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Hydrophilic and Lipophilic Antioxidant Activities of Mistletoe (*Viscum album*) as Determined by FRAP Method

Simona VICAS[1)], József PROKISCH[2)], Dumitrita RUGINA[3)], Carmen SOCACIU[3)]

[1)] *University of Oradea, Faculty of Environmental Protection, 26 Gen. Magheru St., 410048 Oradea, Romania; sim_vicas@yahoo.com*

[2)] *University of Debrecen, 4032 Debrecen, Böszörményi út 138, Hungary; jprokisch@helios.date.hu*

[3)] *University of Agricultural Sciences and Veterinary Medicine, Department of Chemistry and Biochemistry, 3-5 Manastur St., 400372 Cluj-Napoca, Romania; csocaciu@usamvcluj.ro*

Abstract

Phytochemical antioxidants, found in many medicinal plants, gained an increasing interest nowadays, because of their positive effect demonstrated by epidemiological and in vitro studies. Methanol and acetone extracts of European mistletoe (*Viscum album*) leaves and stems collected from five host trees (*Acer campestre, Malus domestica, Fraxinus excelsior, Populus nigra and Robinia pseudoacacia*) were subjected to antioxidant activity measurements. 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, the "lipophilic" (HAA) and "hydrophilic" (HAA) antioxidant capacity has been measured comparatively, based on the reducing power of such antioxidants against the ferric tripyridyltriazine (Fe(III)-TPTZ) complex (method FRAP). Among the selected plants, methanole extract of *V. album* leaves collected from *Malus domestica* (VAM) exhibited the highest antioxidant activity (0.14 ± 0.12 mg/l vitamin C equivalent / g of fresh leaves). A lipophilic antioxidant activity of mistletoe was around 100 times lower compared to hydrophilic antioxidant activities. HAA was positively correlated with total phenol concentration from leaves and stems (R^2 = 0.9363, respectively R^2 = 0.7337), but not with carotenoid content (R^2 = 0.168). Meanwhile, the correlation of LAA with carotenoid was more significant (R^2 = 0.6327). The antioxidant capacity proved to be dependent on the host trees, VAM being a recommendable good source, either in water or alcoholic extract. No significant differences were noticed between the antioxidant content and activity of different plant parts of mistletoe (stems versus leaves). The host tree of mistletoe may play a significant role in the elaboration of specific mistletoe antioxidants and becomes important parameter in the assessment of the mistletoe as a raw material for phytopharmaceutical formulas.

Keywords: *Viscum album*, antioxidant, phenolics, β-carotene, FRAP assay

Introduction

European mistletoe (*Viscum album* L.) is an evergreen, semiparasitic 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 (*Phoradendron serotinum* or *Phoradendron flavescens*), the European (*Viscum album* L.), and the Korean (*Viscum album* L. *coloratum*), most investigative work has been done on European mistletoe. A number of biological effects, such as anticancer, apoptosis–inducing, antimicotic, antibacterial, antiviral, and immunomodulatory activities have been reported (Hajtó *et al.,* 2005).

The phytochemical profile of mistletoe depends of the host trees of this plant (Luczkiewicz *et al.,* 2001). The main bioactive compounds found in mistletoe are lectins (glycoproteins with affects on cell-proliferation), viscotoxin (low protein molecule of 5 kDa) (Romagnoli *et al.,* 2000), as well acidic arabinogalactan with a rhamnose-galactoronic acid backbone and highly branched arabinose-galactose side chains attached by the rhamnose residue to the backbone (Edlund *et al.,* 2000), low alkaloid concentratins dependent on the host tree type (Peng *et al.,* 2005). The antioxidant molecules found in mistletoe are represented by flavonoids (quercetin and quercetin methyl ethers, accumulated on the plant surface, occasionally also the flavonol kaempferol and its methyl derivatives, and rarely naringenin) (Haas *et al.,* 2003) and phenolic acids, such as digallic and o-coumaric acid in the free or glycosilated forms (Luczkiewicz *et al.,* 2001).

Although, morphological and phytochemical differences among mistletoes collected from different species of host trees were reported recently, its taxonomical characteristics are not clear (Ochocka and Piotrowski, 2002).

The European mistletoe extracts are used in an adjuvant cancer therapy due to their simultaneous immunostimulatory and cytotoxic properties. These effects were usually more evident for the whole extracts than for purified mistletoe lectins and viscotoxins alone (Eggenschwiler *et*

al., 2007). One reason can be the potential of antioxidants found in many plants with medicinal effects.

Otherwise, many plant extracts exhibit efficient antioxidant properties due their phytoconstituents, such as phenolics (Aqil *et al.,* 2006; Miliauskas *et al.,* 2004) and carotenoids. To evaluate the antioxidant capacities of plant extracts, numerous *in vitro* methods have been developed. ORAC (oxygen radical absorbance capacity), Trolox equivalent antioxidant capacity (TEAC), total radical-trapping antioxidant capacity (TRAP), 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).

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, the "lipophilic" (HAA) and "hydrophilic" (HAA) antioxidant capacity has been measured comparatively,, based on the reducing power of such antioxidants against the ferric tripyridyltriazine (Fe(III)-TPTZ) complex (method FRAP). Statistical correlations between their phenolic or carotenoid concentrations and hydrophilic/ lipophilic antioxidant activities, in relation to their location (leaves versus stems) are also reported.

Materials and methods

Plant material

Different variants of *V. album* plants were harvested in December 2008, from five different host trees located in North-West of Romania country. They were labelled according with the host trees, thus: *Acer campestre* (VAJ), *Malus domestica* (VAM), *Fraxinus excelsior* (VAF), *Populus nigra* (VAP) and *Robinia pseudoacacia* (VAS) for easy identification.

Extraction

Mistletoe extracts for total phenolic content and hydrophilic and lipophilic antioxidant activity were prepared as presented in Fig. 1: 10 g leaves or stems were mixed with 25 ml methanol (MeOH), and then the slurries were kept at 4°C for 12 hours. After centrifugation for 20 minutes, the supernatant was recovered and stored at -20°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°C until assayed.

HLA and LAA antioxidant activities determined by FRAP assay

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 ml 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 it has been use 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.

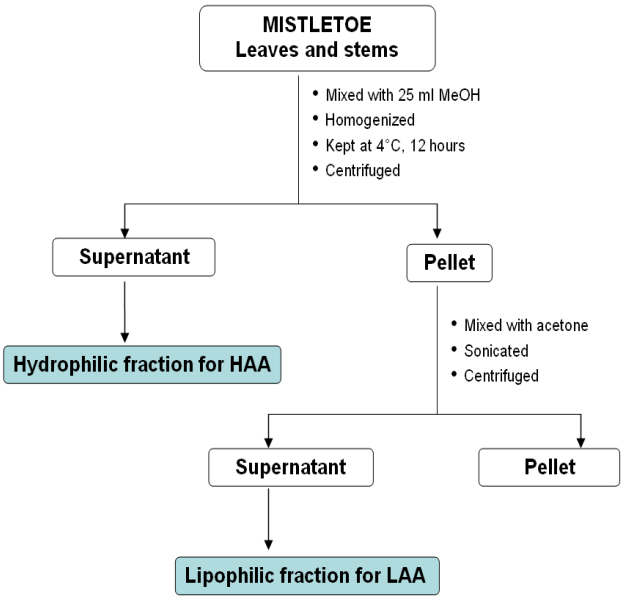


Fig. 1. Flow sheet of extraction of mistletoe extract for the determination of antioxidant activity of hydrophilic and lipophilic fractions (HAA and LAA, respectively)

Evaluation of phenolic and carotenoid contents of mistletoe leaves and stems

Total phenolic concentration was determined by the Folin-Ciocalteu method, which was adapted from Thaipong *et al.* (2005). Shortly,it has been mix 100 µl mistletoe hydrophilic and lipophilic fraction, 2000 µl distillated

water and 200 µl Folin-Ciocalteu reagent; then mixed well using a Vortex. The mixture was allowed to react for 3 minutes, then 1 ml of 15% Na_2CO_3 solution was added and mixed. The samples were incubated at room temperature, in dark for 2 hrs. The absorbance was measured at 725 nm using a JASCO 611 spectrophotometer. In parallel a linear standard curve was obtained using 0.1-0.5 mg/ml gallic acid. The phenolic concentrations were expressed in gallic acid equivalents (mg GAE /g fresh weight).

Total carotenoid 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 carotenoid content was expressed based on β-carotene equivalents (β-carotene; mg/g fresh weight).

Results and discussion

HLA and LAA antioxidant activities, as determined by FRAP assay

To estimate hydrophilic (HAA) and lipophilic (LAA) antioxidant activities, it has been use he FRAP assay and the results are expressed as ascorbic acid (AA) equivalents per gram of fresh leaves or stems (Tab. 1.) it has been notice no significant differences between the HAA values from leaves and stems hydrophilic fractions, but mistletoe leaves extract originating from *Malus 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, it has been observebetter antioxidant capacity for VAF and VAM.

Tab. 1. The hydrophilic (HAA) and lipophilic (LAA) antioxidant activities of different fresh leaves and stem mistletoe extracts fractions

Samples	Antioxidant activity (mg/l AA equivalent/g fresh weight)*	
	HAA	LAA
Leaves		
VAF - *Fraxinus excelsior*	0.13 ± 0.11	0.012 ± 0.03
VAP - *Populus nigra*	0.08 ± 0.08	0.006 ± 0.05
VAS - *Robinia pseudoacacia*	0.09 ± 0.06	0.004 ± 0.10
VAM - *Malus domestica*	0.14 ± 0.12	0.007 ± 0.07
VAJ - *Acer campestre*	0.10 ± 0.14	0.003 ± 0.20
Stem		
VAF - *Fraxinus excelsior*	0.13 ± 0.28	0.005 ± 0.20
VAP - *Populus nigra*	0.14 ± 0.72	0,012 ± 0.40
VAS - *Robinia pseudoacacia*	0.14 ±0.20	0,003 ± 0.10
VAM - *Malus domestica*	0.14 ± 0.20	0.012 ± 0.11
VAJ - *Acer campestre*	0.14 ± 0.87	0,005 ± 0.42

*Data values are presented as mean ± SD (n=3)

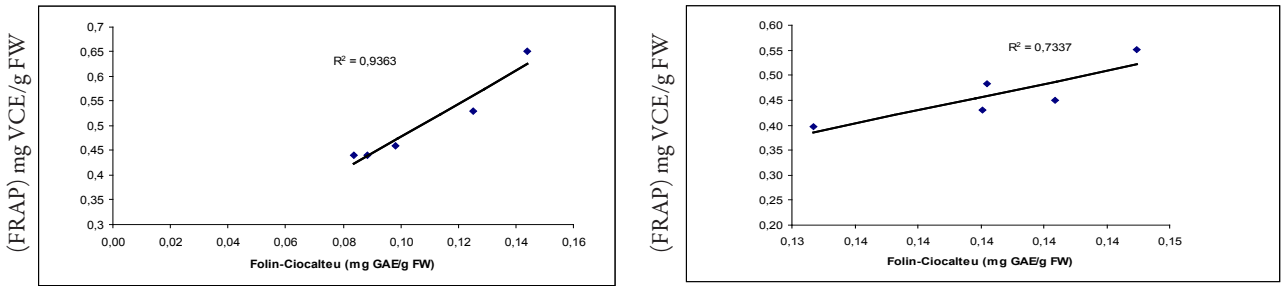
A reason for low LAA values (which can be due to low carotenoid concentrations found in acetone extract) can be also the overlapping of carotenoid absorption (450 nm) and the colour developed during FRAP method (UV-Vis absorption at 595 nm), observed also by other authors who studied vegetable extracts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

Phenolic and carotenoid contents of mistletoe leaves and stems

The total phenolic and carotenoid content measured in methanol and acetone extracts are shown in Tab. 2. Methanolic extract showed relatively high phenolic content (between 0.65 and 0.40 mg GAE/g fresh weight),

Tab. 2. Total content of phenolics and carotenoids in mistletoe in both hydrophilic and lipophilic fractions used for HAA and LAA determinations, respectively

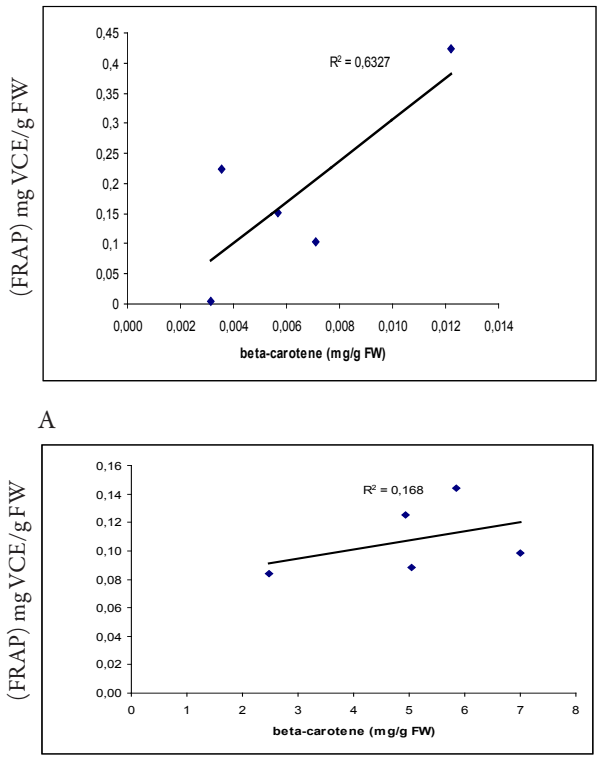
Sample	Total phenolic content for HAA (mg GAE /g fresh weight)		Total carotenoid content for LAA (mg β-carotene mg/g fresh weight)	
	Methanolic extract	Acetonic extract	Methanolic extract	Acetonic extract
Leaves				
VAF - *Fraxinus excelsior*	0.53 ± 0.30	0.005 ± 0.07	4.93 ± 0.01	0.42 ± 0.01
VAP - *Populus nigra*	0.44 ± 0.09	0.005 ± 0.10	2.47 ± 0.03	0.15 ± 0.16
VAS - *Robinia pseudoacacia*	0.44 ± 0.07	0.006 ± 0.06	5.04 ± 0.21	0.22 ± 0.06
VAM - *Malus domestica*	0.65 ± 0.19	0.009 ± 0.12	5.85 ± 0.07	0.10 ± 0.08
VAJ - *Acer campestre*	0.46 ± 0.10	0.003 ± 0.55	7.00 ± 0.15	0
Stem				
VAF - *Fraxinus excelsior*	0.45 ± 0.12	0.002 ± 0.12	1.31 ± 0.23	0
VAP - *Populus nigra*	0.43 ± 0.17	0.004 ± 0.30	0.22 ± 0.01	0
VAS - *Robinia pseudoacacia*	0.48 ± 0.23	0.013 ± 0.10	0	0
VAM - *Malus domestica*	0.40 ± 0.19	0.006 ± 0.08	0.52 ± 0.06	0
VAJ - *Acer campestre*	0.55 ± 0.27	0.015 ± 0.10	0	0



A B

Fig. 2. Correlations found between the HAA, as determined by FRAP method, and total phenolic concentration values (GAE units) of mistletoe leaves (A) and stems (B)

which are known as the major natural hydrophilic antioxidants. The phenolic content did not semnificativ differ between leaves and stems in methanolic extract. But, in the acetonic extract the content in the phenolic compounds are 100 time lower comparing to the methanolic extract



A

B

Fig. 3. A. Correlation between LAA, as determined by FRAP method, and carotenoid content (β-carotene values) in the acetone extract of mistletoe leaves. B. Correlation between HAA, as determined by FRAP method, and carotenoid content in the methanol extract of mistletoe leaves

(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 carotenoid.

Mistletoe leaves originating *Acer campestre* (VAJ), followed by VAM and VAF showed higher concentrations of phenolics, and also carotenoids especially in methanol.

Correlations between HAA, LAA and phenolics/ carotenoids content

The HAA values, as determined by FRAP method, were significantly correlated with the values of phenolic 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. 2.

The LAA values, as determined by FRAP method, were slightly correlated with the carotenoid content ($R^2 = 0.6327$) (Fig. 3A), and meanwhile HAA were no correlated with carotenoids ($R^2 = 0.168$) (Fig. 3 B).

Conclusions

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. 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 carotenoid 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

reason for low extraction rate in acetone. Also, to consider that in leaves carotenoids are represented mainly by lutein and not β-carotene, which would have higher antioxidant activity than other carotenoids.

Our recent results (Vicas *et al.*, 2008) showed that the aqueous *V. album* leaf extracts (VAM and VAS) which contains exclusively phenolics and no carotenoids, have antioxidant activity (as determined by TEAC, ORAC and DPPH assays) and can be considered as a good source for medicinal applications. The antioxidant capacity proved to be dependent on the host trees, VAM being a recommendable good source, either in water or alcoholic extract. No significant differences were noticed between the antioxidant content and activity of different plant parts of mistletoe (stems *versus* leaves).

In conclusion, the host trees of mistletoe may play a significant role in the elaboration of specific mistletoe antioxidants and becomes important parameter in the assessment of the mistletoe as a raw material for phytopharmaceutical formulas.

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