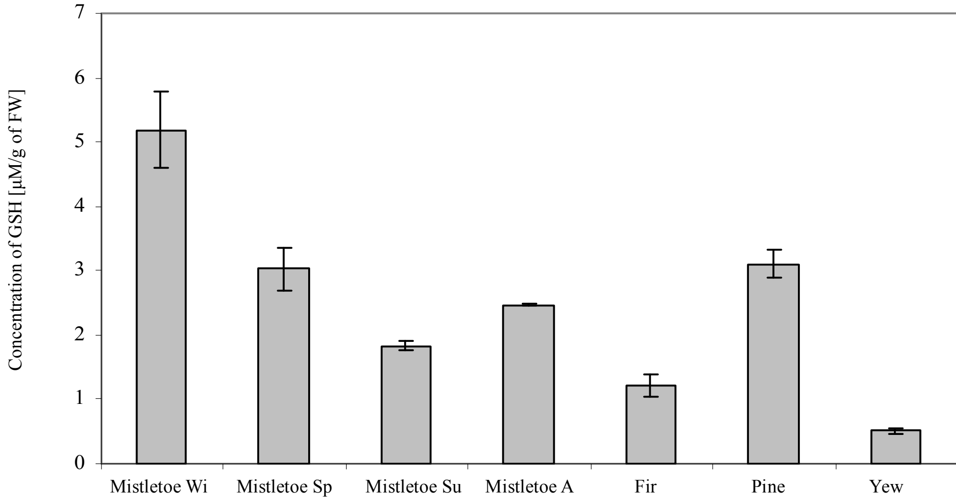

Figure 4.
The content of reduced glutathione (GSH) in aqueous extracts from the mistletoe, fir, pine and yew. The data represent mean values ± SE, n=5.
Wi – winter, Sp – spring, Su – summer, A – autumn.

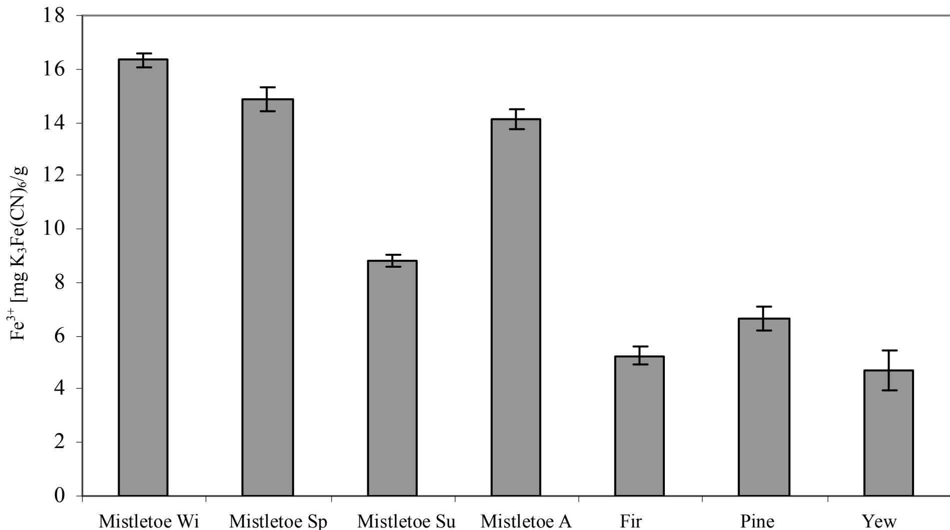

Figure 5.
The level of reduced iron ion ($K_3Fe(CN)_6$) in aqueous extracts of mistletoe, fir, pine and yew. The data represent mean values ± SE, n=5.
Wi – winter, Sp – spring, Su – summer, A – autumn

Carotenoids, vegetable dyes present in the chloroplasts and chromophores, play an auxiliary role in the process of photosynthesis. In addition, they also have a protective function against photo oxidation processes. Carotenoids present in the tissues of studied plants may also act as scavengers of free radicals. Effectively inactivate singlet oxygen, and also react with organic free radicals produced during the process of lipid peroxidation [14]. The highest level of β-carotene was found in the extract of mistletoe collected in summer (fig. 6). Due to favorable conditions for photosynthesis, in summer increase production of chloroplasts, and thus carotenoids increases. Fir and yew also exhibit high levels of this antioxidant in their tissues.

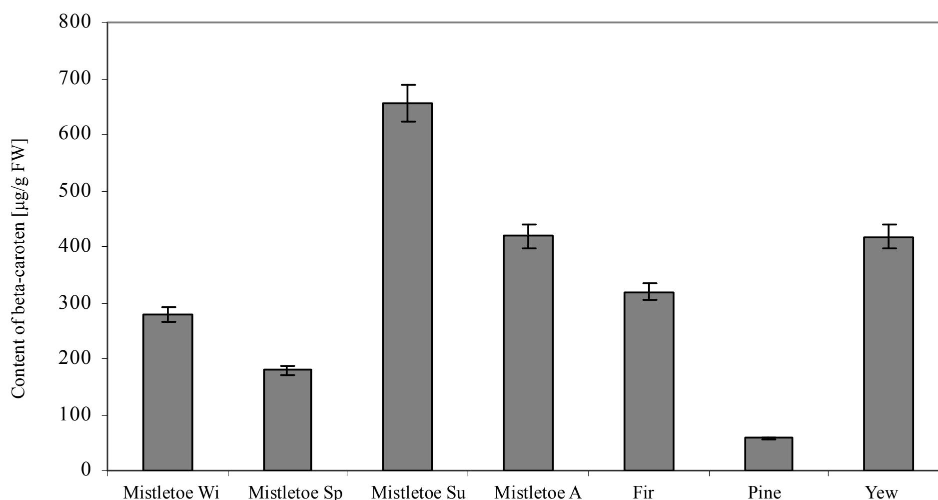

Figure 6.
The content of β-carotene in the tissues of mistletoe, fir, pine and yew. The data represent mean values ± SE, n=5.
Wi – Winter, Sp – spring, Su – summer, A – autumn

Both data from literature and those presented in this paper reveal that mistletoe extracts have high antioxidant activity. The antioxidant properties of studied extracts are mainly due to the content of non-enzymatic antioxidants: glutathione, ascorbic acid and carotenoids. The highest concentration of these compounds was observed in tissues of mistletoe harvested during the winter. This suggests that the extracts obtained from plants in this season should have the greatest therapeutic effect. The analysis of the tissues of hosts showed that, in contrast to mistletoe extracts, antioxidant enzymes level is higher than the level of non-enzymatic antioxidants.

CONCLUSIONS

The results obtained allow to conclude that the mistletoe tissues are extremely rich source of non-enzymatic antioxidants.

- The content of enzymatic antioxidants (SOD and POX) in tissues of potential hosts for mistletoe is much higher than in the tissues of mistletoe.
- High levels of ascorbic acid in the mistletoe tissues does not depend on the harvesting season.
- The highest concentration of reduced glutathione was found in the tissues of winter mistletoe.
- Carotenoids, protecting plants against photooxidation, reach the highest concentration in the tissues of summer mistletoe.

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ZAWARTOŚĆ ANTYOKSYDANTÓW W WYCIĄGACH Z JEMIOŁY (*VISCUM ALBUM* L.), JODŁY (*TAXUS BACCATA* L.), SOSNY (*PINUS SYLVESTRIS* L.) I CISU (*ABIES ALBA* MILL.)

ANNA BARBASZ, BARBARA KRECZMER*, ELŻBIETA RUDOLPHI-SKÓRSKA, APOLONIA SIEPRAWSKA, DANUTA WOŹNICA

Zakład Biochemii, Biofizyki i Biotechnologii
Instytut Biologii
Uniwersytet Pedagogiczny im. KEN
ul. Podchorążych 2, 30-084 Kraków

*autor, do którego należy kierować korespondencję: e-mail: basiakreczmer@gmail.com

S t r e s z c z e n i e

Zaburzenie równowagi między natężeniem procesów oksydacyjnych, które indukują powstawanie reaktywnych form tlenu a przeciwdziałającym mu systemem antyoksydacyjnym określa się mianem stresu oksydacyjnego. Większość zmian patologicznych w organizmach związana jest z procesami kancerogenezy indukowanymi działaniem wolnych rodników. Stan równowagi utrzymywany jest dzięki obecności enzymów antyoksydacyjnych (np. dysmutazy ponadtlenkowej, peroksydazy) oraz innych substancji biologicznie czynnych, takich jak np. glutation, kwas askorbinowy czy β-karoten. Związki te umożliwiają usuwanie nadmiaru reaktywnych form tlenu z komórek.
Celem pracy było zbadanie aktywności oksydacyjnej ekstraktów jemioły oraz jej potencjalnych żywicieli, jodły i sosny oraz cisu, który również ma właściwości terapeutyczne. Przeprowadzone analizy zawartości enzymatycznych antyoksydantów (dysmutazy ponadtlenkowej i peroksydazy) pozwalają stwierdzić, iż ich aktywność w tkankach jemioły jest znacznie niższa niż w tkankach jodły, sosny i cisu. Stwierdzono jednak znacznie wyższą zawartość antyoksydantów nieenzymatycznych, tj. kwasu askorbinowego, glutationu czy β-karotenu w tkankach jemioły w stosunku do pozostałych analizowanych roślin. Ekstrakty z jemioły są więc bogatym źródłem antyoksydantów łatwo przyswajalnych dla pobierających je organizmów.

Słowa kluczowe: *jemioła, jodła, sosna, cis, stres oksydacyjny, antyoksydanty*