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Antioxidant Activity of European Mistletoe (*Viscum album*)

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1. Introduction

European mistletoe (*Viscum album L.*) is an evergreen, hemi-parasitic plant, normally found growing on a variety of trees, especially pine, poplar, apple trees, locus trees etc. Although there are many varieties of mistletoe, including the American (*Phorandendron serotinum* or *Phorandendron flavescens*), the European (*Viscum album L.*), and the Korean (*Viscum album L. coloratum*), most investigative work has been done on European mistletoe.

Traditionally, the genus *Viscum*, has been placed in its own family *Viscaceae*, but recent genetic research by APG II (The Angiosperm Phylogeny Group, 2003) system shows this family to be correctly placed within family Santalaceae (Fig. 1).

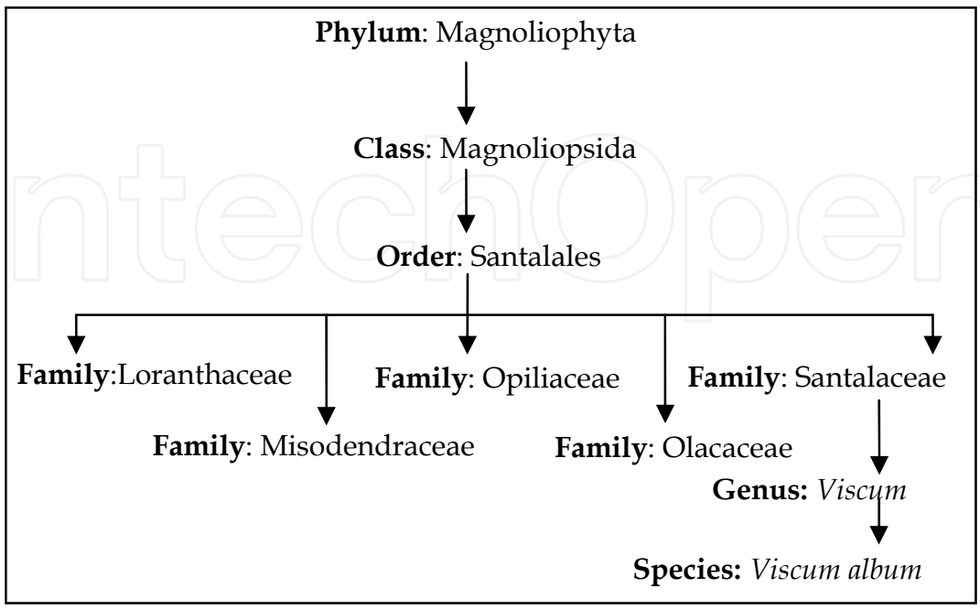


Fig. 1. Scientific classification of species *Viscum album* (according to APG II system).

Different species of *Viscum* are capable of parasitizing a large number of host species. From a review of literature, Barney et al., 1998, identified 452 host species of *Viscum album* (96 genera and 44 families). For *V. album* ssp. *album* 190 hosts have been identified, for *V. album* ssp. *abieties* 10 hosts, and for *V. album* ssp. *austriacum* 16 hosts are known (Zuber, 2004).

The genus *Viscum* are woody hemi-parasitic shrubs with branches 15–80 cm long, which grow on different host trees. Mistletoe tend to form a globular shape (Fig. 2, a) which may reach over 1 m in diameter. The foliage is dichotomously or verticillately branching, with opposite pairs of green leaves (Fig 2, c and d) which perform some photosynthesis (minimal in some species, notably *V. nudum*), but with the plant drawing its mineral and water needs from the host tree. The leaves are narrowly obovate (Fig. 2, b), approximatelly three or four times as long than broad. The color of leaves are dark green, and present three distinct veins and two less distinct veins running parallel to the leaf margin (Fig. 2, b). In young leaves, the veins are visible on both sides, while in older leaves they are mainly seen on the underside (Büssing, 2000). *V. album* is dioecious, i.e. part of the plants is female, the other part is male. Flowers are highly reduced and inconspicuous, greenish-yellow color, and 1–3 mm diameter (Fig. 2, c). The fruit is a berry, white, when mature, containing one seed embedded in very sticky juice (Fig. 2, e).

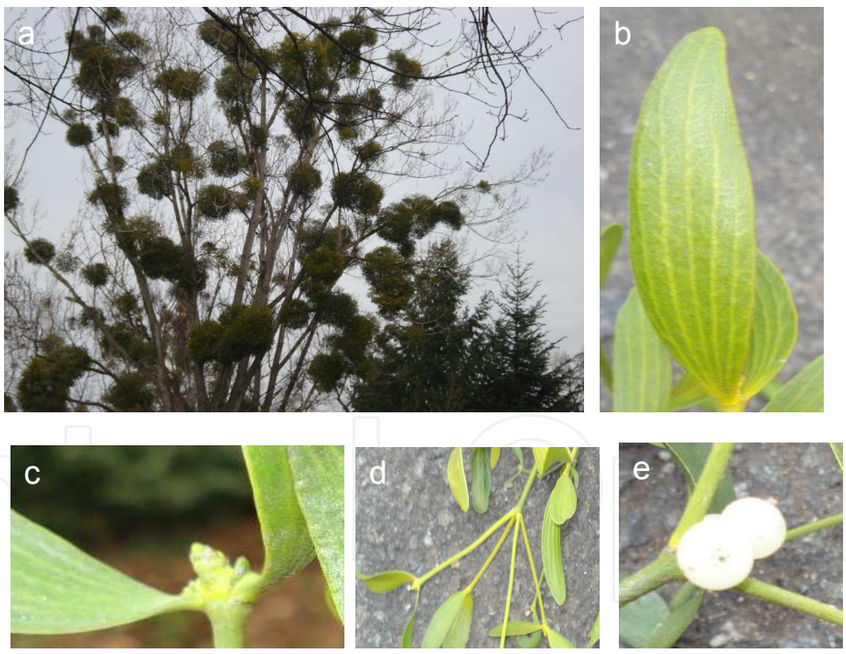


Fig. 2. *Viscum album* morphology. a. Image of *Viscum album* growing on poplar (*Populus nigra*); b. Aspect of leave; c. The flower of mistletoe; d. Stem branched; e. Adult berry containing one seed (Vicas et al., 2009a).

In recent years, antioxidants derived from natural resources, mainly from plants, have been intensively used to prevent oxidative damages. Natural antioxidants have also some advantages over synthetic ones, being obtained easily and economically and have slight or negligible side effects.

Aqueous extracts of the European mistletoe have been widely used for decades as alternative treatment and adjuvant cancer therapy, particularly in Germany, Austria and Switzerland. The European mistletoe extracts are used in an adjuvant cancer therapy because of their immunostimulatory and simultaneously cytotoxic properties. These effects are usually more evidence for the whole extracts than for purified mistletoe lectins and viscotoxins alone (Eggenschwiler et al., 2007).

Different mistletoe preparations are available for the treatment of cancer. Abnobaviscum®, Helixor, Iscador®, Iscucin® and Isorel® are produced according to anthroposophical methods; other mistletoe preparations include Cefalektin®, Eurixor® and Lektinol®. Iscador was first proposed for the treatment of cancer in 1920, by Rudolf Steiner, PhD, (1861-1925), founder of the Society for Cancer Research, in Arlesheim, Switzerland and introduced in the treatment of human cancer as early as 1921. The anthroposophical preparations are available from different host trees such as oak, apple, pine and others. Harvesting procedure is standardized, and the juices from summer and winter harvests are mixed together. The total extract of mistletoe extracts is considered essential for full effectiveness and concentration of its compounds is ensured through process standardization (http://wissenschaft.mistel-therapie.de/- Mistletoe in cancer treatment).

Mistletoe can biosynthesize their own compounds, but it can take some nutrients from the host trees. It has been suggested that pharmacologically active compounds may pass from the host trees to the parasitic plants (Büssing and Schietzel., 1999).

The main ingredients of the *Viscum album* extract are its three ribosome inactivating proteins or lectins (mistletoe lectins, ML) ML-1, ML-2, and ML-3 (Hajtó et al., 2005), the glycoprotein binding with D-galactose and N-acetyl-D-galactosamine, viscotoxins (VT) (Urech et al., 2006), as well as, oligo- and polysaccharides, alkaloids (Khwaja et al., 1980). The flavonoid patterns of *V. album* from various hosts were investigate by Becker and Exner (1980). They identified quercetin and a series of quercetin methyl ethers, which may be assumed to be accumulated on the plant surface. The epicuticular material of the *V. album* contains preferably the flavonol quercetin and its methyl derivatives, occasionally also the flavonol kaempferol and some of its methyl derivatives, and rarely the flavanone naringenin (Haas et al., 2003).

It has been suggested that pharmacologically active compounds may pass from the host trees to the parasitic plants (Büssing and Schietzel., 1999).

In recent years, the research studies were focused on the antioxidant activity of mistletoe (Onay-Ucar et al. 2006; Leu et al., 2006; Yao et al., 2006; Shi et. al., 2006; Vicas et al., 2009b; Choudhary et al., 2010).

Many plant extracts exhibit efficient antioxidant properties due their phytoconstituents, such as phenolics, especially phenolic acids and flavonoids (Aqil *et al.,* 2006; Miliauskas *et al.,* 2004) and carotenoids (Stahl and Sies, 2003).

To evaluate the antioxidant capacities of plant extracts, numerous *in vitro* methods have been developed. DPPH (2,2-diphenyl-1-picrylhydrazyl), ORAC (oxygen radical absorbance capacity), Trolox equivalent antioxidant capacity (TEAC), and ferric-reducing ability (FRAP) are among the more popular methods that have been used (Wu *et al.,* 2004). The advantages and disadvantages of these methods have been fully discussed in several

reviews (Cao and Prior, 1998; Frankel and Meyer, 2000; Prior and Cao, 1999; Sánchez-Moreno, 2002).

The aim of our research studies are focused, the first, on screening of bioactive compounds (phenolic acid and flavonoids) from leaves and stems of *V. album* that are growing on different host trees, and the second we evaluated the antioxidant activity of *V. album'* leaves and stems.

2. HPLC fingerprint of bioactive compounds from *Viscum album*

To analyze the bioactive compounds, leaves and stems of *V.album* were harvested in July 2009, from five different host trees located in the Borod-Gheghie region, North-West of Romania country. The mean annual rainfall is 500-700 mm/an. The mean annual minimum and maximum temperatures were -22.3^{0}C and 35^{0}C, respectively (20-yr averages). The mean annual air temperature is 9.2 ^{0}C. The area is opened to West, the frequent air mass is oriented to western circulation, transporting oceanic air, cold and humid. Multi-annual average wind speed is 4.1 m/sec. The mistletoe samples have been harvested at the approximate same height, the trees where from it has been harvested having the same soil (brown soil) and climate conditions. The host trees were located in semi-shaded to sunny area (Vicas et al., 2010). The plant materials were labeled according to the host trees, thus: *Acer campestre* (VAA), *Mallus domestica* (VAM), *Fraxinus excelsior* (VAF), *Populus nigra* (VAP) and *Robinia pseudoacacia* (VAR) for easy identification. A voucher specimen of the plants was deposited in the herbarium of the Environmental Protection Faculty from University of Oradea.

The leaves and stems of *V. album* from different host trees were dried rapidly, in an oven at 90^{0}C, for 48 hours, in order to prevent enzymatic degradation (Markham, 1982). The dried plant material was stored in a sealed plastic bag for HPLC analysis. After weighing out a portion of the dried material (approximately 1 g), extraction was carried out with ethanol 70% (1:10, w/v). The mixture was stirred for 24 hours in the dark, and then it was centrifuged for 5 minutes, at 3000 rpm. The ethanol fraction of the supernatant was removed using a rotary evaporator. Further, the aqueous extract was subjected to acid hydrolysis (1N HCl) for 2 hours, at 80^{0}C. The aglycones were extracted 3 times with ethyl acetate by continuous stirring and then centrifuged at 5000 rpm, for 5 minutes. The solvent was removed by flushing the samples with nitrogen. The residue resulting after evaporation was dissolved in ultrapure water (300 µl), filtered throught 0.45 µm filters (Millex-LG, Millipore), and subjected to HPLC analysis.

A Shimadzu HPLC system equipped with a LC20AT binary pump, a degaser, a SPD-M20A diode array detector (Shimadzu Corp., Kyoto, Japan) and a SUPELCOSIL ™ LC-18 column (Sigma-Aldrich Co), 5µm, 25 cm x 4.6 mm was used. Gradient elution was performed with mobile phase A, composed of methanol: acetic acid: double distilled water (10:2:88 v/v/v) and mobile phase B, comprising methanol: acetic acid: double distilled water (90:3:7 v/v/v), at a flow rate of 1.0 ml/min. All solvents were HPLC grade solvents, filtered through a 0.45-μM membrane (Millipore, U.S.A.) and degassed in an ultrasonic bath before use. The chromatograms were monitored at 280 and 360 nm. The following pure standards were used to quantify the bioactive compounds in the leaves and stems of mistletoe: betulinic acid, gallic acid, protocatechuic acid, gentisic acid,

chlorogenic acid, p-OH benzoic acid, caffeic acid, syringic acid, salicilyc acid, p-coumaric acid, ferulic acid, sinapic acid, trans-cinamic acid, naringenin, quercetin, kaempherol and rosmarinic acid. The quantification was made by comparison to calibration curves with pure standards, in the range 0.48 to 500.0 μg/ml. The regression coeficients of calibration curves ranged between 0.9812 and 0.9999. Integration and data analysis were made using Origin 7.0 software.

Quantitative data regarding the phenolic compounds composition of mistletoe extracts are shown in Tab. 1.

We identified and quantified 17 compounds from mistletoe samples (Tab. 1), including a pentacyclic triterpene (betulinic acid), 12 phenolic acids (gallic acid, protocatechuic acid, gentisic acid, chlorogenic acid, p-OH benzoic acid, caffeic acid, syringic acid, salicilyc acid, p-coumaric acid, ferulic acid, sinapic acid, and trans-cinamic acid) and 4 polyphenols (naringenin, quercetin, kaempherol and rosmarinic acid). The Fig. 3 presents the chemical structure of bioactive compounds investigated.

These compounds were identified according to their retention time and the spectral characteristics of their peaks compared with standards, as well as by spiking the sample with individual standards. Phenolic compounds are found usually in nature as esters and rarely as glycosides or in free form. Thus, hydrolysis was needed for their identification and quantitative determination. Flavonoids are also present in plants in the form of glycosides. Each flavonoid may occur in a plant in several glycosidic combinations. For this reason, hydrolysis was used to release the aglycones which were further investigated by HPLC.

Quantitative HPLC analysis of *V. album* showed a higher content of bioactive compounds in the leaves compared with stems (Tab. 1). In the case of leaves from *V. album* hosted by *Acer campestre* (VAA), seven phenolic acids and three polyphenols were identified, while in the stem of mistletoe we found only one phenolic acid (trans-cinnamic acid). Caffeic acid was the dominant compound (13.61 μg/g dry matter) in the leaves of mistletoe. Kampherol and rosmarinic acid were presented in both, leaves and stems, while quercetin was identified only in leaves.

The mistletoe hosted by *Fraxinus excelsior* (VAF) contains nine phenolic acids, and two flavonoids. HPLC chromatograms of phenolic acids from leaves and stems of *Viscum album* hosted by *Fraxinus excelsior* are presented in Fig. 4. Concentration of para-coumaric acid in the VAF sample was 1.82 μg/g dry matter, but we have not identified it in other mistletoe extracts. Caffeic acid was found to have the higest values both in leaves (13.98 μg/g dry matter) and stems (15.86 μg/g dry matter). Kaempherol was also present both in leaves (7.30 μg/g dry matter) and stems (3.66 μg/g dry matter), while quercetin was present only in leaves (6.05 μg/g dry matter).

In case of *V. album* collected from *Populus nigra* (VAP), ferrulic acid was a dominant compound in the set of phenolic acids both in leaves (11.52 μg/g dry matter) and in stems (6.14 μg/g dry matter). Salycilic acid was also present in VAP leaves (8.4 μg/g dry matter) and stems (2.3 μg/g dry matter), while in the other mistletoe samples it was detected only in leaves.

The HPLC chromatogram of mistletoe hosted by *Mallus domestica* (VAM) showed seven phenolic acids in leaves. Betulinic acid was present only in this mistletoe, both in leaves (1.87 μg/g dry matter) and stems (2.05 μg/g dry matter). Also, like in mistletoe hosted by *P.*

COMP. NO.	BIOACTIVE COMPOUNDS	VAA *Acer campestre*		VAF *Fraxinus excelsior*		VAP *Populus nigra*		VAM *Mallus domestica*		VAR *Robinia pseudocacia*	
		leaves	stems	leaves	stems	leaves	stems	leaves	stems	leaves	stems
Pentacyclic triterpene											
1.	Betulinic acid	nd	nd	nd	2.35 ±0.03	nd	nd	1.87 ±0.20	2.05 ±0.05	nd	nd
Phenolic acids											
2.	Gallic acid	nd	nd	nd	nd	nd	nd	nd	nd	39.93 ±0.4	nd
3.	Protocatechuic acid	nd	nd	5.06 ±0.03	3.87 ±0.01	2.58 ±0.01	0.45 ±0.01	4.10 ±0.32	nd	nd	2.01 ±0.2
4.	Gentisic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
5.	Chlorogenic acid	4.70 ±0.01	nd	2.74 ±0.02	1.27 ±0.2	nd	nd	nd	nd	nd	nd
6.	para-OH benzoic acid	10.16 ±0.1	nd	10.81 ±0.02	nd	1.25 ±0.02	nd	1.02 ±0.11	nd	nd	nd
7.	Cafeic Acid	13.61 ±0.04	nd	13.98 ±0.01	15.86 ±0.03	nd	5.34± 0.03	6.39 ±0.23	6.81 ±0.004	nd	nd
8.	Syringic acid	nd	nd	1.11 ±0.04	12.13 ±0.01	nd	nd	nd	1.32 ±0.02	nd	nd
9.	Salicilyc acid	6.70 ±0.03	nd	2.70 ±0.03	nd	8.4 ±0.01	2.3 ±0.05	1.80 ±0.01	nd	nd	nd
10.	para-coumaric acid	nd	nd	1.82 ±0.001	nd	nd	nd	nd	nd	nd	nd
11.	Ferulic acid	7.58 ±0.001	nd	8.99 ±0.02	8.06 ±0.02	11.52 ±0.1	6.14 ±0.11	7.81 ±0.01	6.88 ±0.01	9.93 ±0.01	nd
12.	Sinapic acid	5.41 ±0.3	nd	12.35 ±0.01	4.82 ±0.04	7.17 ±0.3	nd	2.11 ±0.02	1.13 ±0.01	19.32 ±0.01	nd
13.	Trans-cinnamic acid	5.41 ±0.04	1.23 ±0.03	nd	3.07 ±0.05	nd	nd	nd	nd	nd	nd
Flavonoids											
14.	Naringenin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
15.	Quercetin	0.93 ±0.01	nd	6.05 ±0.02	nd	3.25 ±0.01	nd	0.36 ±0.02	nd	7.90 ±0.01	nd
16.	Kampherol	2.74 ±0.01	3.32±0. 001	7.30 ±0.01	3.66 ±0.01	nd	nd	nd	nd	7.58 ±0.01	6.38 ±0.01
Polyphenol											
17.	Rosmarinic acid	1.94 ±0.002	1.81	nd	1.27 ±0.01	0.8 ±0.2	2.0 ±0.001	1.12 ±0.01	1.08 ±0.02	nd	nd
Total phenolic acids		53.57	1.23	59.56	49.08	30.92	14.23	23.23	16.14	69.18	2.01
Ratio leaves/stems of phenolic acid		43.51:1		1.2:1		2.2 : 1		1.4:1		34.4:1	
Total phenolic acid (leaves +stem)		54.80		108.64		45.15		39.37		71.19	
Total flavonoids (leaves +stem)		6.99		17.01		3.25		0.36		21.86	

Table 1. Quantitative HPLC analysis of bioactive aglicones of phenolics (μg/g dry matter*) from leaves and stems of *V. album* harvested from different hosts, on July 2009 (Vicas et al.,2011a).

nigra, ferulic acid was the main compound in leaves and stems (7.81 µg/g dry matter, and 6.88 µg/g dry matter, respectively) of VAM samples.

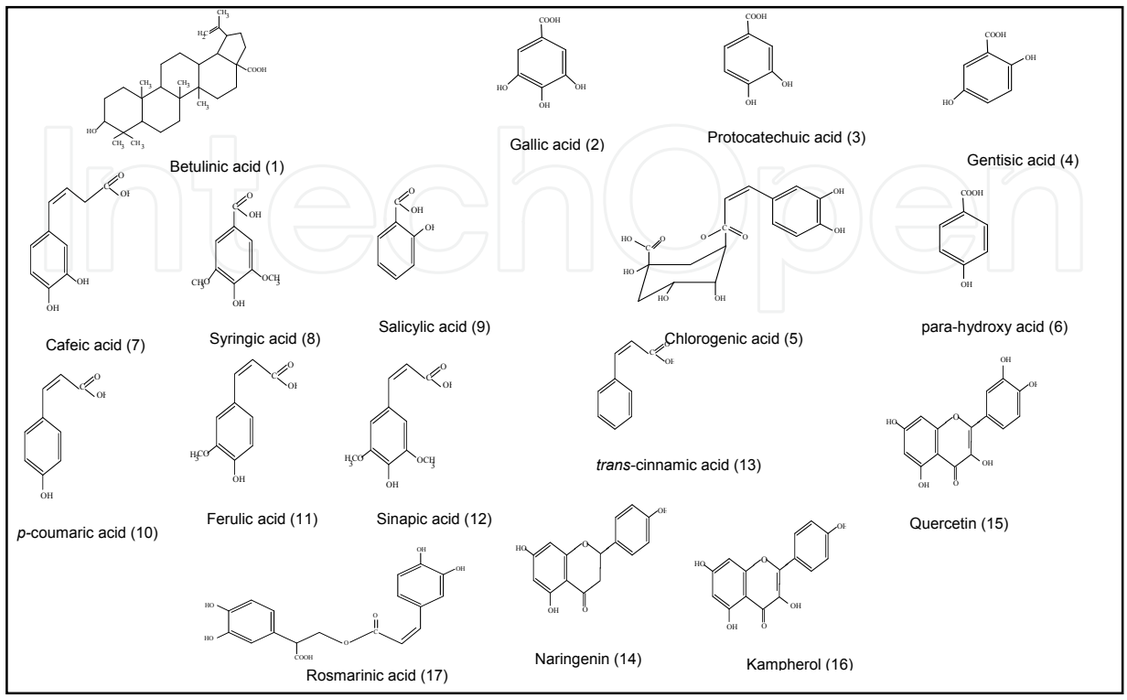


Fig. 3. Bioactive compounds investigated in the leaves and stem of *V.album.*

The main compound in *V. album* hosted by *Robinia pseudoacacia* (VAR) was gallic acid (39.93 µg/g dry matter), which has not been found in the other samples studied.

We did not detect gentisic acid in any sample, nor naringenin, while quercetin was identified only in stems.

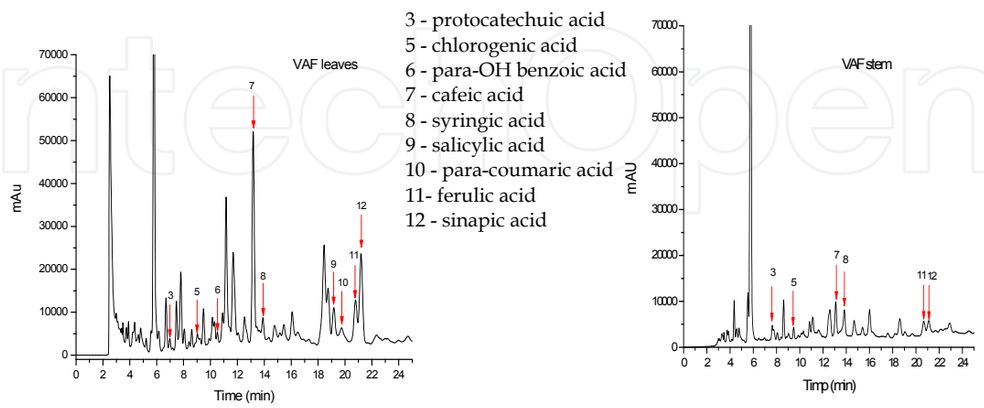


Fig. 4. HPLC chromatograms, used to fingerprint and evaluate quantitatively phenolic acids from leaves and stems of *V.album* harvested from *Fraxinus excelsior* (VAF) (Vicas et al., 2011a).

Phenolic acids represent the major fraction of bioactive compounds in all *V. album*. A high variability of phenolic acid rations between leaves and stems was observed. While, VAA and VAR had high ratios (43.51:1 and 34.41:1, respectively) the lowest ratios were observed in the case of VAF and VAM (1.21:1 and 1.44:1, respectively).

In our study, the mistletoe hosted by *Fraxinus excelsior* (VAF) proved to be the richest in phenolic acids (108.64 μg/g dry matter), followed by VAR (71.19 μg/g dry matter), VAA (54.80 μg/g dry matter), VAP (45.15 μg/g dry matter) and VAM (39.37 μg/g dry matter).

The total polyphenols from leaves and stems of *V. album* decreased in the following order: VAR > VAF > VAA > VAP > VAM.

Luczkiewicz *et al.* (2001), analyzed the phenolic acids present in mistletoe plants hosted by six different hosts. They found that in mistletoe hosted by *Mallus domestica*, the main compound was rosmarinic acid (17.48 mg %), while in mistletoe hosted by *Populus nigra*, the dominant component was chlorogenic acid (12.34 mg %).

Condrat *et al.*, (2009) investigated also nine phanerogam plants (including the European mistletoe) for their flavonoid content and antioxidant activity. Quercetin and kaempferol concentrations were found to be very low in mistletoe extracts (0.20 μmol/g dry matter, and 0.16 μmol/g dry matter, respectively).

Our study revealed that the flavanone naringenin was not present in all the varieties of *Viscum album* investigated. This result is in agreement with the study of Haas *et al.,* (2003) that did not find naringenin in all subspecies of *V. album* analysed, but they found it, rarely, in epicuticular waxes, in *V. cruciatum*. They also found flavonols (quercetin and occasionally kaempherol, along with some of their methyl derivatives) in epicuticular material of *V. album*.

3. Antioxidant activity of *Viscum album*

Phenolic compounds have attracted the interest of many researchers because they are powerful antioxidants and can protect the human body from oxidative stress. The antioxidant activity of phenolics is mainly due their redox properties.

The present study inquired a variety of *in vitro* tests, based on the capacity to scavenge free radicals. On the basis of the chemical reactions involved, major antioxidant capacity can be divided into two categories: i) hydrogen atom transfer (HAT) and ii) single-electron transfer (SET) reaction – based assay. HAT-based procedures measure the classical ability of an antioxidant to quench free radicals by hydrogen donation (ORAC method):

$$\mathbf{X^{\cdot} + AH \rightarrow XH + A^{\cdot}},\ \text{where AH = any H donor}$$

SET-based method detects the ability of a potential antioxidant to transfer one electron to reduce a species, including metals, carbonyls, and radicals (TEAC method):

$$X^{\cdot} + AH \rightarrow X^{-} + AH^{+}$$

$$AH^{+} + H_2O \leftrightarrow A^{\cdot} + H_3O^{+}$$

$$X^{-} + H_3O^{+} \rightarrow XH + H_2O$$

$$M(III) + AH \rightarrow AH^{+} + M(II)$$

For the determination of antioxidant activity of mistletoe extract through different methods, we used fresh leaves and stems (2 g) that were homogenized with 10 ml distilled water, or with 10 ml 98% ethanol using an Ultra Turax homogenizator, for 1 minute. This mixture was centrifuged (10 000 rpm, at 4^0C, for 10 minutes) and the supernatants were filtered through a filter paper. The filtrate was used for the antioxidant activity measurements and total phenolics content.

3.1 DPPH inhibition by mistletoe extracts

A rapid, simple and inexpensive method to measure antioxidant capacity of plant extracts involves the use of the free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH). 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 (Fig.5) that can be easily monitored using a spectrophotometer at 515 nm.

The DPPH radical-scavenging activity was determined using the method proposed by Brand-Williams *et al.,* (1995). The reaction was performed in 12 well-plate. 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:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A\ s) \times 100\ ] / A_0 \quad (1)$$

Where, A_0 is absorbance of the blank, and A_S is absorbance of the samples at 515 nm

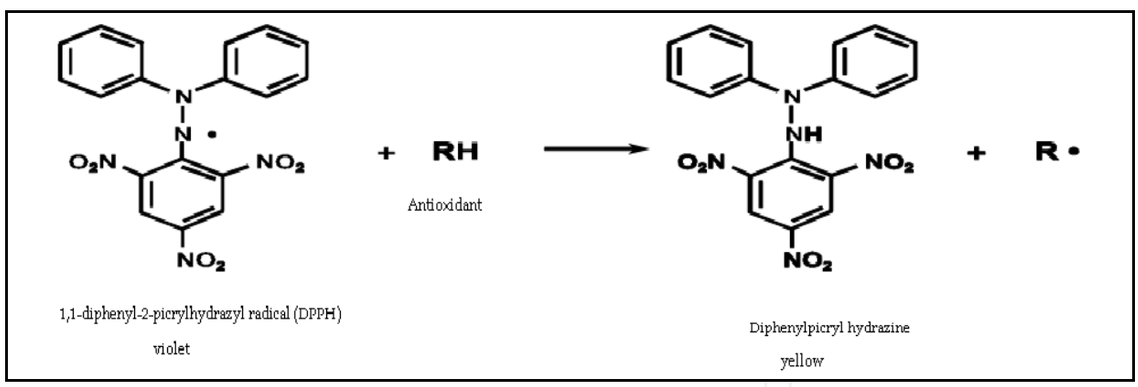


Fig. 5. The structure of DPPH radical and its reduction by an antioxidant.

The comparative antioxidant activity of *V.album* leaves hosted by different host trees, evaluated by the DPPH method shown significantly differences ($p < 0.001$) regarding to DPPH scavenging effect (%) of aqueous and ethanol extracts in leaves and stems (Vicas et al., 2011a).

The results showed that DPPH scavenging effect of aqueous extracts from mistletoe leaves varied between 11.49 %, in the case of mistletoe growing on *Robinia pseudoacacia* (VAR), to 2.22 % in the case of VAM (mistletoe growing on *Mallus domestica*). Higher DPPH

scavenging effect was observed in the ethanol extracts, with values ranging from 77.19% (VAF) to 50.47 % (VAA).

The DPPH scavenging effect of extracts of mistletoe stems was lower than that of leaf extracts. No antioxidant activity was detected in aqueous extracts of VAF and VAM stems.

In all samples, stem extracts have lower antioxidant activity than the corresponding leaf extracts, also in the case of ethanol extracts.

Similar results were obtained by Önay-Uçar *et al.,(*2006), who investigated the antioxidant activity of methanol extracts of *V.album* grown on different host trees. Their results showed that mistletoe hosted by *Robinia pseudocacia* (VAR) exhibited 73.44% inhibition of DPPH, and mistletoe hosted by *Acer campestre* (VAA) presented 59.52% inhibition of DPPH. The slight differences between our results and theirs can be assigned to the solvent used for extraction and/or to environmental factors.

Sharma and Bhat (2010), showed that the absorbance profiles of DPPH were highest in a buffered methanol solution, followed by methanol and ethanol solutions. Higher absorbance in methanol solutions implies better sensitivity vis-à-vis ethanol solutions of DPPH.

Roman *et al.,* (2009) investigated the efficiency of ultrafiltration process on the antioxidant activity of aqueous extract of *V. album*. The values obtained by the DPPH assay ranged between 66.2% and 88.2% DPPH inhibition for mistletoe concentrated extracts. The correlation coefficient between data of DPPH inhibition and total protein content was 0.94, suggesting that, besides the phenolic compounds of *Viscum* extracts, viscolectins have a great contribution to the radical scavenging activity.

Other research paper (Oluwaseun and Ganiyu, 2008) investigated the antioxidant properties of methanol extracts of *V. album* isolated from cocoa and cashew trees in the South Western part of Nigeria. The scavenging ability of each methanol extract against DPPH followed a dose-dependent pattern (0-10 mg/ml). The free radical scavenging ability of the *V. album* extract from cocoa tree performs better than that from cashew tree, a fact that is in agreement with the total phenol content of the two extracts (182 mg /100 g, and 160 mg /100 g, respectively).

Papuc et al., 2010, investigate the free radicals scavenging activity of ethanol extract from mistletoe. The scavenging activity calculated for *V. album* in % inhibition was 7.2%.

When the activities of the same type of mistletoe extracts, collected from the same host tree, but in different seasons, were compared using the DPPH assay, it was found that the antioxidant activity was, in general, higher in spring (Vicaş *et al.,* 2008). The values obtained in May 2008 by the DPPH assay varied from 42.2 % DPPH inhibition for *V. album* growing on *Robinia pseudocacia* (VAR) to 17.4 % DPPH inhibition for *V. album* growing on *Populus nigra* (VAP). In July, the VAR extracts exhibited the highest capacity to scavenge free radicals (46.91%), but the VAA and VAP extracts lost their antioxidant activity. The differences may be explained by the different environmental factors (temperature, water, irradiation, etc.).

The antioxidant activity of the *V. album* extract from *Robinia pseudoacacia* has the highest, especially in the case of DPPH method (11.49 ±0.04 % for VAR leaves aqueous extracts harvesting in July; 76.60± 0.02% for VAR leaves ethanol extracts harvesting December) and this is also in agreement with total phenolic content (Vicas *et al.,* 2011b).

3.2 ORAC method

The ORAC method measures antioxidant inhibition of peroxyl radical-induced oxidations, and thus reflects classical radical chain breaking antioxidant activity by hydrogen atom transfer. The ORAC assay was performed essentially as described by Huang *et al.,*(2002). A volume of 150 µl of working solution of sodium fluorescein (4×10^{-3} mM) was added to 25 µl samples, in a 12 well-microplate. The plate was allowed to equilibrate by incubating it for a minimum of 30 minutes in the Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT) at 37°C. Reaction was initiated by the addition of 25 µl of 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) solution (153 mM) and the fluorescence was then monitored kinetically with data taken every minute, at 485 nm, 20 nm bandpass excitation filter, and a 528 nm, 20 nm bandpass emission filter. ORAC values were calculated as described by Cao and Prior (1999). The area under the curve (AUC) and the Net AUC of the standards and samples were determined using equations 2 and 3 respectively.

$$AUC = 0.5 + (R2/R1) + (R3/R1) + (R4/R1) + 0.5(Rn/R1) \quad (2)$$

$$\text{Net AUC} = AUC_{sample} - AUC_{blank} \quad (3)$$

Where R1- fluorescence value at the initiation of reaction and Rn - fluorescence value after 30 min.

The standard curve was obtained by plotting the Net AUC of different Trolox concentrations against their concentration (6.25 – 100 µM). ORAC values of samples were then calculated automatically using Microsoft Excel to interpolate the sample's Net AUC values against the Trolox standard curve.

The values obtained by ORAC assays varied from 10.73 ± 1.90 mM Trolox equivalents/g fresh matter for the VAP ethanol leaf extract, to 1.52 ± 1.25 mM Trolox equivalents/g fresh matter for the VAM aqueous stem extract. According to the results obtained in the ORAC assay, there was no significant differences between the antioxidant capacity of leaves and stems for all variants of mistletoe investigated, except for the aqueous leaf extracts of VAA *vs* VAM ($p < 0.01$), and for the aqueous stem extracts of VAA *vs* VAM ($p < 0.05$). The highest values were recorded in the case of VAA aqueous leaf extract (5.49 mM Trolox equivalents/g fresh matter) and VAP ethanol leaf extract (10.73 mM Trolox equivalents/g fresh matter) (Vicas et al. 2011a).

3.3 TEAC method

The TEAC is a spectrophotometric method, widely used for the assessment of antioxidant activity of various substances. This method measures the ability of compounds to scavenge the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical cation in relation to Trolox. The blue/green $ABTS^{+}$ chromophore radical is produced through the reaction

between ABTS and potassium persulfate. In the presence of an antioxidant the $ABTS^+$ radical changes from blue/green to colorless depending on antioxidant capacity of compound, its concentration and the duration of the reaction. ABTS was dissolved in distilled water to a 7 mM concentration. $ABTS^+$ was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand, in the dark, at room temperature for 12-16 h before use. ABTS stock solution was diluted with ethanol in order to obtain an absorbance of 0.70 ± 0.02 at 734 nm. After addition of 17 µl of extract to 170 µl of diluted $ABTS^+$, the interaction between the antioxidants and the $ABTS^+$ was monitored spectrophotometrically at 734 nm (Arnao *et al.* 2001). The results were expressed in mM Trolox equivalent/g fresh matter.

Based upon the conducted research, it has been found that all mistletoe extracts (aqueous or ethanol, leaf or stem) have the ability of scavenging cation-radicals $ABTS^+$. According to the results obtained with TEAC assays, there were significant differences ($p < 0.001$) between all the extracts investigated (Vicas et al. 2011a). The highest level of scavenging radicals was detected in water extracts, and ethanol extracts had the lowest deactivation level. Aqueous leaf and stem extracts of mistletoe growing on *Acer campestre* (VAA) recorded the highest TEAC values (678.72 ± 0.00 mM equivalent Trolox/g fresh matter, and 577.94 ±0.01 mM equivalent Trolox/g fresh matter, respectively), while for the ethanol extracts the highest level of scavenging cation-radicals $ABTS^+$ was recorded for leaves from VAF (461.09 ±0.11 mM equivalent Trolox/g fresh matter) and for stems from VAP (306.68 ± 0.01 mM equivalent Trolox/g fresh matter). We may suppose that water extracts had the highest antioxidant activity because they contain more bioactive compounds with the ability of scavenging cation-radicals $ABTS^+$, as compared to ethanol extracts (Vicas et al. 2011a).

3.4 Folin-Ciocalteu method

Total phenolic content was determined by the Folin-Ciocalteu method (Singleton *et al.*, 1999). Mistletoe extract (23 µl) was mixed with 1817 µl distillated water, 115 µl Folin-Ciocalteu reagent (dilution 1:10, v/v) and 345 µl of 15% Na_2CO_3 solution, and the mixture incubated at room temperature, in the dark, for 2 hours. The absorbance was measured at 765 nm using a spectrophotometer (BioTek Synergy). The calibration curve was linear for the range of concentrations between 0.1-0.5 mg/ml gallic acid. The results were expressed in mg gallic acid equivalents (GAE)/g fresh matter). In aqueous leaf extracts, the highest polyphenolic content was found in VAR (200.51 ± 0.00 mg GAE/g fresh matter, while the lowest value was 176.87 ± 0.003 mg GAE/g fresh matter for VAM. The values obtained for total phenolics in both, aqueous and ethanol extracts, decreased in the order: VAR > VAF > VAP > VAA > VAM. The mistletoe stem extracts contained lower levels of phenolics than the leaf extracts, in both solvents. The lowest level of total phenolics was recorded for VAF and VAA aqueous stem extracts (58% and 54,97% less that leaves, respectively). In the other extracts (VAM or VAR), the differences between leaves and stems were not significant.

In a recent research paper Vicas et al., (2011 b) presented comparatively the total phenolic content from leaves of mistletoe, in three different periods (May, July and December). Generally, the content of total phenolic was higher in aqueous extract comparative with ethanol extract. In aqueous leaves extract, the highest phenolic content was found in VAR

(209.51 ± 0.01 mg GAE/ g fresh matter), harvesting in May, while the lowest value was 83.93 ± 0.001 mg GAE/ g fresh matter for VAF, harvesting in July. The mistletoe stem extracts contained lower levels of phenolics, comparing with leaves, in both solvents. In the ethanol extract, the highest phenolic content was found in VAM (58.97 ± 0.009 mg GAE/ g fresh matter), harvesting in December, followed by VAA extract (51.96 ± 0.006 mg GAE/ g fresh matter). These results can be explained by the influence of harvesting time on the chemical composition and antioxidant activity.

There are many research studies that have established a correlation between the total phenol content of plants and their antioxidant properties (Kılıçgün and Altıner, 2010; Song *et al*., 2010; Tosun *et al*., 2009; Alali et al., 2007).

3.5 FRAP method

Considering the antioxidant potential of European mistletoe components (leaves and stems) due to their content in phenolic derivatives (phenolic acids and flavonoids) and carotenoids, and their specific hydrophilic and lipophilic character, respectively, we measured comparatively the „lipophilic" and hydrophilic" antioxidant capacity, based on the reducing power of such antioxidants against the ferric tripyridyltriazine (Fe(III)-TPTZ) complex (Fig.6). Statistical correlations between their phenolic or carotenoid concentrations and hydrophilic / lipophilic antioxidant activities, in relation to their location (leaves *versus* stems) are also reported.

Mistletoe extracts for total phenolic content and hydrophilic and lipophilic antioxidant activity were prepared as presented in Fig. 7. Shortly, 10 g leaves or stems were mixed with 25 ml methanol (MeOH), and then the slurries were kept at 4^0C for 12 hours. After centrifugation for 20 minutes, the supernatant was recovered and stored at – 20^0 C until the hydrophilic antioxidant activity (HAA) was assayed. The pellet was dissolved in acetone, homogenized and sonicated to extract the lipophilic components submitted to lipophilic antioxidant activity (LAA) analysis. The homogenates were centrifuged for 20 minutes, and the supernatant was recovered and stored at – 20^0 C until assayed

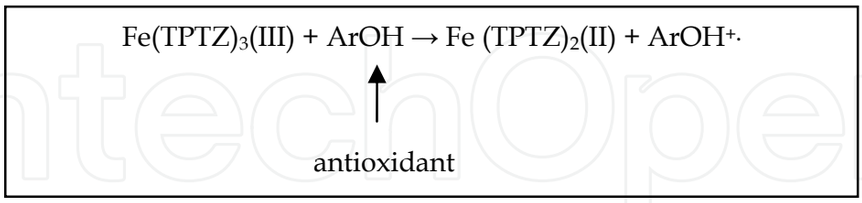


Fig. 6. Reducing power of an antioxidants against the ferric tripyridyltriazine (Fe(III)-TPTZ) complex.

The ferric reducing antioxidant power (FRAP) assay was used to determine both hydrophilic and lipophilic antioxidant activities. The assay was determined according to the method of Benzie and Strain (1996) with some modifications. The FRAP assay consists in the ferric tripyridyltriazine (Fe(III)-TPTZ) complex reduction to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by an antioxidant at low pH. The stock solutions included: 300 mM acetate buffer; 250 mg $Fe_2(SO_4)_3 \cdot H_2O$ dissolved in 50 ml distillated water; 150 mg TPTZ and 150 µl HCl, dissolved in 50 ml distillated water. The working FRAP solution was freshly prepared

by mixing 50 ml acetate buffer, 5 ml $Fe_2(SO_4)_3 \cdot H_2O$ solution and 5 ml TPTZ solution. Mistletoe extracts (100 µl) were allow to react with 500 µl FRAP solution and 2 ml distilled water, for 1 h, in dark. The final colored product (ferrous tripyridyltriazine complex) was quantified by VIS absorption at 595 nm. As positive antioxidant control we used ascorbic acid (AA) and obtained a standard linear curve, between 5 and 100 mg/l vitamin C. The antioxidant activity (HAA and LAA) was expressed in mg/l AA equivalents/ g fresh weight.

We noticed no semnificative differences between the HAA values from leaves and stems hydrophilic fractions, but mistletoe leaves extract originating from *Mallus domestica* (VAM) and *Fraxinus excelsior* (VAF) and all stem extracts have shown the highest antioxidant activity, (0.14 ± 0.12 and 0.13 ± 0.11mg/l vitamin C equivalent / g of fresh leaves).

Meanwhile, LAA is significantly lower (around 100 times) comparing to HAA, in both leaves and stems. No significant differences were noticed between stem and leaves of mistletoe extract. Overall, we observed better antioxidant capacity for VAF and VAM.

A reason for low LAA values (which can be due to low carotenoid concentrations found in acetone extract) can be also the overlapping of carotenoids absorption (450 nm) and the color developed during FRAP method (UV-Vis absorbtion at 595 nm), observed also by other authors who studied vegetable extracts (Ou *et al.*, 2002).

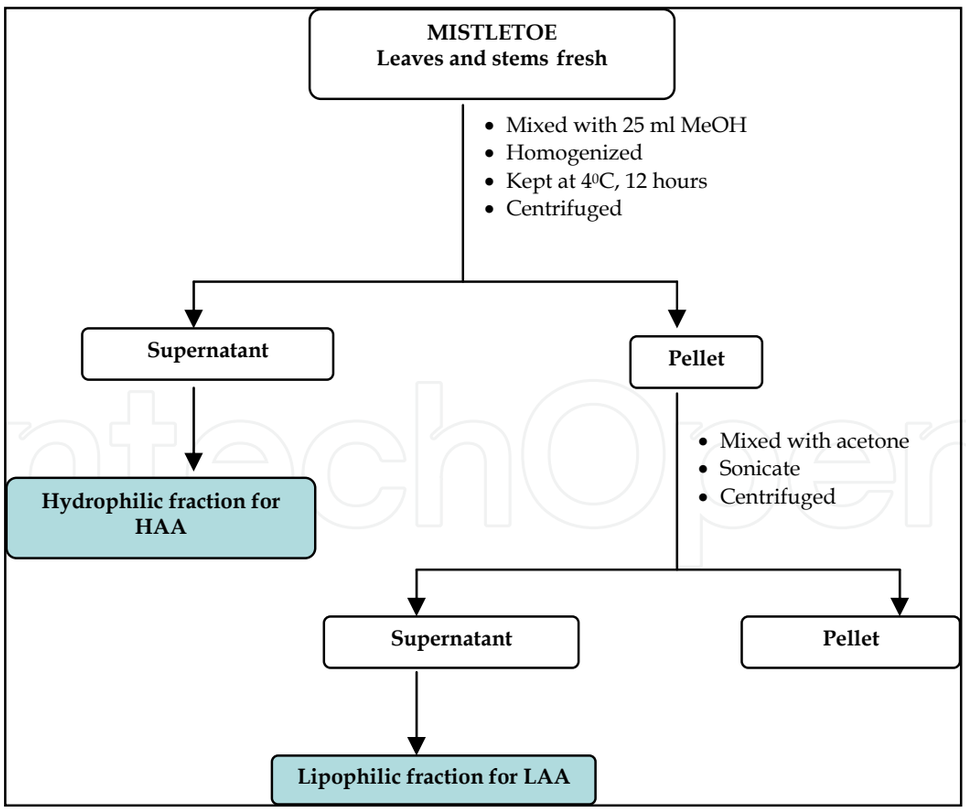


Fig. 7. Flow sheet of extraction of mistletoe extract for the determination of antioxidant activity of hydrophilic and lipophilic fractions (HAA and LAA, respectively). (Vicas et al., 2009b).

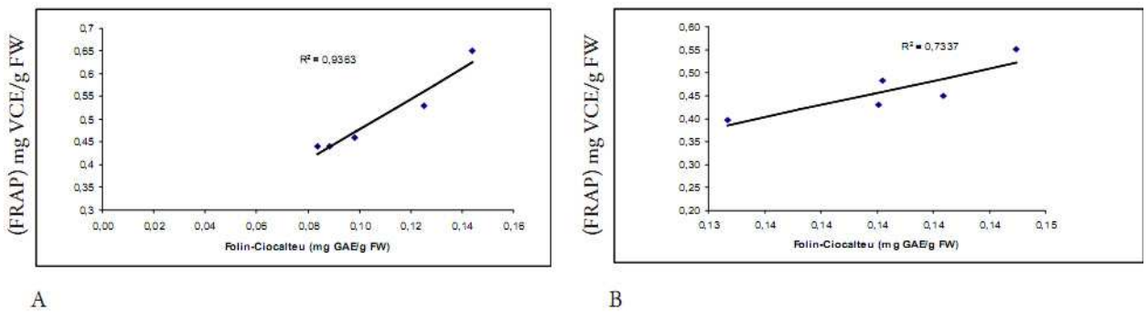
Total phenolics concentration was determined by the Folin-Ciocalteu method, and total carotenoids content from the lipophilic and hydrophilic fraction was determined by the VIS absorption at 470 nm, using a β-carotene (0.001-0.004 mg/ml) standard curve. The total carotenoids content was expressed based on β-carotene equivalents (β-carotene; mg/g fresh weight).

The total phenolics and carotenoids content were measured in methanol and acetone extracts. Methanol extract showed relatively high phenolics content (between 0.65 and 0.40 mg GAE/g fresh weight), which are known as the major natural hydrophilic antioxidants.

The phenolics content did not differ significantly between leaves and stems in the methanol extract. But, in the acetone extract the content in the phenolic compounds are 100 time lower comparing to the methanol extract (from 0.015 to 0.002 mg GAE/g FW). Generally, the acetone extracts gave lower values of phenolics and carotenoids than methanol extract. The leaves had, in both solvents, higher concentrations of phenolics and carotenoids, comparing with stems. The stem acetone extract did not contain any carotenoids. Mistletoe leaves originating *Acer campestre* (VAJ), followed by VAM and VAF showed higher concentrations of phenolics, and also carotenoids especially in methanol.

The HAA values, as determined by FRAP method, were significantly correlated with the values of phenolics content, as determined by Folin-Ciocalteu assay (R^2 = 0.9363) in the case of leaves, and R^2 = 0.761 in the case of stems, as shown in Fig. 8. The LAA values, as determined by FRAP method, were slightly correlated with the carotenoids content (R^2 = 0.6327) (Fig. 8A), and meanwhile HAA were no correlated with carotenoids (R^2 = 0.168) (Fig. 8 B) (Vicas et al., 2009b).

The methanol extracts of *V. album* demonstrated to be rich in phenolic compounds, potential antioxidants with ferric reducing ability. Mistletoe leaves originating from *Acer campestre* (VAJ), followed by VAM and VAF showed higher concentrations of phenolics, and also carotenoids, superior to acetone extracts. Meanwhile, VAF and VAM showed higher HAA and LAA activities. These data suggest that the antioxidant capacity slightly differs depending on the host trees.



A B

Fig. 8. Correlations found between the HAA, as determined by FRAP method, and total phenolics concentration values (GAE units) of mistletoe leaves (A) and stems (B) (Vicas et al., 2009b).

Other authors (Onay-Ucar et al., 2006) also reported that antioxidant capacity of *V. album* extract differ depending on the time of harvest and nature of the host trees. Similar results

were obtained by Oluwaseun and Ganiyu (2007), who evaluated the antioxidant activity of methanol extract of *V. album* leaves from two hosts (cocoa and cashew trees), showing that mistletoe from cocoa tree had higher total phenol content (182 mg/100g) than that from cashew tree (160 mg/100g), the main reason of their antioxidant capacity. Therefore, that the total phenolic content, more than carotenoids content can serve as a useful indicator for the antioxidant activities of mistletoe extracts. Carotenoids are less available also for extraction, being linked to proteins in the photosynthetic apparatus in leaves, a possible.

4. Conclusion

The influence of the host tree may have a key role in the phenolic composition of mistletoe leaves or stems. The mistletoe hosted by *Fraxinus excelsior* (VAF) proved to be the richest in phenolic acids (108.64 μg/g dry matter), followed by VAR (71.19 μg/g dry matter), VAA (54.79 μg/g dry matter), VAP (45.15 μg/g dry matter) and VAM (39.37 μg/g dry matter), as determined by HPLC. The total polyphenols from leaves and stems of *V. album* decreased in the following order: VAR > VAF > VAA > VAP > VAM.

In aqueous leaf extracts, the highest polyphenol content was found in VAR (200.51 ± 0.00 mg GAE/ g fresh matter, while the lowest value was 176.87 ± 0.003 mg GAE/ g fresh matter for VAM. The values obtained for total phenols in both, aqueous and ethanol extracts, decreased in the order: VAR > VAF > VAP > VAA > VAM. The mistletoe stem extracts contained lower levels of phenols, as compared to leaves, in both solvents.

The bioactive compounds and the antioxidant activity are present in leaves and also in stems, in all the mistletoe samples examined (aqueous and ethanol). Of the samples examined, the best results were obtained with ethanol extract of VAF, followed by VAR.

As it has been observed by other authors (Cao and Prior, 1998), the values obtained for the antioxidant capacity of an extract depend greatly on the methodology used. The antioxidant potential is reflected by a more complex synergy of active molecules, not only phenols.

The differences in antioxidant activity between leaves and stems of mistletoes harvested from different trees can be attributed to environmental factors such as season, climate and temperature which can significantly affect the accumulation of the antioxidant components in the plant tissue.

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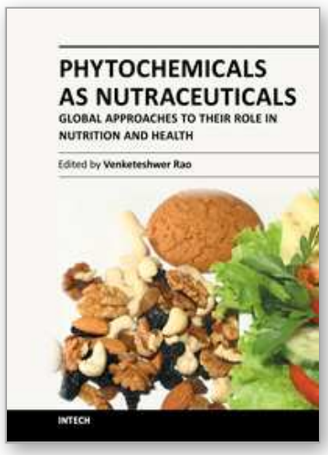
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Phytochemicals as Nutraceuticals - Global Approaches to Their Role in Nutrition and Health

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