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# Determination of phenolic content and antioxidant properties of methanolic extracts from *Viscum album* ssp. *album* Beck.

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**Abstract:** Content of phenolic compounds and antioxidant properties of methanolic extracts from *Viscum album* ssp. *album* Beck. leaves and stems was determined. Mistletoe was collected from four different hosts (*Betula* L., *Tilia cordata* Mill., *Robina pseudoacacia* L, and *Salix alba* L.). Folin-Ciocalteu method, $AlCl_3$ method, method with Arnow reagent, and acid-butanol assay were used for determination of total phenols, flavonoids, phenolic acids and proanthocyanidins respectively. Antioxidant properties of the extracts were investigated with DPPH, ABTS and FRAP method. Total phenols were in range 7.02-13.52 mg GAE/g DW, flavonoids 2.29-5.05 mg RE/g DW, phenolic acids 0.62–2.84 mg caffeic CAE/g DW and proanthocyanidins 0.63 -4.83 mg LCE/g DW. Content of flavonoids and proanthocyanidins was higher in leaves than the stems. The highest antioxidant activity in leaves (68.93-86.89 μmol Trolox equivalents/g DW) and in stems (67.28-81.72 μmol Trolox equivalents/g DW) with DPPH, ABTS and FRAP method had mistletoe collected from *Robina pseudoacacia* L. Also, high correlation was obtained between total phenols, phenolic acids and proanthocyanidins content and antioxidant activity.

## INTRODUCTION

*Viscum album* L. (Loranthaceae) also known as European mistletoe or Common mistletoe is an evergreen semi-parasitic plants which grows on various host trees and shrubs. In Bosnia, the plant is represented by three subspecies: ssp. *album* Beck, ssp. *abietis* (Wiesb.) Abromeit. and ssp. *austriacum* (Wiesb.) Vollmann and can be found on approximately 46 different trees. The most abundant is *Viscum album* ssp. *album* Beck which has been identified on 42 leafy tree and shrubs in Bosnia (Treštić, 2015). Different pharmacological effects of *V. album* has been reported such as: antioxidant and antidiabetic (Orhan, Aslan, Sendogdu *et al*., 2005; Gray and Flatt, 1999), antiepileptic, antipsychotic and sedative (Gupta, Kazmi, Afzal, *et al.,* 2012), vasodilator and antihypertensive properties (Tenorio-Lopez, del Valle, Gonzalez, *et al.*, 2005; Ofem, Eno, Imoru, *et al.*, 2007). Also, some studies cover immunostimulant, antimutagenic and anticancer effects of *V. album* samples (Yesilada, Deliorman, Ergun, *et al*., 1998; Hong and Lyu, 2012; Sabova, Pilatova, Szilagy, *et al*., 2010; Siegle, Fritz, MacClellan, 2001).The main bioactive compounds in *V. album* are polysaccharides, phenylpropanes, lecitins, viscotoxins, alkaloids, flavonoids, caffeic and other acids (Ergun and Deliorman, 1995). Phytochemical composition depends on host tree and as the main antioxidant flavonoids and phenolic acids are reported (Luczkiewich, Cisowski, Kaiser, *et al.,* 2001). In order to estimate antioxidant activity of plant extracts different methods were used: TEAC (Trolox equivalent capacity), FRAP (ferric-reducing ability), TRAP (total radical trapping capacity, ORAC (oxygen radical absorbance capacity) (Wu, *et al*., 2004).

In this work, for the first time, we investigated total content of phenols, flavonoids, phenolic acids and proanthocyanidins in *V. album* ssp. *album* Beck. leaves and stems collected from several different hosts. Antioxidant activity of the extracts was determined with three methods: DPPH, ABTS and FRAP using Trolox as a standard. Correlations between investigated compounds and antioxidant activity in relation to their location (leaves and stems) are also reported.

## EXPERIMENTAL

All chemicals used in this work were highest purity grade obtained from Sigma-Aldrich Chemical Company (Germany).

***Plant material***

Leaves and stems were collected from four different hosts in area of Sarajevo at localities Vraca and Miljevići in November 2015. According to the host tree, plant material was marked as follows: VAR *(Robina pseudoacacia)*, VAT (*Tilia cordata*), VAB (*Betula* L.), and VAS (*Salix alba*). The plant material was dried in a ventilated place at room temperature and stored in paper bags until use. A voucher specimens of the plants were deposited at herbarium of Department of Forest Ecology at Faculty of Forestry.

***Extraction***

Powdered dry sample of leaves or stems (0.5 g) was extracted with 80% aqueous methanol (2 x 12 mL) during 30 minutes in an ultrasonic bath (Elmasonic S 60H). The mixture was centrifuged for 15 min at 3000 rpm (Centric 322 B, Techtnica). Obtained supernatants were combined, filtrated and volume of the extract was adjusted with extraction mixture up to 25 mL. Extracts were stored at -20ºC until analysis.

***Determination of total phenols***

Total phenolic content was determined with Folin-Ciocalteu method (Singelton, Orthofer, Lamuela-Raventos, 1974). Briefly, 0.1 mL of diluted sample was mixed with 7.9 ml of distillated water and 0.5 mL of Folin-Ciocalteu reagent was added. Approximately, after 5 minutes, 20% $Na_2CO_3$ (1.5 mL) was added to the reaction mixture, and it was left to stand for 30 minutes in a water bath at 40ºC. Blank was prepared by using distillated water instead extract. Absorbance of the colored product was measured at 765 nm and a calibration curve was prepared with gallic acid as a standard. Final results are expressed as mg gallic acid equivalents (GAE) per gram of dry sample. All spectrophotometric measurements were done with Shimadzu UV-mini 1240 spectrophotometer.

***Determination of total flavonoids***

Total flavonoids were determined with $AlCl_3$ method (Quettier, Gressier, Vasseur, *et al*., 2000) using rutin as a standard. Equal volumes of extracts and reagent were mixed and left to stand at room temperature for 1 hour, and absorbance of the colored product was measured at 415 nm against the blank. Sample blank was also used in the analysis. Final results are expressed as mg rutin equivalents (RE) per gram of dry sample.

***Determination of total phenolic acids***

Quantification of total phenolic acid was done with Arnow reagent (Gawlic-Dziki, 2012). Briefly, 1 mL of diluted sample was mixed with 5 mL of water, 1 mL HCl (0.5 M), 1 mL of Arnow reagent and 1 mL of NaOH (1 M). Calibration curve with caffeic acid standards was established. Absorbance was measured at 490 nm, and the results are expressed as mg caffeic acid equivalents (CAE) per gram of dry sample.

***Determination of total proanthocyanidins***

Proanthocyanidins were determined by butanol-HCl assay (Hagerman, Harvey-Mueller, Makkar, 2000b). 0.5 mL of diluted extracts was mixed with 3.0 mL of the butanol-HCL reagent (butanol-HCl 95:5 *v/v)* and 0.1 ml of ferric reagent (2% ferric ammonium sulfate in 2 M HCl). Samples were heated at boiling water bath for 60 minutes. Absorbance was measure before and after heating at 550 nm against blank. Results are expressed as mg leucocyanidin equivalents (LCE) using specific absorbance of leucocyanidin 460.

***Determination of antioxidant capacity***

***DPPH assay***

Antioxidant activity of the extracts was measured by the method of Brand-Williams, Cuvelier, Berset, (1995) and Thaipong, Boonprakob, Crosby, *et al.* (2006). The extracts were dissolved in methanol while DPPH stock solution (0.094 M) was prepared in methanol on a daily basis and diluted to absorbance of 1.1±0.02 at 515 nm. Aliquots of extracts (0.1 mL) were mixed with 1.9 mL of DPPH solution and left at room temperate in the dark for 30 minutes. Standard solutions of Trolox were used for preparation of a calibration curve. Final results are expresses as μmol of Trolox equivalents (TE) per gram of dry sample.

***ABTS assay***

To determine ABTS assay, the method of Ree, Pellegrini, Proteggente, *et al.* (1999) modified by Thaipong, *et al.* (2006) was used. In brief, stock solutions of 7 mM ABTS and 2.45 mM potassium persulfate were mixed in equal volumes and left to stand in the dark for 12-16 hours in order to obtain ABTS radical cation solution ($ABTS^{\bullet+}$). An aliquot of freshly prepared $ABTS^{\bullet+}$ solution was diluted with methanol to obtain absorbance of 1.1 ±0.02 units at 734 nm. Plant extracts (0.1 mL) were allowed to react with 1.9 mL of working $ABTS^{\bullet+}$ solution for 6 minutes after what the reduction in absorbance was measured. Standard solutions of Trolox were used to prepare a calibration curve, and the results are expressed as μmol of Trolox (TE) per gram of dry sample.

***FRAP assay***

Ferric reducing antioxidant power (FRAP) was determined by method of Benzie and Strain (1999). FRAP reagent was prepared by mixing 300 mM acetate buffer, pH= 3.6; 10 mM TPTZ (2,4,6-tripiridil-s-triazine) in 40 mM HCl acid and 20 mM $FeCl_3$ in the ratio 10:1:1.

Obtained solution was heated at 37ºC for 30 minutes in a water bath. Plant extracts (0.1 mL) were mixed with 1.9 mL of working FRAP solution and left in the dark for additional 30 minutes. Absorbance of the formed blue complex was measured at 593 nm against a blank. Standard solutions of Trolox were used to prepare a calibration curve, and the results are expressed as μmol of Trolox (TE) per gram of dry sample.

## RESULTS AND DISCUSSION

Quantitative content of investigated bioactive compounds in leaves and stems of mistletoe samples hosted by different trees: *Robina pseudoacacia (VAR), Tilia cordata (VAT), Betula* L. (VAB), *Salix alba (*VAS) are presented in Table 1. In leaves, total phenols were in range 8.75-13.52 mg GAE/g DW, total flavonoids 3.34-5.05 mg RE/g DW, total phenolic acids 1.19-2.39 mg CAE/g DW, and total proanthocyanidins 2.29-4.83 mg LCE/g DW. In stems, total phenols were in range 7.02-13.51 mg GAE/g DW, total flavonoids 2.29-2.84 mg RE/g DW, total phenolic acids 0.62-2.84 mg CAE/g DW, and total proanthocyanidins 0.63-1.19 mg LCE/g DW. Generally, higher content of flavonoids and proanthocyanidins was found for leaves extracts compared with stems for all samples. Mistletoe leaves hosted by *Salix alba* (VAS) had higher content of all investigated compounds than the stems. However, stems of mistletoe from VAT and VAB were richer in the content of total phenols and total phenolic acids than their leaves. The highest content of total phenols (13.52 mg GAE/g DW), total flavonoids (5.05 mg RE/g DW) and total phenolic acids (2.23 mg CAE/g DW) was found in mistletoe leaves from VAR, while the lowest levels of phenols (8.75 mg GAE/g DW), flavonoids (3.34 mg RE/g DW) and phenolic acids (1.19 mg CAE/g DW) was determined in mistletoe leaves from VAS. The highest content of proanthocyanidins was found in mistletoe leaves fromVAB while the lowest was in mistletoe leaves from VAR.

The mistletoe stems extracts hosted by *Salix alba* (VAS) had the lowest content of all investigated compounds while the highest content was found in mistletoe stems hosted by VAR for phenols (13.51 mg GAE/g DW), phenolic acids (2.84 mg CAE/g DW) and proanthocyanidins (1.19 mg LCE/g DW). Content of flavonoids in mistletoe stems from VAR and VAT were very similar 2.82 and 2.84 mg RE/g DW respectively.

Vicaş, Rugina, Leopold, *et al.* (2011) pointed that influence of the host tree may have a key role in the phenolic composition of mistletoe leaves or stems. Orhan, Senol, Hosbas, *et al.* (2014) investigated *V. album s*pp *album* hosted by different trees and found that *V. album* from *Robina pseudoacacia* L. had higher content of flavonoids (5.53 ±0.74 mg QE/g extract) than *V.album* from *Salix sp.* (4.66 ±0.73 mg QE/g extract).

**Table 1**: Total phenols (TP), total flavonoids (TF), total phenolic acids (TPA), total proanthocyanidins (TPC) in leaves (l) and stems (s) of mistletoe

| Samples | **TP** (mgGAE/g) | **TF** (mgRE/g) | **TPA** (mgCAE/g) | **TPC** (mgLCE/g) |
|---|---|---|---|---|
| | | Leaves | | |
| **VAR(l)** | 13.52±0.01 | 5.05±0.02 | 2.39±0.1 | 2.29±0.2 |
| **VAT(l)** | 10.34±0.01 | 4.56±0.05 | 1.31±0.19 | 4.11±0.23 |
| **VAB(l)** | 9.28±0.01 | 3.44±0.02 | 1.62±0.01 | 4.83±0.16 |
| **VAS(l)** | 8.75±0.006 | 3.34±0.01 | 1.19±0.02 | 4.78±0.07 |
| | | Stems | | |
| **VAR(s)** | 13.51±0.02 | 2.82±0.02 | 2.84±0.002 | 1.19±0.2 |
| **VAT(s)** | 11.32±0.01 | 2.84±0.02 | 1.59±0.003 | 1±0.32 |
| **VAB(s)** | 11.11±0.01 | 2.6±0.003 | 1.96±0.001 | 0.83±0.13 |
| **VAS(s)** | 7.02±0.007 | 2.29±0.02 | 0.62±0.005 | 0.63±0.03 |

Mistletoe from VAR-*Robina pseudoacacia*; VAT- *Tilia cordata*; VAB-*Betula* L.; VAS- *Salix alba*

On the other hand, the content of phenols was higher in plant hosted by *Salix sp.* (29.41 ±1.45 mg GAE/g extract) than *R. pseudoacacia* (27.08 ±1.87 mg GAE/ g extract). As it was observed by Vicaş, Rugina, Socaciu (2011) differences in phenolic contents highly depends on harvesting seasons and generally they are higher for samples harvested in spring than in autumn. The same authors found that leaves of mistletoe are richer in phenolic contents (total phenols and phenolic acids) than the stems which is similar to our results. (Vicaş, Rugina, Leopold, *et al.*, 2011).

### *Antioxidant activity*

Antioxidant activity of mistletoe leaves and stems extracts were investigated by three methods: DPPH, ABTS and FRAP method. The results are presented in Table 2.

**Table 2.** Antioxidant activity in mistletoe leaves (l) and stems (s) determined with DPPH, ABTS and FRAP method.

| | **DPPH** (µmolTE/g) | **ABTS** (µmolTE/g) | **FRAP** (µmolTE/g) |
|---|---|---|---|
| | | Leaves | |
| **VAR(l)** | 68.93±0.12 | 72.21±0.10 | 86.89±0.60 |
| **VAT(l)** | 34.01±0.86 | 43.39±0.11 | 54.39±0.51 |
| **VAB(l)** | 32.12±0.27 | 41.20±0.14 | 48.13±0.18 |
| **VAS(l)** | 31.25±0.19 | 43.79±0.31 | 42.78±0.24 |
| | | Stems | |
| **VAR(s)** | 67.28±0.12 | 67.28±0.42 | 81.72±0.11 |
| **VAT(s)** | 42.41±0.29 | 51.59±0.41 | 67.71±0.44 |
| **VAB(s)** | 45.00±0.20 | 46.92±0.46 | 59.12±0.15 |
| **VAS(s)** | 30.13±0.21 | 36.86±0.26 | 38.04±0.34 |

Mistletoe from VAR-*Robina pseudoacacia*; VAT- *Tilia cordata*; VAB-*Betula* L.; VAS- *Salix alba*

The comparative antioxidant activity of *V. album* leaves is presented in Figure 1 and for stems in Figure 2. In mistletoe leaves, antioxidant activity varied from 31.25 to 68.93 µmol Trolox/g DW for DPPH, from 41.20 to 72.21 µmol Trolox/g DW for ABTS, and from 42.78 to 86.89 µmol Trolox/g DW for FRAP method (Table 2).

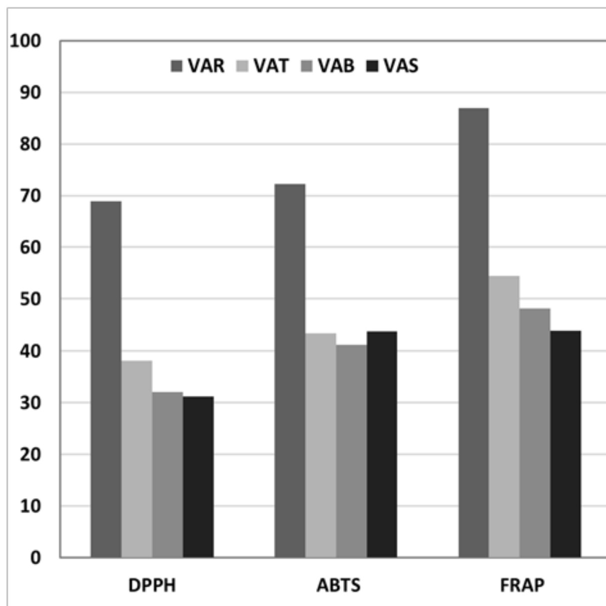
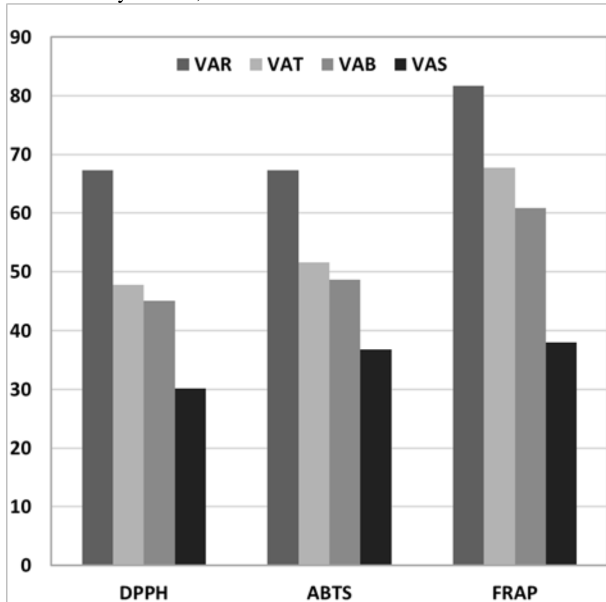



**Figure 1**: Antioxidant activity (µmol TE/g DW) of mistletoe leaves determined by DPHH, ABTS and FRAP method.

Generally, antioxidant activity decreased in the order: FRAP>ABTS>DPPH. (Figure 1). This can be explained by the fact that methanolic extracts of *V. album* harvested in autumn were richer in phenolic as antioxidant with ferric reducing ability (Vicaş,Prokisch, Rugina, *et al.*, 2009). Antioxidant activity of mistletoe leaves extracts from VAR and VAS were higher than the corresponding stems extracts. Interestingly, antioxidant activity of mistletoe leaves extracts collected from VAT and VAB were lower than corresponding stems extracts. This can be explained by higher contents of phenols and phenolic acids found in mistletoe stems from VAT and VAB than in leaves which may contribute to higher values of antioxidant activity in stems. (Figure 2).

**Figure 2**: Antioxidant activity (µmol TE/g DW) of mistletoe stems determined by DPHH, ABTS and FRAP method.




The highest antioxidant activity was determined in mistletoe leaves VAR while the lowest values were found for mistletoe leaves from VAS (DPPH and FRAP) and VAB (ABTS). These results are in agreement with other investigators who found that antioxidant capacity differs depending on the host trees (Onay-Ucar, Karagoz, Arda, 2006; Oluwaseun and Ganiyu, 2007; Vicaş *et al.,* 2009).

Antioxidant activity for stems varied from 30.13 to 67.28 µmol Trolox/g DW for DPPH, from 36.86 to 67.28 µmol Trolox/g DW for ABTS and from 38.04 to 81.72 µmol Trolox/g DW for FRAP (Table 2). Antioxidant activity for stems decreased in the following order: VAR >VAT> VAB >VAS for all three methods (Figure 2).

The highest values of antioxidant activity were determined for mistletoe stems from VAR and the lowest for stems from VAS.

Correlations investigated between antioxidant activity and bioactive compounds in leaves and stems are given in Table 3.

**Table 3**: Correlations between antioxidant activity and phenolic compounds in mistletoe leaves and stems

| | DPPH | ABTS | FRAP |
|---|---|---|---|
| | | Leaves | |
| **TP** | 0.9768 | 0.9004 | 0.9931 |
| **TF** | 0.7338 | 0.5918 | 0.7724 |
| **TPA** | 0.848 | 0.8346 | 0.8701 |
| **TPC** | 0.9667 | 0.9354 | 0.9802 |
| | | Stems | |
| **TP** | 0.9146 | 0.9116 | 0.9812 |
| **TF** | 0.688 | 0.6999 | 0.873 |
| **TPA** | 0.9316 | 0.9199 | 0.8912 |
| **TPC** | 0.9399 | 0.9495 | 0.965 |

TP- total phenols, TF-total flavonoids, TPA – total phenolic acids, TPC-total proanthocyanidins

We found strong correlation between phenols, phenolic acids, proanthocyanidins and antioxidant activity. In case of leaves, correlation coefficients were in range $r^2$= 0.9004-0.9768 for phenols; $r^2$= 0.8346-0.8701 for phenolic acids and for proanthocyanidins $r^2$= 0.9354-0.9802. In case of stems, they were in range 0.9116-0.9812 for phenols, 0.8912-0.9316 for phenolic acids and 0.9399-0.965 for proanthocyanidins. The lowest correlation coefficients were found for flavonoids $r^2$= 0.5918–0.7724 in leaves and for stems $r^2$= 0.688-0.873 which suggest that these compounds also contribute to the antioxidant activity of the extracts but in some less extend. Other investigators found that phenolics and flavonoids may contribute mostly to antioxidant activity of mistletoe extracts (Papuc, Crivineanu, Goran, *et al.,* 2010; Pietrzak, Nowak, Olech, 2014; Orhan *et al.,* 2014).

## CONCLUSION

In all investigated samples, flavonoids and proanthocyanidins were present in higher amounts in leaves than the stems. Mistletoe from VAR was the richest in the content of phenols and phenolic acids. Mistletoe from VAS had the lowest content of phenols, flavonoids and phenolic acids in leaves and lower content of all investigated compounds in stems compering with other samples.

Mistletoe from VAT and VAB had higher content of total flavonoids and proanthocyanidins in leaves while stems were richer in content of total phenols and phenolic acids.

Antioxidant activity of *V.album* spp *album* leaves and stems generally were similar and decreased in the order: FRAP>ABTS>DPPH for leaves and stems. The highest antioxidant activity was found for mistletoe leaves and stems from VAR. This indicate that host three is important parameter in assessment of the mistletoe as a row material in medical and pharmaceutical application.
Antioxidant activity was highly correlated with content of total phenols, proanthocyanidins and phenolic acids. Moderate correlation was noticed between antioxidant capacity and total flavonoid content.

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**Summary/Sažetak**

Određivan je sadržaj fenolskih jedinjenja i antioksidacijska svojstva metanolnih ekstrakata listova i grančica imele *Viscum album* ssp. *album* Beck. Imela je prikupljana sa četiri različita domaćina (*Betula* L., *Tilia cordata* Mill., *Robina pseudoacacia* L, and *Salix alba* L.). Folin –Ciocalteu metoda, $AlCl_3$ metoda, metoda sa Arnow reagensom i kiselinsko-butanolna metoda korištene su u određivanju ukupnih fenola, flavonoida, fenolskih kiselina i proantocijanidina respektivno. Antioksidacijska svojstva ekstrakata ispitivana su upotrebom DPPH, ABTS i FRAP metode. Ukupni fenoli kretali su se u granicama 7,02-13,52 mg GAE/g s.u., flavonoidi 2,29-5,05 mg RE/ g s.u., fenolske kiseline 0,61-2,84 mg CAE/g s.u. i proantocijanidini 0,63-4,83 mg LCE/g s.u. Dobiveni sadržaj flavonoida i proantocijanidina je bio veći u listovima u odnosu na grančice. Najveća antioksidacijska aktivnost (68,93-86,89 µmol ekvivalenata Troloxa/g s.u. za listove i 67,28-81,72 µmol ekvivalenata Troloxa/g s.u. za grančice) za DPPH, ABTS i FRAP metodu određena je za imelu prikupljenu sa *Robina pseudoacacea* L. Takođe, dobivena je visoka korelacija između sadržaja ukupnih fenola, fenolskih kiselina, proantocijanidina i antioksidacijske aktivnosti.