# Biologically active substances in plant extracts from mistletoe *Viscum album* and trees: fir *(Abies alba* Mill.*)*, pine **(***Pinus sylvestris L.***)** and yew (*Taxus baccata* L.)

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S u m m a r y

Taking into account the growing number of reports which confirm the medicinal effects of preparations of plant origin, the paper analyzed the biochemical composition of extracts obtained from mistletoe parasitizing on fir and extracts from fir, pine and yew trees was analyzed. The mistletoe samples were collected in winter, spring, summer and autumn. Mistletoe's extract is a source of viscotoxines – substances used in a treatment of heart diseases. Yew is also used as a plant which tissues contain substances of high therapeutic value. Extracts obtained from tissues of this plant are used for cancer treatment. The content of protein, soluble sugars and proline were determined in all samples. The biological activity of mistletoe may be connected with high concentrations of almost all investigated metabolites. The level of these compounds were higher than that of extracts from trees. An increased level of proline and malondialdehyde in the tissues of spring mistletoe is probably related to summary effect of environmental stresses in this season (temperature, insolation intensity and duration). Among polyamines, the highest concentration of spermidine in extracts from mistletoe and of spermine in extracts from trees may be a sign of specific metabolism pathway in investigated plants. Among extracts from trees tissues, fir was the richest in studied bioactive substances. The less amounts of all analyzed substances were found in tissues of pine.

**Key words:** *mistletoe, fir, pine, yew, protein, proline content*

## INTRODUCTION

Despite significant progress in medicine, some illnesses represent a major cause of death of people in the world, particularly in developed countries. Every year the cancer death rate increases by 2–3% worldwide [1]. Heart disease, especially hypertension is also the cause of an increasing number of deaths. The challenge for modern science is the search for new substances of pharmacological activity. In search of effective therapeutic agents greater attention is focused on plant extracts. The basis of their action is the inhibition of proliferation of cancer cells, blocking the receptors for their adhesion, as well as mobilization of the immune system. In many countries the cultivation of medicinal plants and food supplements is one of the branches of agriculture.

The evidence of the effectiveness of medicinal plants in cancer treatment is fact that about 25% of cytostatics are of plant origin [2, 3]. Pharmaceuticals developed on the basis of plant extracts include, inter alia, Taxol, vincristine and vinblastine. Many medications used in prevention of the development of heart disease are based on extracts from plants e.g.: Iscador and Helixor (4). Diterpene pseudoalkaloid – Taxol is produced mainly from the bark of western yew *Taxus brevifolia*. Its effectiveness has been demonstrated against such cancers: ovarian, breast, bowel, lung, and also as an adjunctive medicine in the treatments of Alzheimer's, cardiovascular diseases and as an anti-virus agent. The mechanism of Taxol action on cancer cells consists in preventing the processes of cell division, leading to acceleration of their apoptosis [5, 6].

Viscumins and viscotoxins were found in mistletoe (*Viscum album*) - the plant parasitizing on various species of trees[7]. In addition to these substances, the extract of mistletoe contains about 40 biologically active components. All these chemicals occur in pharmaceuticals such as Iscador and Helixor [8-10].

Medicinal properties of preparations obtained from mistletoe depend on the host species (tree): for example, Iscador P – from plants parasitizing on the pine, Helixor –extracted from mistletoe parasitizing on fir [10]. These relations between healing attributes of extracts from mistletoe and its host tree tempt to compare the amount of bioactive compounds in both plants. The aim of the work was to determine the contents of metabolites such as proteins, sugars, amino acids (proline), polyamines, and the ions, which are necessary for the establishment of the osmotic conditions of cells. All these compounds are essential for the course of the key biochemical processes. Analysis were performed for samples of tissue of mistletoe collected in different growing seasons (spring, summer, autumn, winter) and of tissues of trees (fir and pine), which parasitizes mistletoe.

All these extracts contain biologically active substances, used for prophylactic therapy such as cancer. Obtained in this work results will be used in the experiments carried out on the white mice of Swiss line to compare the effect of the tested plant extracts and pharmaceutical products derived from them on the physiological parameters of animal cells

## MATERIAL AND METHODS

Leaves of white mistletoe (*Viscum album L.*), and the needles of fir (*Abies alba Mill.*), yew (*Taxus baccata L. Fastigiata*) as well as Scots pine (*Pinus sylvestris L.*) were used in the study. Mistletoe, fir and pine were collected in Czasław (Racichowice community near Kraków). The 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) from the same fir tree each time. Needles of fir, yew and pine coming from the spring growth were collected in April-May. Plant material was frozen with liquid nitrogen and kept at – 80° C for biochemical analysis.

### Determination of protein contents

Proteins were determined according to Bradford [11], using bovine serum albumin as a standard. The 0.01cm$^3$ of plant extract (1 g×cm$^{-3}$) was added to 0.2 cm$^3$ Coomassie dye-based (Bradford) solution in 96-well plate and incubated 15 minutes. The dye exihibits a maximum of absorption spectrum at 595 nm, and sample absorbance was detected using Tecan Sunrise microplate reader.

### Determination of soluble sugars

The content of carbohydrates was determined in 80% ethanol extracts of leaf tissues according to the method described by Keles and Öncel [12] A sample of tissue (0.1 g) was shaken in 10 ml 80% (v/v) ethanol. Fraction of soluble sugars was centrifuged at 5 000 x g for 10 min. Glucose was analyses by reacting (0.5 cm$^3$ of extract) with 2.5 cm$^3$ freshly prepared anthrone (400 mg anthrone + 154 cm$^3$ $H_2SO_4$ + 48 cm$^3$ $H_2O$) and placed in a boiling water bath for 5 min. After cooling the absorbance at 625 nm was determined with spectrophotometer.

### Measurement of proline content

0.4 g of plant material was homogenized in 10 cm$^3$ of 3% aqueous sulfosalicylic acid. The homogenate was filtered through Whatman 2 filter paper. Two ml of filtrate was mixed with 2 cm$^3$ ninhydrine and 2 cm$^3$ of glacial acetic acid in a test tube for 1 hour at 100°C. The reaction was stopped by inserting the sample into ice bath. 4 cm$^3$ of toluene added to the reaction mixture was mixed vigorously for 15–20 s with a test tube stirrer. After warming to room temperature sample

absorbance at 520 nm was read using toluene as a blank sample. Proline concentration was determined from the calibration curve and counted on the weight of fresh sample.

## Malondialdehyde (MDA) concentration determination

The degree of lipid peroxidation in plant tissues was determined as 2-thiobarbituric acid (TBA) reactive metabolites, mainly malondialdehyde (MDA) [13]. Plant samples were extracted in 0.25% TBA made in 10% trichloroacetic acid. The mixture was heated at 95°C for 30 min and, after cooling, centrifuged at 10 000 x g. The absorbance of supernatant was measured at 532 nm and the non-specific absorbance at 600 nm was subtracted. The MDA content was calculated using the extinction coefficient of 155 $mM^{-1}/cm^{-1}$.

## Determination of lipids

Lipids (sum of phospholipids, galactolipids and neutral lipids, mainly triacylglycerols) were extracted according to the modified method of Bligh and Dyer (1959) (14): at first boiling isopropanol, then chloroform:methanol mixture (1:1, v/v) containing an antioxidant – butylhydroxytoluol were added to the sample. Thereafter, the mixture was re-extracted with chloroform. Chloroform extract was evaporated under nitrogen and the mass of lipids was determined.

## Polyamines determination using HPLC

About 0.2 g of fresh tissue was mixed with 2 $cm^3$ of 5% (w/v) of cold perchloric acid and centrifuged at 14 000 x g for 15 minutes. The supernatants were analyzed for polyamines content. The polyamines were derivatized according to the methods of Flores and Galston (1982) (15). 0.4 $cm^3$ aliquots of the supernatant was added to 0.4 $cm^3$ saturated sodium carbonate and 0,8 $cm^3$ of dansyl chloride in acetone (5 $mg \times cm^{-3}$). The mixture was incubated at room temperature for 12 h in the dark. 0,4 $cm^3$ of proline was added to the mixture to remove the excess dansyl chloride. After 0,5 h, the polyamines were extracted with 2 $cm^3$ of toluene by vortexing for 30 s. The mixture separated into two phases, aqueous and organic. The organic phase, containing the polyamines, was dried under nitrogen. The polyamines residue was dissolved in 0.3 $cm^3$ of methanol and ultrafiltered through nylon membranes (0,2 $\mu$m pore).

A high-pressure liquid chromatography (HPLC) analysis was performed using system consisting of UV-detector (L-7400 LaChrom HPLC System MERCK) and linked to a data system (D-7000 HPLC System Manager) used for data acquisition. The chromatographic signal of 330 nm was detected. LiChrospher 100 RP-18

(5$\mu$m) (Merck) column was used for the separation of the amine derivatives. A water-methanol mixture (40:60 v/v) was used as the eluent in a gradient elution mode at a flow rate of 2 ml/min. The gradient used was 60% of methanol at zero time to 90% after 15 min. All reagents were of HPLC purity. Standard solution of polyamine of concentration 0,1 $mg \times cm^{-3}$ was prepared by dissolving the pure compound in water. All solutions were stored in the dark at 4ºC.

## Determination of $K^+$, $Cl^-$, $Ca^{2+}$ ions and pH

1.5 g of fresh plant tissue was homogenized in 15 $cm^3$ of water. The samples were centrifuged at 10 000 x g for 15 min. Ion contents in water extract were determined using ion-selective electrodes (Sentek).

## RESULTS AND DISCUSSION

The results obtained show that, IN studied plants the highest concentrations of main metabolites: protein, proline (tab. 1), lipids (fig. 2) and ions (potassium and chloride) (tab. 2) were found in the mistletoe tissues. The content of sugars was also largest in mistletoe but only in samples collected in summer and autumn. The highest concentration of soluble sugars observed in extracts from summer and autumn samples correlates with periods of increased photosynthesis in plants. The protein content in mistletoe did not change significantly in different seasons. Proline amount in plant tissues is usually closely linked to their potential ability to response to stress factors. It was observed that under stress conditions accumulation of proline in the tissues grows much faster than other amino acids [16]. The high level of proline observed in tissues of spring mistletoe may be correlated with summary effect of increased temperature, insolation intensity and duration, acting as stress factors which in turn stimulate synthesis of this substance. Increased MDA content found in spring mistletoe indicates the reaction of plant onto stress condition. (fig.1). Higher level of malondialdehyde (MDA) point to the lipid peroxidation by reactive oxygen species produced at larger quantities under stress conditions [17]. This may justify the high biological activity of extracts from this plant. sugars (in plants collected in summer and autumn).

Lipid content increases during mistletoe development from winter to autumn (fig. 2). It seems that the greater amount of lipids detected in summer or autumn mistletoe may be linked to changes in cell metabolism toward the accumulation of these substances for the winter. An increased level of sugars, observed in these mistletoe vegetation periods confirms this assumption.

Potassium and chloride ions are considered the most important inorganic substances involved in osmotic regulation of plant cells and tissues [18]. Potassium is involved in basic metabolic processes, such as activation of enzymes, protein

synthesis, photosynthesis, transport of organic substances (including assimilates). Along with chloride ions play an important role in maintaining cation-anion balance in the cells [19, 20]. Relatively high levels of potassium, found in extracts of mistletoe as compared to other plants (tab. 2), may be related to the influence of this ion on protein synthesis. As it was shown. mistletoe contains relatively large quantities of proteins in relation to other studied plants. High levels of both ions (potassium and chloride) and proline may correspond with maintaining the osmotic balance in changing environmental conditions.

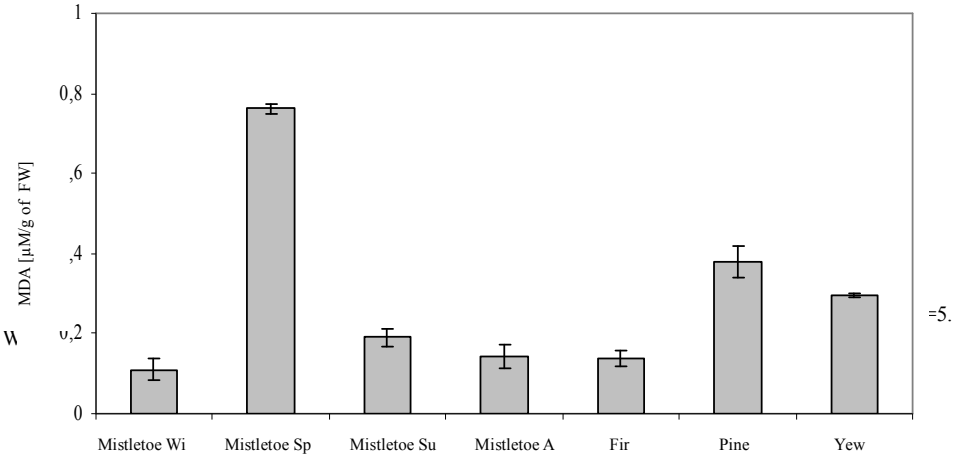


Figure 1.
Contents of malondialdehyde (MDA) in mistletoe, fir, pine and yew. The data represent mean values ±SE, n=5.
Wi – Winter, Sp – spring, Su – summer, A – autumn

**Table 1.**

Contents of total proteins, soluble sugars and proline in aqueous extracts obtained from mistletoe, fir, pine and yew. The data represent mean values ± SE, n = 5.

| Sample | Total proteins [mg/g of fresh weight] | Carbohydrates [mg/g of fresh weight] | Proline [$\mu$M/g of fresh weight] |
|---|---|---|---|
| Mistletoe winter | 15.213±0.831 | 1.435±0.270 | 0.869±0.222 |
| Mistletoe spring | 16.308±0.653 | 1.259±0.317 | 1.381±0.109 |
| Mistletoe summer | 15.378±0.737 | 2.436±0.010 | 0.632±0.149 |
| Mistletoe autumn | 16.220±0.730 | 2.588±0.076 | 0.940±0.236 |
| Fir | 2.399±0.420 | 1.795±0.196 | 0.089±0.018 |
| Pine | 1.050±0.364 | 0.424±0.005 | 0.026±0.001 |
| Yew | 1.154±0.910 | 1.199±0.209 | 0.515±0.071 |

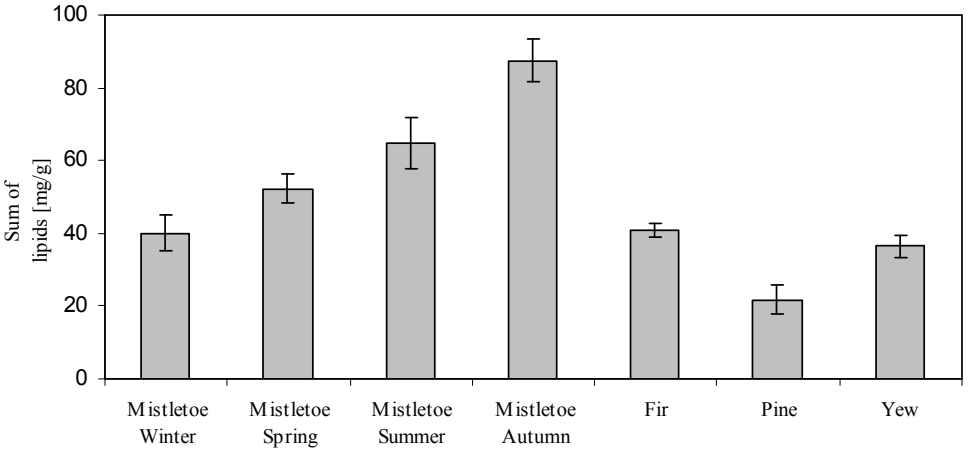


Figure 2.
Content of lipids in extracts from mistletoe, fir, pine and yew. The data represent mean values ± SE, n=5.

**Table. 2.**

The levels of potassium, chloride and calcium ions of the water extracts obtained from mistletoe, fir, pine and yew. The data represent mean values ±SE, n=5

| Sample | $k^+$ [mM/g of fresh weight] | $Cl^-$[mM/g of fresh weight] | $Ca^{2+}$ [mM/g of fresh weight] |
|---|---|---|---|
| Mistletoe winter | 13.734±1.044 | 5.955±0.899 | 0.142±0.024 |
| Mistletoe spring | 11.919±0.965 | 5.397±1.009 | 0.020±0.005 |
| Mistletoe summer | 9.363±1.003 | 2.487±0.225 | 0.107±0.059 |
| Mistletoe autumn | 13.051±2.008 | 4.666±0.873 | 0.160±0.034 |
| Fir | 4.264±0.405 | 1.089±0.105 | 0.233±0.092 |
| Pine | 3.113±0.239 | 0.449±0.065 | 0.032±0.008 |
| Yew | 4.063±0.821 | 1.057±0.099 | 0.023±0.002 |

Calcium ions are also involved in the ensuring of homeostasis of cells. These ions are considered to be secondary signals` transmitters and they are regarded as a universal response to environmental stimuli [21, 22]. A significant decrease in calcium ion content in the spring mistletoe (tab. 2) may be associated with the involvement of this ion in the defense processes occurring in the cells under stress.

Polyamines are low molecular weight organic compounds, biogenic, formed in all cells of pro-and eukaryotic organisms. Changes in the level of these substances are related to both to developmental processes and stresses [23, 24]. Putrescine,

spermine and spermidine were found in extracts obtained from tissues of studied plants (fig. 3). The largest amount of polyamines found in the spring mistletoe correlates with an increased proline content. This may prove that production of larger amount of polyamines is related both to stressful conditions and enhanced development of the plants occurring in spring. It was shown that, of the three analyzed polyamines, the level of spermidine was the highest. This means that the end product of polyamines biosynthesis in mistletoe was spermidine.

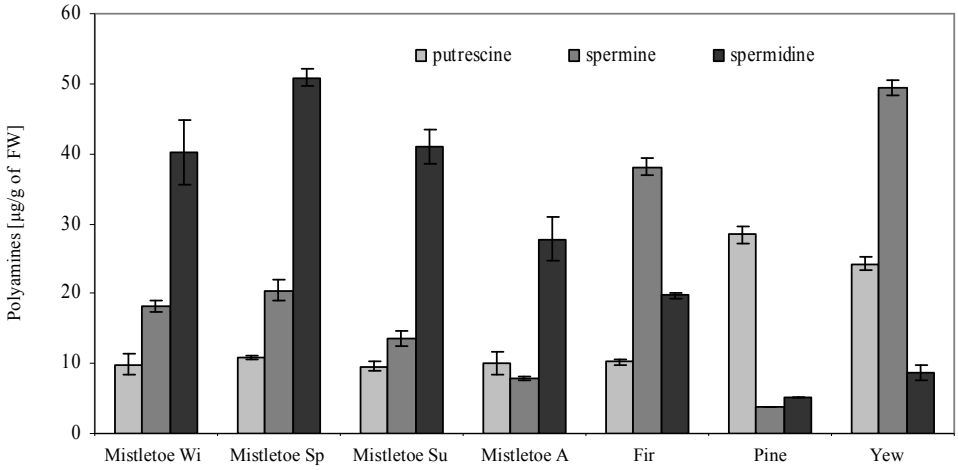


Figure 3.
The level of polyamines (putrescine, 1,6-diaminoheksanu, spermidine and spermine) in plant tissues. The data represent mean values ± SE, n=5. Wi – winter, Sp – spring, Su – summer, A – autumn

Among the host trees of mistletoe, fir tissues contain higher amounts of all tested substances as compared to the tissues of pine. Thus, mistletoe parasitizing on fir can gather higher amounts of these biologically active substances. Extracts from mistletoe parasitizing on fir are a more valuable source of a variety of bio-components. Their presence may enhance the increase of the bioavailability of previously acquired pharmaceutical preparations of mistletoe origin (Iscador or Helixor).

Use of extracts from fir, pine and yew trees, being a mixture of biologically important metabolites, can also be useful in the medical prophylaxis. Comparing the two trees, extracts from fir are a richer source of bioactive substances than the these from pine. Larger amount of spermine than spermidine found in the tissues of trees, as compared to mistletoe, may indicate the specificity of polyamine metabolism in woody plants. The levels of all studied substances in the cells of the yew were intermediate as compared to other studied trees (with exception of significantly lower concentration of calcium ions).

The comparison of the content of biologically active species of pine and fir is an important part benchmarking the output to mistletoe tissue. Described in the work concerning fir, mistletoe clearly demonstrates the effect of host on the content of bioactive substances in mistletoe tissues. The studies represent a starting point for further research into the composition of mistletoe parasitic on the pine.

The analysis of biologically active substances in aqueous extracts of mistletoe and yew was designed to check whether the plants of a similar therapeutic effect are characterized by a similar biochemical composition. According to Ji Ram and Kumari (2001) [25] pharmaceuticals based on extracts from yew (Taxol) have a therapeutic effect similar to those produced on the basis of extracts of mistletoe (Iscador). Thus, valuable material for production of preparations rich in bioactive substances can be obtained from controlled cultivation of mistletoe and of fir and yew trees.

## CONCLUSIONS

The presented results allow to conclude that the period of plant vegetation has an impact on the biochemical composition of mistletoe tissues as well as on changes in these parameters in the tissues of its host.

- The mistletoe tissues exhibit significantly higher protein levels than the other samples.
- Sugar levels are quite similar in analyzed plant samples.
- The level of proline in the tissues of mistletoe is associated with the accessibility of water.
- The increase in malondialdehyde content increases with exposure of mistletoe to strong sunlight, which results in increased oxidative stress.
- The concentration of potassium ions in the tissues of mistletoe is much higher as compared to the other tested samples. This is closely related to the observed pH values, which, in fir, pine and yew is lower by two pH units as compared to the tissues of mistletoe.
- Calcium ion concentration is significantly lower in the spring, which correlates with the exposure of plants to oxidative stress, as confirmed by the increase of MDA in the tissues.
- The content of polyamines in plant tissues is closely associated with the other parameters analyzed, and the possibility of water stress occurrence.

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# SKŁADNIKI BIOLOGICZNIE AKTYWNE W WYCIĄGACH Z JEMIOŁY JODŁY *(ABIES ALBA* MILL.*)*, SOSNY (*PINUS SYLVESTRIS* L.) I CISU (*TAXUS BACCATA* L.)

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

Biorąc pod uwagę rosnącą liczbę doniesień potwierdzających lecznicze działanie preparatów roślinnych, w niniejszej pracy analizie poddano parametry biochemiczne ekstraktów pozyskiwanych z jemioły (ze zbiorów w okresie zimowym, wiosennym, letnim i jesiennym), jodły, sosny i cisu. Ekstrakt jemiołowy jest bogatym źródłem składników bioaktywnych, takich jak m.in. wiskuminy, wiskotoskyny, aminy biogenne, związki terpenowe, związki fenolowe, alkaloidy czy flawonoidy. Do badań wykorzystano także potencjalnych żywicieli jemioły tj. jodłę i sosnę, oraz cis jako rośliny, których ekstrakty mają dużą wartość terapeutyczną. Dokonano analizy zawartości białka, cukrów rozpuszczalnych oraz proliny. O wysokiej aktywności biologicznej jemioły, może świadczyć fakt, iż w jej tkankach zaobserwowano prawie ośmiokrotnie wyższe stężenie białka, w porównaniu do tkanek jej żywicieli. Poziom proliny w tkankach jemioły jest ściśle związany z dostępnością do wody. Zbadano także stężenie sumarycznych lipidów oraz produktów peroksydacji lipidów (których miarą było stężenie aldehydu dimalonowgo). Obserwowany wzrost zawartości aldehydu dimalonylowego skorelowany jest ze wzrostem nasłonecznienia w okresie wiosennym, co skutkuje zwiększonym stresem oksydacyjnym. Analizie poddano także zawartość bioaktywnych poliamin oraz jonów potasu, chloru i wapnia w tkankach badanych roślin. Na podstawie uzyskanych wyników pomiarów biochemicznych stwierdzono, iż cykl roczny związany z okresem wegetacyjnym rośliny ma wpływ na skład biochemiczny tkanek jemioły i jest powiązany ze zmianami tych parametrów w tkankach jej żywicieli.

***Słowa kluczowe:*** *jemioła, jodła, sosna, cis, białko, cukier, zawartość proliny*