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HPLC Fingerprint of Bioactive Compounds and Antioxidant Activities of *Viscum album* from Different Host Trees

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Abstract

Viscum album L. is a medicinal plants used for many years as a remedy in the traditional medicine and in complementary cancer therapies. We investigated the influence of some host trees: *Acer campestre* (VAA), *Fraxinus excelsior* (VAF), *Populus nigra* (VAP), *Malus domestica* (VAM), and *Robinia pseudoacacia* (VAR), on the chemical composition and antioxidant activity of leaves and stems from *V. album*, as well the influence of the solvent (water and ethanol) used for extraction on biological activity. HPLC with photodiode array detector analysis of bioactive compounds from leaves and stems of mistletoes (*V. album*) hosted by five differents trees was performed. Antioxidant activities, determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH), Oxygen Radical Absorbance Capacity (ORAC), (Trolox-equivalent antioxidant capacity (TEAC) methods were compared with the quantification of total phenolics using the Folin -Ciocalteu reagent. *V. album* hosted by *Fraxinus excelsior* (VAF), recorded the highest level of total phenolic acids (108.64 μg/g dry matter) while the mistletoe hosted by *Malus domestica* (VAM) had the lowest level of total polyphenols (39.37 μg/g dry matter). In general, higher antioxidant activity was detected in the alcoholic than in the aqueous extracts. 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, as compared to leaves, in both solvents. Our results suggest that mistletoe extracts possess significant antioxidant activities that may be due to their bioactive compounds content, suggesting that this plant may be an important source of natural products with chemopreventive and chemotherapeutic activities.

Keywords: *Viscum album*, HPLC-fingerprint, DPPH, ORAC, TEAC, total polyphenols

Introduction

In search of novel sources of antioxidants, medicinal plants, especially those used in traditionally folk medicine, have been extensively studied in the last years for their antioxidant activity (Li *at al.*, 2008; Borneo *et al.*, 2009; Antal, 2010; Djeridane *et al.*, 2010; Kratchanova *et al.*, 2010). 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 (*Phorandendron serotinum* or *Phorandendron flavescens*), the European (*Viscum album* L.), and the Korean (*Viscum album* L. *coloratum*), most research 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 flavonoids and phenolic acids present in the plants are natural antioxidants. The most investigated chemical property of the phenolic compounds is their antioxidant activity. Quercetin, a flavonol, has been demonstrated to display a very strong antioxidant activity, often accompanied by antiviral and antibacterial activity (Materska, 2008). Phenolic acids are a ubiquitous plant constituents that arises from the metabolism of phenylalanine and tyrosine. Due to their phenolic nucleus and an extended side hain conjugation, they readly forms a resonance stabilized phenoxy radical which accounts for their potent antioxidant potential (Graf, 1992).

The phytochemical profile of mistletoe depends on the host trees of this plant (Luczkiewicz *et al.*, 2001). The main bioactive compounds found in mistletoe are lectins (glycoproteins with effects on cell-proliferation) and viscotoxin (a small protein molecule of 5 kDa) (Edlund *et al.*, 2000; Romagnoli *et al.*, 2000). Alkaloid concentration is usually low, and dependent on the host tree type (Peng *et al.,* 2005). It is considered that, flavonols like quercetin, kampherol and their methyl derivatives, and flavonone, naringenin, are the antioxidant compounds in mistletoe (Haas *et al.,* 2003). Among the phenolic acids presents in mistletoe, digallic and *o*-coumaric acid in the free or glycosilated forms (Luczkiewicz *et al.,* 2001) are also considered to be compounds with antioxidant activity.

The antioxidant capacity of many plant extracts is related to the presence of phenolic compounds (Miliauskas *et al.,* 2004; Aqil *et al.*, 2006; Roman *et al.,* 2009).

ÖnayUçar *et al.* (2006) investigated the antioxidant activity of methanol *V. album* extracts, using DPPH, ferric thiocyanate, and thiobarbituric acid methods. The authors showed that the antioxidant activity of mistletoe extracts depends both on the harvesting time and on the host trees. Also, the antioxidant effects of *Viscum coloratum* were found to be associated to the flavonoid fraction (Leu *et al.*, 2006; Shi *et al*., 2006; Yao *et al*., 2006).

Choudhary *et al*. (2010) isolated from the methanol extract of *V. album* six compounds which were found to posses anti-glycation activity, whereas 3-(4-acetoxy-3,5-dimethoxy)-phenyl-2E-propenyl-β-D-glucopyranoside and 4',5-dimethoxy-7-hydroxy flavanone exhibited antioxidant activity.

Viscum album aqueous extracts are used in complementary and alternative medicine as immunomodulators in cancer therapy. Based on this fact, we decided to evaluate the antioxidant activity of aqueous and ethanol extracts from leaves and stems of mistletoe.

The objective of the current study was to investigate the influence of host trees on the phenolics composition, and to compare the antioxidant activities of *V. album* samples originating from five different host trees.

Materials and methods

Plant material

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 plant materials were labeled according to the host trees, thus: *Acer campestre* (VAA), *Malus 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.

Extraction methods

Extraction and isolation of bioactive compounds for HPLC analysis

The leaves and stems of *V. album* from different host trees were dried rapidly, in an oven at 90^0C, 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 rotatory evaporator. Further, the aqueous extract was subjected to acid hydrolysis (1N HCl) for 2 hours, at 80^0C. 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.

Preparation of aqueous and ethanol mistletoe extracts for antioxidant activity assay

Fresh leaves and stems (2 g) were homogenized with 10 ml distilled water, or with 10 ml 98% ethanol using a 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.

HPLC separation of phenolic compound

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.

Determination of antioxidant activities

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

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:

$$\textbf{DPPH scavenging effect (\%)} = [(A_0 - A_s) \times 100]/A_0$$

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

Oxygen Radical Absorbance Capacity (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 x 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 (1998). The area under the curve (AUC) and the Net AUC of the standards and samples were determined using equations 1 and 2 respectively.

$$AUC=0.5+(R2/R1)+(R3/R1)+(R4/R1)+...+0.5(Rn/R1) \quad (Eq.1)$$

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

$$\text{Net AUC}=AUC_{sample}-AUC_{blank} \quad (Eq.2.)$$

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.

Trolox Equivalents Antioxidant Capacity (TEAC) assay

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. 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 $ABTS^{\cdot+}$ was monitored spectrophotometrically at 734 nm (Arnao *et al.* 2001). The results were expressed in mM Trolox equivalent/g fresh matter.

Total phenolics determinated by the 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).

Statistical analysis

All data were expressed as mean ± standard deviation (SD) of three replications for each mistletoe extract tested. The data obtained from the antioxidant activity tests were analyzed statistically by the one-way analysis of variance (ANOVA) and Tukey's Multiple Comparison Test to compare each extract to VAA extract. The probability level of less than 0.05 was accepted as significant. An overall antioxidant potency composite index was determined by assigning an index value of 100 to the best score for each test, and then calculated an index score for all other samples within the test as follows: antioxidant index score = [(sample score/best score) x 100] (Seeram *et al.,* 2008). The average of all four tests for each mistletoe extract was then taken for the antioxidant potency composite index.

Results and discussion

Evaluation of phenolics content by HPLC analysis

Quantitative data regarding the phenolic compounds composition of mistletoe extracts are shown in Tab. 1. HPLC chromatograms of phenolic acids from leaves and stems of *Viscum album* hosted by different trees: *Acer campestre* (VAA), *Fraxinus excelsior* (VAF*), Populus nigra* (VAP*), Malus domestica* (VAM), and *Robinia pseudoacacia* (VAR) are presented in Fig. 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). 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 com-

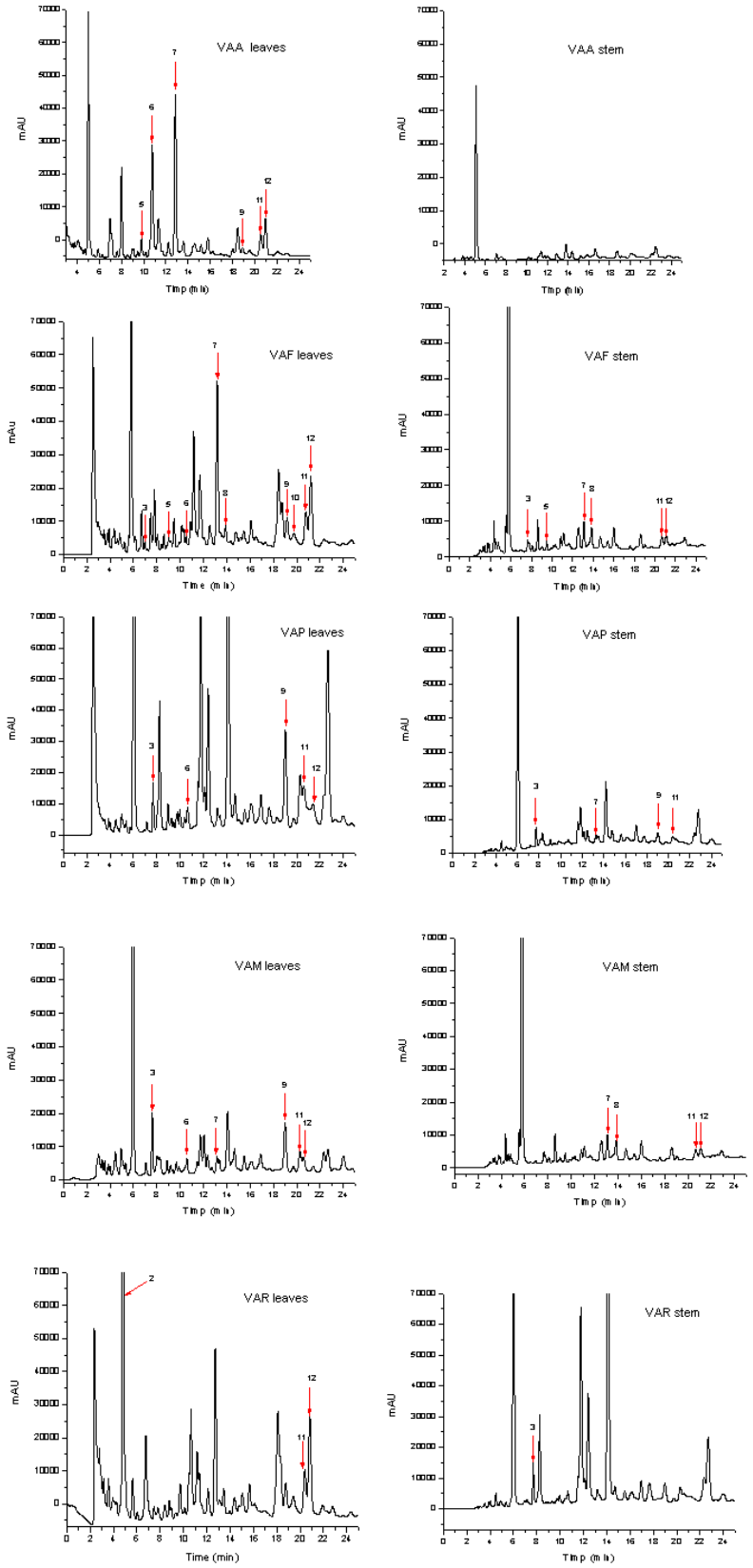

Fig. 1. HPLC chromatograms, used to fingerprint and evaluate quantitatively phenolic acids from leaves and stems of *V.album* harvested from different host trees : VAA –mistletoe from *Acer campestre*, VAF - mistletoe from *Fraxinus excelsior* , VAP mistletoe from *Populus nigra* , VAM - mistletoe from *Malus domestica* and VAR- mistletoe growing on *Robinia pseudoacacia*. The peak numbers are identified in Tab. 1

pared 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. Concentration of para-coumaric acid in theVAF 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 *Malus 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. 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.

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.

it has been not detect gentisic acid in any sample, nor naringenin, while quercetin was identified only in stems.

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

Tab. 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. For abbreviations, see materials and methods

Com. no.	Bioactive compounds	VAA *Acer campestre*		VAF *Fraxinus excelsior*		VAP *Populus nigra*		VAM *Malus 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	

*mean ± standard deviation (n=3)

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 *Malus 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*.

Antioxidant activity

Antioxidant properties of aqueous and ethanol extracts from leaves and stems of mistletoe were determined by three methods: their ability to deactivate stable $DPPH^{\cdot}$ radical, to inhibit peroxyl radical and deactivation of cation radicals $ABTS^{\cdot+}$.

DPPH inhibition by mistletoe extracts

The comparative antioxidant activity of *V.album* leaves hosted by different host trees, evaluated by the DPPH method is presented in Fig. 2. Beetwen all the samples examined, we observed significantly differences ($p < 0.001$) regarding to DPPH scavenging effect (%) of aqueous and ethanol extracts in leaves and stems, except the aqueous mistletoe samples from leaves and stems of VAP.

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 *Malus 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 ÖnayUçar *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.

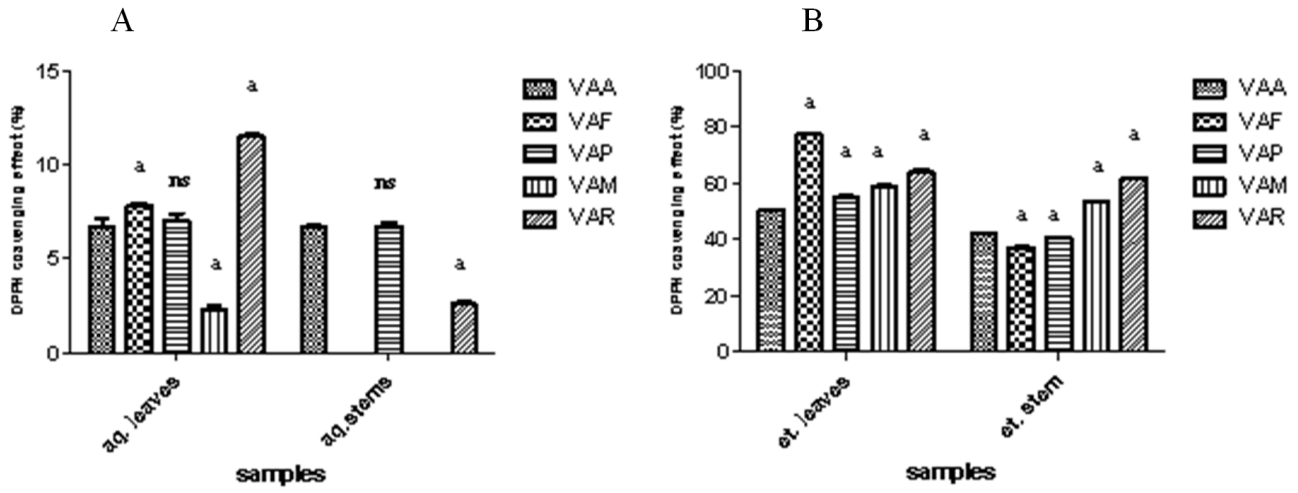

Fig. 2. DPPH scavenging effect (%) of aqueous (aq.) (A) *versus* ethanol (et.) (B) extracts of mistletoe leaves and stems harvested in July 2009, from five different host trees. The data were expresed as means ± standard deviation (n=3) and evaluated by one-way ANOVA to compare each extract to VAA extract. Differences were considered to be statistically significant if $p < 0.05$; ns- no significant differences; a- $p < 0.001$

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).

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.)

ORAC method

The values obtained by ORAC assays are shown in Tab. 2, and 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).

Our results showed that the ethanol extracts exhibit the highest ability to inhibit peroxyl radicals. These results are in concordance with data obtained by Al-Duais *et al.* (2009), who reported 103.3±2.5 mmol/100 g Trolox equivalents in ethanol leaf extracts of *Cyphostemma digitatum* (*Vitaceae*), as compared to 16.7 mmol/100 g Trolox equivalents in water extracts.

Tab. 2. Antioxidant capacity (as determined by the ORAC assay) of fresh leaves and stems from mistletoe (in water and ethanol extracts)

Mistletoe samples	ORAC (mM equivalents Trolox/ g fresh matter)*	
	Aqueous extract	Ethanol extract
Leaves		
VAA (*Acer campestre*)	6.33±1.42	9.42±0.25
VAF *(Fraxinus excelsior)*	3.55±2.65^{ns}	10.04±0.40^{ns}
VAP *(Populus nigra)*	3.96±0.31^{ns}	10.73±1.90^{ns}
VAM *(Malus domestica)*	1.87±1.25**	9.30±0.55^{ns}
VAR *(Robinia pseudoacacia)*	3.43±1.57^{ns}	9.97±1.84^{ns}
Stems		
VAA (*Acer campestre*)	5.49 ±2.73	9.37±0.14
VAF *(Fraxinus excelsior)*	2.42±3.68^{ns}	9.88±1.24^{ns}
VAP *(Populus nigra)*	2.07±0.27^{ns}	9.19±1.78^{ns}
VAM *(Malus domestica)*	1.52±1.25*	9.29±1.40^{ns}
VAR *(Robinia pseudoacacia)*	3.25±2.19^{ns}	9.79±0.87^{ns}

*The data were expresed as means ± standard deviation (n=3) and evaluated by one-way ANOVA to compare each extract to VAA extract. Differences were considered to be statistically significant if $p < 0.05$. ns-no significant differences; $^{*}p < 0.05$; $^{**}p < 0.01$

TEAC assay

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 $ABTS^{*+}$ (Tab. 3). According to the results obtained with TEAC assays, there were significant differences ($p < 0.001$) between all the extracts investigated.

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 higest antioxidant activity because they contain more bioactive compounds with the ability of scavenging cation-radicals $ABTS^{*+}$, as compared to ethanol extracts.

When aqueous extracts of green tea were compared with methanol extracts, Drużyńska *et al*. (2007) found that the former had the highest level of activity for scavenging cation-radicals $ABTS^{*+}$, because catechins are hydrosoluble. The authors supposed that catechins conferred the ability to scavenge cation-radicals to the green tea extracts.

Folin Ciocalteu method

Total phenolic content, determined by reaction with the Folin-Ciocalteu reagent, was expressed as mg gallic

acid equivalent/g fresh matter (GAE/FW). The results are shown in Tab. 4. Significant differences ($p < 0.001$) in the total phenolic content of aqueous and ethanolic extracts were observed. 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.

Tab. 3. Antioxidant potential (as determined by the TEAC assay) of fresh leaves and stems from mistletoe (in water and ethanol extracts)

Mistletoe Samples	TEAC (mM equivalents Trolox/g fresh matter)*	
	Aqueous extract	Ethanol extract
Leaves		
VAA (*Acer campestre*)	678.72±0.00	312.18±0.00
VAF *(Fraxinus excelsior)*	496.32±0.01***	461.09±0.11***
VAP *(Populus nigra)*	401.81±0.01***	365.78±0.01***
VAM *(Malus domestica)*	473.09±0.01***	215.28±0.01***
VAR *(Robinia pseudoacacia)*	592.05±0.01***	417.78±0.03***
Stem		
VAA (*Acer campestre*)	577.94±0.01	281.60±0.02
VAF *(Fraxinus excelsior)*	333.77±0.00***	258.00±0.00***
VAP *(Populus nigra)*	363.95±0.01***	306.68±0.01***
VAM *(Malus domestica)*	325.04±0.00***	213.92±0.02***
VAR *(Robinia pseudoacacia)*	541.84±0.01***	209.59±0.01***

* The data were expresed as means ± standard deviation (n=3) and evaluated by one-way ANOVA to compare each extract to VAA extract.. Differences were considered to be statistically significant if $p < 0.05$. *** $p < 0.001$

Tab. 4. Total phenolic content of *V. album* leaves and stems (aqueous and ethanol extracts)

Samples	mg GAE/g fresh matter	
	Aqueous extract	Ethanol extract
Leaves		
VAA (*Acer campestre*)	192.65±0.002	32.16±0.003
VAF *(Fraxinus excelsior)*	199.88±0.002***	46.85±0.005***
VAP *(Populus nigra)*	195.03±0.007***	45.03±0.02***
VAM *(Malus domestica)*	176.87±0.003***	31.32±0.008***
VAR *(Robinia pseudoacacia)*	200.51±0.00***	65.30±0.002***
Stem		
VAA (*Acer campestre*)	166.94±0.003	23.86±0.013
VAF *(Fraxinus excelsior)*	152.11±0.001***	36.22±0.014***
VAP *(Populus nigra)*	189.77±0.005***	42.45±0.008***
VAM *(Malus domestica)*	161.41±0.005***	30.22±0.001***
VAR *(Robinia pseudoacacia)*	186.93±0.003***	27.96±0.002***

** The data were expresed as means ± standard deviation (n=3) and evaluated by one-way ANOVA to compare each extract to VAA extract.. Differences were considered to be statistically significant if $p < 0.05$. *** $p < 0.001$

In a recent research paper (Vicaş *et al*., 2009), it was shown that methanolic extracts of *V. album,* harvested in December 2008, were rich in phenolics, potential antioxidants, with ferric reducing ability. In that case, mistletoe leaves originating from *Acer campestre* (VAA), showed the highest concentration of phenolics, followed by VAM and VAF. These results can be explained by the influence of harvesting time on the chemical composition and antioxidant activity.

The data presented in Tab. 5 show that the highest antioxidant index score was recorded from ethanol extracts, particulary for leaves VAF (97.85 %), followed by VAR (88.77%). For the aqueous extracts, the highest antioxidant index score was registered for VAA (85.93%), followed by VAR (80.47%). Both, leaf and stem extracts exhibited antioxidant activity, but generally it was higher for leaves.

The antioxidant potential determined by the ORAC and TEAC methods (Tab. 5) were combined into a single, mean antioxidant index score and correlated with the plenolics concentration, determined by the Folin-Ciocalteu method, as presented in Fig. 3. We found a weak correlation beetwen this index and the phenolics' content, non-significant for the aqueous extract ($R^2 = 0.167$) or the ethanolic extract ($R^2 = 0.62$).

Conclusions

The influence of the host tree may have a key role in the phenolic composition of mistletoe leaves or stems. In this study, 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 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, as compared to leaves, in both solvents. Our research paper, showed that the bioactive compounds and the antioxidant activity are present in leaves and also in stems, in all the misteltoe samples examinated (aqueous and ethanol). Of the samples examined, the best results were obtained with ethanolic 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. In our measurements, no correlations were established between the antioxidant score determined by DPPH, ORAC and TEAC methods. In the case of hydrosoluble extracts, DPPH measurements, based on free radi-

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Tab. 5. Individual Antioxidant index scores *and Mean antioxidant index scores** calculated index in leaves and stems of mistletoe (aqueous and ethanol extracts). The values are compared with total phenolics***, as determined by the Folin-Ciocalteu method (last columns)

Sample	Antioxidant index score *									
	DPPH		ORAC		TEAC		Mean score**		Total phenolics***	
	Aqueous extract	Ethanol extract	Aqueous extract	Ethanol extract	Aqueous extract	Ethanol extract	Aqueous extract	Ethanol extract	Aqueous extract	Ethanol extract
Leaves										
VAA	57.79	65.38	100.00	87.79	100.00	67.70	85.93	73.63	96.08	49.25
VAF	68.15	100.00	56.12	93.55	73.13	100.00	65.80	97.85	99.68	71.74
VAP	60.75	71.33	62.55	100.00	59.20	79.33	60.83	83.55	97.27	68.69
VAM	19.32	76.51	29.53	86.66	69.70	46.69	39.52	69.95	88.21	47.96
VAR	100.00	82.80	54.19	92.92	87.23	90.61	80.47	88.77	100.00	100.00
Stem										
VAA	57.79	54.46	86.73	87.33	85.15	61.07	76.56	67.62	83.26	36.54
VAF	0.00	48.18	38.21	92.08	49.18	55.95	29.13	65.40	75.86	55.47
VAP	58.49	52.70	32.67	85.66	53.62	66.51	48.26	68.29	94.64	65.01
VAM	0.00	69.45	23.96	86.59	47.89	46.39	23.95	67.48	80.50	46.28
VAR	22.28	79.93	51.34	91.28	79.83	45.46	51.15	72.22	93.23	42.82

*antioxidant index score = [(sample score/best score) x 100]; ** mean antioxidant index score by cumulating the DPPH, ORAC and TEAC scores

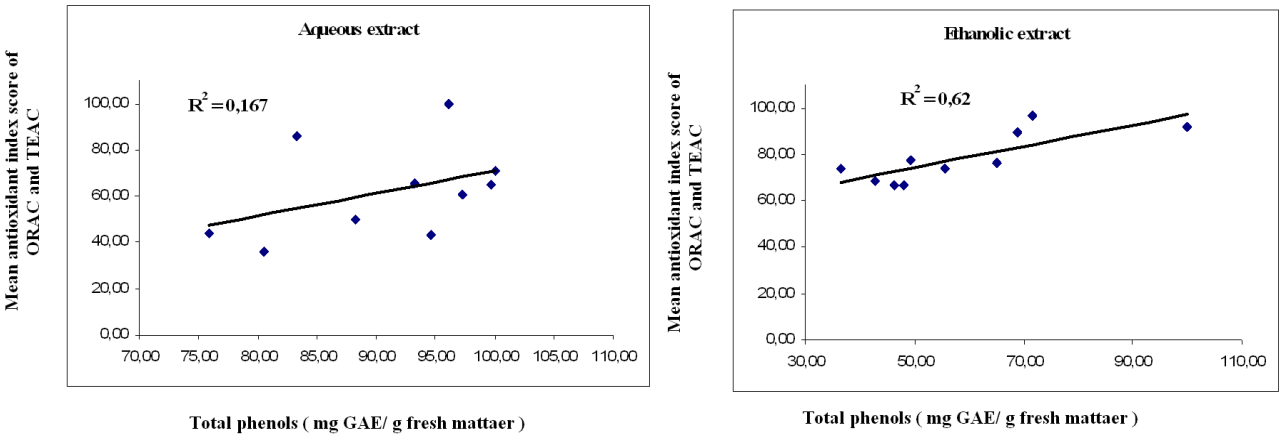

Fig. 3. Correlation between mean antioxidant index scores (determined in aqueous and ethanolic extracts), obtained by combination of the two antioxidant methods used (ORAC and TEAC), and total concentration of phenolics (mg GAE/g fresh matter) determined by the Folin-Ciocalteu method

cal scavenging, seem to be non-relevant, while the methods based on cation redox changes are more relevant for ethanol or aqueous extracts. So, we combined into a single, mean antioxidant index, the score obtained by the ORAC and TEAC methods. No significant correlations with the plenolics concentration were obtained, suggesting that the antioxidant potential is reflected by a more complex synergy of active molecules, not only phenolics. 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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