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Research Article

Investigation of leafy shoots and tea products of European mistletoe (*Viscum album* L.) with special focus on their antioxidant capacity

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Antioxidants are compounds that inhibit combustion (oxidation) processes. Antioxidants are vital components of our body, which can be obtained in part through plant nutrition. Therefore, it is very important to study species that have significantly higher antioxidant capacity than other species. The aim of the study was to investigate the antioxidant capacity of total polyphenols (TPC) of European mistletoe (*Viscum album* L.) leafy shoots collected from different species of trees (black locust, European ash, white poplar, field maple and black walnut) based on different methods; DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (ferric reducing antioxidant power) and TPC (total phenolic contents). The results proved that the antioxidant effect of leafy shoots from European ash (*Fraxinus excelsior*) against hydroxyl radicals (ROS) showed significantly higher values than those of the other four tree species. We found that the DPPH, FRAP and TPC methods show significant differences in antioxidant effect of European white mistletoe leafy shoots on the studied tree species and tea brands. However, the FRAP method shows higher sensitivity for trees but for tea brands, the DPPH method is more sensitive. The reason for the difference might be explained by the different methods of drying. In the future, we consider it feasible to plant ash groves at an altitude of at least 80 m above sea level in a closed area, where we can start growing European white mistletoe as an herb. Based on the results obtained European white mistletoe can be recommended as an herb to natural medicine for supplementary treatment of several cancer diseases.

Keywords: European mistletoe (*Viscum album* L.), leafy shoot, antioxidant capacity, polyphenols

1 Introduction

In some European countries, aqueous plant extracts from the leaves and stems of white mistletoe have been used as an immunomodulator to treat cancer diseases for more than 60 years (Ziska & Franz, 1985). However, the active ingredient responsible for this was only detected in mistletoe 10 years ago (Vicas, Rugina & Socaciu, 2012). It is a galactoside-specific sugar-binding protein (lectin) which applied as a plant extract caused a loss of immunomodulatory effect in *in vivo* experiments (Sharon, 1984; Hajtó, 2002). As a result, research on European white mistletoe over the past 10 years has focused mainly on the lectins (*Viscum album agglutinin*) (Dietrich et al., 1992). The white mistletoe (*Viscum album*) lectin (VAA-I) consists of two chains (Hajtó et al., 2005). The 29-kd 'A-chain' due to N-glycosidase activity is a potent ribosome inactivator while the sugar-binding 'B-chain' with a molecular weight of 34 kd is responsible for the immunomodulatory effect of the lectin molecule (Hajtó, 2002). Lectin of *Viscum album*, often used in anthroposophic medicine, reduces tumor growth and metastasis, while significantly increasing quality of life (Karnofsky index) and even survival. Its area of application depends on the tree species (Steiner, 1994) (Table 1). A few studies have been published on the effectiveness of other bioactive polyphenol substances of leafy shoots against oxidants so far, and the earliest determined the total polyphenol capacity of European mistletoe by Ochocka and Piotrowski (2002) and Vicas, Rugina & Socaciu (2012) (Table 2).

In biological systems, oxidation processes produce free radicals (ROS, namely: reactive oxygen species). Free radicals have one or more unpaired electrons and are therefore highly reactive and thus short-lived particles that can cause damages in the human body. Antioxidants are most often understood as natural or

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Table 1 Application of lectin of *Viscum album* in anthroposophist medicine

Tree species	Area of application
Scotch pine (*Pinus sylvestris*)	Lymphpoma
Norway maple (*Acer platanoides*)	hepatocellular carcinoma, pancreas carcinoma
English oak (*Quercus robur*)	gastrointestinal carcinoma, cholecysta tumor, male reproductive system tumor
European ash (*Fraxinus excelsior*)	for intensification of lectin
White birch (Betula pendula)	skin cancer
Russian elm (*Ulmus laevis*)	respiratory system tumor
Apple (*Malus domestica*)	mamma carcinoma, female reproductive system tumor

Source: Steiner, 1994

Table 2 Bioactive components of leaf shoots of European white mistletoe

Bioactive components	
Pentacyclic triterpene	betulinic acid
Phenolic acids	gallic acid
	protocatechuic acid
	gentisic acid
	chlorogenic acid
	para-OH bensoic acid
	cafeic acid
	syiringic acid
	salicylic acid
	para-coumaric acid
	ferulic acid
	sinapic acid
	trans-cinnamic acid
Flavonoids	naringenin
	quercetin
	kampherol
Polyphenol	rosmarinic acid

Source: Ochocka & Piotrowski, 2002; Vicas, Rugina & Socaciu, 2012

artificial compounds found in living organisms or in food, which have the function of inhibiting or delaying chemical oxidation processes (Gillich & Krüzselyi, 2014; Lantos, 2015). Another point to discuss is whether the massive European white mistletoe is a valuable herb or a parasite that also endangers orchards.

The European white mistletoe (*Viscum album* L.) is an evergreen, flowering parasitic plant that can be a hemiparasite of various tree species (Szepessy, 1977). According to the botanical division, three species are distinguished: American (*Phorandendron serotinum*), Korean (*Viscum album* L. *coloratum*), and European gene-centered (*Viscum album* L.) mistletoe (Barney, Hawksworth & Geils, 1998). The European white mistletoe (*Viscum album* L.) is a hemiparasite that belongs to the family of Sandalwood (Santalaceae), the genus Viscum (Fig. 1), which grows on the stems of other trees. It has stems of 300–1,000 mm long with dichotomous branching. The leaves are in opposite pairs, strap-shaped, entire, leathery-textured, 20–80mm long, 8–25 mm broad and are yellowish-green in colour. This species is dioecious, and the insect-pollinated flowers are inconspicuous, yellowish-green, 2–3 mm. The fruit is a white or yellow berry containing one (very rarely several) seed embedded in the very sticky, glutinous fruit pulp (Zuber, 2004). In spreading of its seeds in Europe the thrushes (*Turdus visciorus*, *Turdus pilaris*, *Turdus iliacus*) and blackcaps (*Sylvia atricapilla*) have prominent role. In Hungary, it occurs beyond 80 m altitude above sea level in the western areas of the Carpathian Basin (Varga, 2013). In Scandinavia, mistletoe is grown on trees (fir, maple, almond, birch, hawthorn, ash, apple, pine, and oak) on hundreds of hectares of land for the production of certain homeopathic products. For medicine/medicament preparation the mistletoe is to be taken from different trees because their composition and effect also depend on the host plant (Rácz, 2004).

Development of mistletoe is indirectly climate-dependent. The weather of the period before the beginning of the vegetation period has a detectable effect on the host plant, including the development of mistletoe (Makra et al., 2016).

The objective of the study is (1) to investigate and compare two methodologies measuring antioxidant capacities of five tree species, as well as (2) to analyze differently treated, processed, and marketed mistletoe leaf shoots as different branded five teas. In addition, polyphenol contents of (1) and (2) are compared and evaluated.

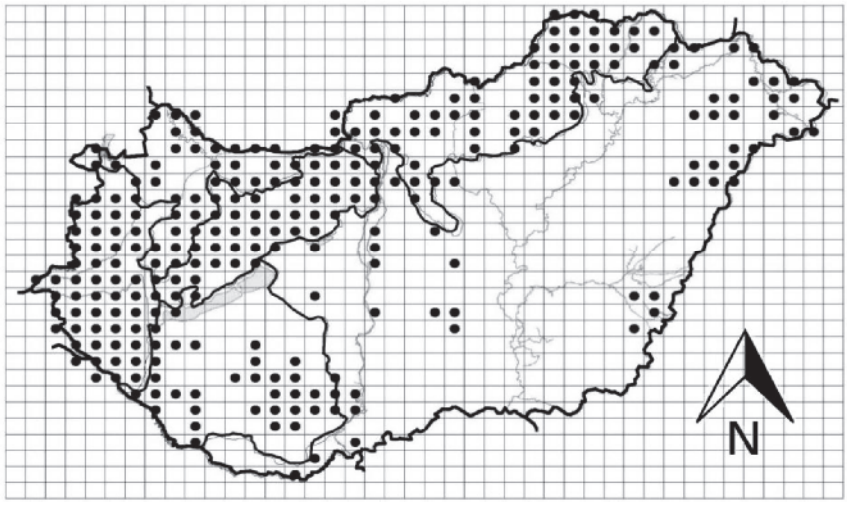

Figure 1 The spread of white mistletoe in Hungary in the mid-1990s in the basic fields of floristics
Source: Bartha & Mátyás, 1995

2 Material and methods

We selected leaf shoots of five tree species, namely: white poplar, European ash, black walnut, black locust, and field maple. In addition, mistletoe leaf shoots as five different branded teas (Herbária, György tea, Adamo, Fitodry and Herbs) were also chosen.

Leaf shoots for the study were collected in October of 2019–2020 from five different altitudes in hilly and lower areas (Table 3). A sample of 70 g was taken from each tree. The wet leafy shoots were dried for 24 hours at 55 °C in a Memmert UN55 furnace and stored for another 24 hours in a dark, dehumidified place. The tea leafy shoots were dried at room temperature 22–24 °C, longer time (4 months), at maximum 60 % air humidity. Three methods were used to determine the antioxidant capacity of mistletoe samples. They are as follows: DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (ferric reducing antioxidant power) and TPC (total phenolic contents). Samples were tested in 6–6 replicates for all three methods. It should be noted that 50% of vitamin C is broken down in two minutes at 82.5 °C and in half a minute at 90 °C. This process accelerates with increasing temperature and/or time. Decomposition of a half of vitamin C at 50 °C happens approximately in an hour (Svirbelf & Szent-Gyorgyi, 1932).

2.1 Methods of determination of antioxidant capacity

The methods used for measuring antioxidant capacity can be divided into two groups: 1) Determinations based on hydrogen atom transfer (HAT) and 2) Tests based on electron transition (ET – Electron Transfer).

Table 3 Tree species and their occurrence parameters in Hungary

Tree species	Place of origin	Altitude (a. s. l.)*
White poplar (*Populus alba*)	Zalacsány	>240 m
European ash (*Fraxinus excelsior*)	Keszthely	>240 m
Black locust (*Robinia pseudoacacia*)	Bátmonostor	80–85 m
Field maple (*Acer campestre*)	Nagybaracska	90–95 m
Black walnut (*Juglans nigra*)	Dunafalva	90–95 m

*a. s. l. – above sea level

For our research we used two tests based on hydrogen atom transfer [2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP)]. The total polyphenolic content (TPC) method was used to detect all polyphenols.

2.1.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) method

The DPPH radical-scavenging activity was determined using the method proposed by Blois (1958). It is a rapid, simple and inexpensive method to measure antioxidant capacity of plant extracts involving the use of the free radical. DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of extracts. The reaction involves a colour change from violet to yellow that can be easily monitored using a spectrophotometer at 515 nm. The reaction was performed in 12 well-plates. A volume of 200 µl sample and 1.4 ml DPPH solution (80 µM) were added to each microplate well. The decrease in the absorbance of the resulting solution was monitored at 515 nm for 30 min. The percentage of scavenging effect of different extracts against DPPH radicals, was calculated using the following equation:

$$DPPH\ scavenging\ effect\ (\%) = \\ = [(A_0 - A_s) \times 100]/A_0 \qquad (1)$$

where: A_0 – absorbance of the blank; A_s – absorbance of the samples at 515 nm

2.1.2 FRAP (ferric reducing antioxidant power) method

The assay was determined according to the method of Benzie and Strain (1996) with some modifications. The FRAP assay consists of ferric tripyridyltriazine (Fe(III)-TPTZ)

complex reduction to ferrous tripyridyltriazine (Fe(II)-TPTZ) by an antioxidant at low pH. The FRAP assay was used to determine both hydrophilic and lipophilic antioxidant activities. The stock solutions included: 300 mM acetate buffer; 250 mg $Fe_2(SO_4)_3$ H_2O dissolved in 50 ml distilled water; 150 mg TPTZ and 150 µl HCl, dissolved in 50 ml distilled water. The working FRAP solution was freshly prepared by mixing 50 ml acetate buffer, 5 ml $Fe_2(SO_4)_3$ H_2O solution and 5 ml TPTZ solution.

2.1.3 TPC (total phenolic contents) method

Total polyphenolic content of each extract was determined using the Folin-Ciocalteu reagent according to the method of Singleton and Rossi (1965). A volume of 0.5 mL of Folin-Ciocalteu reagent previously diluted with distilled water (1 : 10) was mixed with 0.1 mL of each extract (methanolic or enzymatic). The solution was kept for 5 min at 25 °C before adding 1.7 mL of sodium carbonate solution (20%). Then, 10 mL of distilled water were added to the mixture, and the absorbance was measured at $\lambda = 765$ nm after 20 min of incubation with agitation at room temperature (Huang, Ou & Prior, 2005).

Taking into account the specific "hydrophilic" and "lipophilic" nature of the European white mistletoe components – phenolic derivatives (phenolic acids and flavonoids) and carotenoids – we measured the "lipophilic" and "hydrophilic" antioxidant capacity in a comparative way. The nature of the components was described by Vicas, Rugina & Socaciu (2012) (Table 2).

2.2 Data analyses

One-way analysis of variance (ANOVA) was used to determine whether the means of the different series of measurements characterizing the antioxidant capacity of white mistletoe leaf shoots against hydroxyl radicals from the studied tree species differed significantly from each other. An *F*-test was performed to check whether the average values of the series of measurements of the antioxidant capacity of European white mistletoe from the studied tree species using different methods differ significantly from each other. If the difference is significant, we reject our 0-hypothesis for the similarity of the means (Tukey, 1953). It is then and only then that the Tukey test can be used to determine specifically, which tree species have a significant difference in the antioxidant capacities of European white mistletoe leaf shoots in the pairwise comparisons. The values obtained were then compared in order to determine whether these differences exceed a critical level, i.e., whether they are significant. If the difference between the pairwise averages of the tree species exceeds the threshold, then the actual difference is said to be significant. When comparing the pairwise group averages of the parameters of leafy shoots according to the studied tree species with the Tukey test, not only the individual effect (the effect of the current two tree species) but also the common effect (the effect of the other tree species) is taken into account in the differences of the pairwise group means. When performing the Tukey test, we first determine the differences between the means of all possible group pairs and then compare them with the following statistics:

$$HSD = q\sqrt{\left(\frac{(MSw)}{n}\right)} \qquad (2)$$

where: q – the studentized value set statistics with the appropriate degree of freedom. Its value can be found in the Table (2). The value of *MSw* – the average square deviation within the group, known from the ANOVA procedure, while n – the number of sample elements within the group (Matyasovszky et al., 2011, 2015; Makra et al., 2013; Makra et al., 2016)

3 Results and discussion

We studied and compared antioxidant capacity of European white mistletoe leaf shoots in pairwise comparisons.

First, antioxidant capacity of European white mistletoe leaf shoots was studied in pairwise comparisons of the studied tree species using the DPPH method (Table 4). We found that the antioxidant capacity of European white mistletoe leaf shoots of white poplar and European ash, as well as that of European ash and black walnut, European ash and black locust, and that of the European white mistletoe leaf shoots from European ash and field maple differed significantly (Table 4). The underlined trees show their significantly higher antioxidant capacity in the pairwise comparisons. Performing the same test using the FRAP method (Table 5), we obtained that antioxidant capacity of European white mistletoe leaf shoots of white poplar and European ash, white poplar and black walnut, white poplar and black locust, furthermore white poplar and field maple, as well as that of European ash and black walnut, European ash and black locust, in addition European ash and field maple show significant difference. Among the here-mentioned tree species the underlined ones show significantly higher antioxidant capacity in the pairwise comparisons (Table 5). Finally, using the TPC method for the same study (Table 6), we found that polyphenol concentration of white poplar and European ash, white poplar and black locust, as well as that of European ash and black walnut, Euroepan ash and black locust, furthermore that of European ash

Table 4 Comparison of the antioxidant capacity of European white mistletoe leaf shoots on the studied tree species using the DPPH method based on ANOVA and Tukey test

Legends	Tree species	Place of origin	Altitude (a. s. l.)*	A	B	C	D
A	white poplar (*Populus alba*)	Zalacsány	>240 m				
B	European ash (*Fraxinus excelsior*)	Keszthely	>240 m	X			
C	black locust (*Robinia pseudoacacia*)	Bátmonostor	80–85 m		X		
D	field maple (*Acer campestre*)	Nagybaracska	90–95 m		X		
E	black walnut (*Juglans nigra*)	Dunafalva	90–95 m		X		

X – significant at p <0.01 probability level
*a. s. l. – above sea level

Table 5 Comparison of the antioxidant capacity of European white mistletoe leaf shoots on the studied tree species using the FRAP method based on ANOVA and Tukey test

Legends	Tree species	Place of origin	Altitude (a. s. l.)*	A	B	C	D
A	white poplar (*Populus alba*)	Zalacsány	>240 m				
B	European ash (*Fraxinus excelsior*)	Keszthely	>240 m	X			
C	black locust (*Robinia pseudoacacia*)	Bátmonostor	80–85 m	X	X		
D	field maple (*Acer campestre*)	Nagybaracska	90–95 m	X	X		
E	black walnut (*Juglans nigra*)	Dunafalva	90–95 m	X	X		

X – significant at p <0.01 probability level
*a. s. l. – above sea level

Table 6 Comparison of polyphenol concentration of European white mistletoe leaf shoots on the studied tree species using the TPC method based on ANOVA and Tukey test

Legends	Tree species	Place of origin	Altitude (a. s. l.)*	A	B	C	D
A	white poplar (*Populus alba*)	Zalacsány	>240 m				
B	European ash (*Fraxinus excelsior*)	Keszthely	>240 m	X			
C	black locust (*Robinia pseudoacacia*)	Bátmonostor	80–85 m		X		
D	field maple (*Acer campestre*)	Nagybaracska	90–95 m	X	X		
E	black walnut (*Juglans nigra*)	Dunafalva	90–95 m		X		

X – significant at p <0.05 probability level; X – significant at p<0.01 probability level
*a. s. l. – above sea level

and field maple, in addition that of black locust and field maple differ significantly with substantially higher values of the underlined tree species (Table 6).

Based on our results, using the DPPH and FRAP methods, and all pairwise comparisons, antioxidant capacity of European white mistletoe leaf shots picked from European ash was significantly higher than that of the other tree species. This is consistent with the results of Vicas, Rugina & Socaciu (2012), who found the antioxidant effect attributed to the total phenolic acid content of European ash – derived samples outstanding.

In the following, we compared the antioxidant capacity of European white mistletoe leaf shoots treated differently, processed and marketed as different branded teas on the studied tea varieties using the DPPH method based on ANOVA and Tukey test (Table 7). The examined branded teas, which are available in Hungary, are as follows: Herbária, György tea, Adamo, Fitodry and Herbs. In the pairwise comparisons, the underlined brands show significantly higher antioxidant capacity. Using the DPPH method, the antioxidant capacity of differently treated white mistletoe leaf shoots of tea brands Herbária and György tea, György tea and Adamo, György tea and Fitodry, György tea and Herbs, as well as Adamo and Fitodry, in addition Adamo and Herbs show significant difference. Among the here-mentioned tea brands the underlined ones show significantly higher antioxidant capacity in the pairwise comparisons (Table 7). When using the FRAP method (Table 8), the antioxidant capacity of European white mistletoe leaf shoots treated differently show significant difference in the following pairwise comparisons (with significantly higher antioxidant values for the underlined tea brands): Herbária and György tea,

György tea and Fitodry, György tea and Herbs, in addition Adamo and Fitodry (Table 8). Polyphenol concentration of the differently treated European white mistletoe leaf shoots of the here-mentioned tea brands were also compared (TPC method) (Table 9). We received the following significant differences of the tea brands in the pairwise comparisons, with significantly higher total phenolic contents of the underlined brands: Herbária and György tea, György tea and Adamo, György tea and Fitodry, furthermore György tea and Herbs (Table 9).

When comparing the antioxidant capacity of European white mistletoe leafy shoots on the studied tree species by using the DPPH method (Table 4) and the FRAP method (Table 5), the latter one seems more sensitive in the sense that the FRAP method shows more significant differences (7 vs. 4 significant differences) in the antioxidant capacity of European white mistletoe leaf shoots on the studied tree species in the pairwise comparisons. On the other hand, comparison of the antioxidant capacity of differently treated European white mistletoe leafy shoots marketed as different branded teas, the DPPH method shows higher sensitivity (6 vs. 4 significant differences in the pairwise comparisons). Note that for the studied tree species (tea brands) 4(4) significant differences were common (i.e. occurred for the same pairwise comparisons), while the remaining 3(2) significant differences occurred in other pairwise comparisons (Table 5; Table 6).

Table 7 Comparison of the antioxidant capacity of European white mistletoe leaf shoots treated differently, processed and marketed as different branded teas on the studied tea varieties using the DPPH method based on ANOVA and Tukey test

Legends	Tree species	Place of origin	Altitude (a. s. l.)*	A	B	C	D
A	Herbária	Zalacsány	>240 m				
B	György tea	Keszthely	>240 m	X			
C	Adamo	Bátmonostor	80–85 m		X		
D	Fitodry	Nagybaracska	90–95 m		X	X	
E	Herbs	Dunafalva	90–95 m		X	X	

X – significant at p <0.01 probability level
*a. s. l. – above sea level

Table 8 Comparison of the antioxidant capacity of European white mistletoe leaf shoots treated differently, processed and marketed as different branded teas on the studied tea varieties using the FRAP method based on ANOVA and Tukey test

Legends	Tree species	Place of origin	Altitude (a. s. l.)*	A	B	C	D
A	Herbária	Zalacsány	>240 m				
B	György tea	Keszthely	>240 m	X			
C	Adamo	Bátmonostor	80–85 m				
D	Fitodry	Nagybaracska	90–95 m		X	X	
E	Herbs	Dunafalva	90–95 m		X		

X – significant at p <0.01 probability level
*a. s. l. – above sea level

Table 9 Comparison of polyphenol concentration of European white mistletoe leaf shoots treated differently, processed and marketed as different branded teas on the studied tea varieties based on ANOVA and Tukey test

Legends	Tree species	Place of origin	Altitude (a. s. l.)*	A	B	C	D
A	Herbária	Zalacsány	>240 m				
B	György tea	Keszthely	>240 m	X			
C	Adamo	Bátmonostor	80–85 m		X		
D	Fitodry	Nagybaracska	90–95 m		X		
E	Herbs	Dunafalva	90–95 m		X		

X – significant at p <0.01 probability level
*a. s. l. – above sea level

This is because the DPPH is one of the earliest methods for measuring antioxidant capacity based on stable radical scavenging (Blois, 1958). However, the disadvantage of this method is that it does not use a radical generated during normal metabolism in the cell but a stable radical that is not found in the living organism (Frankel & Meyer, 2000). The FRAP is a moderner iron-reducing method in which the iron-2,4,6-tripyridyl-S-triazine (TPTZ) complex is reduced by antioxidants (AH) but carotenoids do not have iron reducing ability. In the case of fresh leafy shoots samples, the chlorophyll A and B content, – which belongs to the carotenoids – is even less active than in teas due to the drying on higher than 55 °C temperature. So, the antioxidant capacity depends on the drying methods.

4 Conclusions

In our days, the deactivation of harmful substances with oxidant effect is an increasing challenge. In our opinion, also in academic and natural medicine the emphasis must be placed on the using of the herbs. A number of white mistletoe scientific researches proved that some of its compounds have strong antioxidant effect. Our many-year research also proved that the antioxidant capacity depends on the species of trees and the height above sea level of the habitat. The results proved that the antioxidant effect of leafy shoots from European ash (*Fraxinus excelsior*) against hydroxyl radicals (ROS) showed significantly higher values than those of the other four tree species. Based on the results obtained European white mistletoe can be recommended as an herb to natural medicine for supplementary treatment of several cancer diseases.

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