

**PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY OF *VISCUM ALBUM*  
LEAVES AND TWIGS FROM VARIOUS HOST TREES**

**Sadržaj fenola i antioksidacijski kapacitet lišća i grančica *Viscum album* sa različitim  
domaćina drveća**

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**Abstract**

Phenolic compounds content and antioxidant capacity from *Viscum album* ssp. *album* Beck. leaves and twigs extracts were determined. Common mistletoe was collected from four hosts (*Crataegus monogyna*, *Malus domestica*, *Prunus cerasifera*, and *Populus x canadensis*). Folin-Ciocalteu method, AlCl<sub>3</sub> method, method with Arnou reagent, and acid-butanol assay were applied for determination of total phenols, flavonoids, phenolic acids and proanthocyanidins respectively. DPPH, ABTS and FRAP methods were applied in the determination of antioxidant capacity of the extracts. Total phenols were in the range 7.16-11.70 mg GAE/g DW, flavonoids 2.115-4.668 mg RE/g DW, phenolic acids 0.811-1.789 mg caffeic CAE/g DW, and proanthocyanidins 0.71-2.62 mg LCE/g DW. Content of phenols and flavonoids was higher in the leaves than the twigs. The highest antioxidant capacity in leaves (50.86-65.93 μmol Trolox equivalents/g DW) was determined for mistletoe collected from *Crataegus monogyna* while the highest capacity in twigs had mistletoe collected from *Malus domestica* (67.28-81.72 μmol Trolox equivalents/g DW). Also, high correlation has been noticed between total phenols, flavonoids, phenolic acids and antioxidant capacity for *V. album* leaves. For twigs, high correlation was obtained between phenols and antioxidant capacity, and in some moderate extends for proanthocyanidins.

**Key words:** antioxidant activity, phenols, leaves, mistletoe, twigs, *Viscum album*

**INTRODUCTION- Uvod**

European mistletoe or Common mistletoe (*Viscum album* L.) belongs to Loranthaceae family. It is an evergreen semi-parasitic plant which grows on various host trees and shrubs. The plant is represented by three subspecies which can be found on approximately 46 different trees in Bosnia: ssp. *album* Beck., ssp. *abietis* (Wiesb.) Abromeit. and ssp. *austriacum* (Wiesb.) Vollmann. *Viscum album* ssp. *album* is the most abundant and it has been identified on 42 leafy trees and shrubs (TREŠTIĆ, 2015). It has been reported that *V. album* shows various pharmacological effects:

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antioxidant and antidiabetic (GRAY and FLATT, 1999, ORHAN et al, 2005), vasodilator and antihypertensive properties (TENORIO-LOPEZ et al, 2005; OFEM, 2007), antiepileptic, antipsychotic and sedative (GUPTA et al, 2012). According to some studies, *V. album* shows immunostimulant, antimutagenic and anticancer effects (YESILADA et al, 1998; SIEGLE et al, 2001; SABOVA et al, 2010; HONG and LYU, 2012).

Polysaccharides, phenylpropanes, lecithins, viscotoxins, alkaloids, flavonoids, caffeic and other acids appear to be the main bioactive compounds in *V. album* (ERGUN and DELIORMAN, 1995). The main antioxidants are flavonoids and phenolic acids although phytochemical composition mainly depends on a host tree (LUCZKIEWICH et al, 2001). Estimation of antioxidant capacity of the plant extracts can be carried out with different methods: TEAC (Trolox equivalent capacity), FRAP (ferric-reducing ability), TRAP (total radical trapping capacity, and ORAC (oxygen radical absorbance capacity) (WU et al, 2004).

The aim of this work was to determine total content of different phenolic compounds: phenols, flavonoids, phenolic acids and proanthocyanidins in *V. album* ssp. *album* leaves and twigs collected from hawthorn, cherry plum, apple and Canadian poplar trees. Antioxidant activity of the extracts was determined with DPPH, ABTS and FRAP methods with Trolox as a standard. Correlations between investigated compounds and antioxidant capacity of the extracts obtained from leaves and twigs are reported. According to our best knowledge, these are the first results on the investigated species from selected host trees.

## **MATERIAL AND METHODS – Materijal i metode**

### **Plant material – Biljni materijal**

Leaves and twigs were collected from four different hosts in area of Sarajevo in November 2015. Plant material for leaves (l) and twigs (t) was marked as follows: VAC (*Crataegus monogyna*), VAM (*Malus domestica*), VAPR (*Prunus cerasifera*), and VAPO (*Populus x canadensis*). The plant material was dried in a ventilated place at room temperature and stored in paper bags until use. Voucher specimens of the plants were deposited at herbarium of Department of Forest Ecology at Faculty of Forestry University of Sarajevo.

### **Chemicals and reagents – Hemikalije i reagensi**

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

### **Sample extracts preparation - Priprema ekstraktata uzoraka**

Powdered dry sample of leaves or twigs (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, Technica). Obtained supernatants were combined, filtrated and volume of the extract

was adjusted with extraction mixture up to 25 mL. Extracts were stored at -20°C until analysis.

#### **Determination of total phenols - *Određivanje ukupnih fenola***

Folin-Ciocalteu method is used for the determination of total phenols content (SINGELTON et al, 1974). Diluted sample was mixed with 7.9 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent was added. After 5 minutes, 20% Na<sub>2</sub>CO<sub>3</sub> (1.5 mL) was added in the reaction mixture, and it was left to stand for 30 minutes in a water bath at 40°C. Blank was prepared by using distilled 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 (DW). All spectrophotometric measurements were done with Shimadzu UV-mini 1240 spectrophotometer.

#### **Determination of total flavonoids – *Određivanje ukupnih flavonoida***

Determination of total flavonoids was done with AlCl<sub>3</sub> method (QUETTIER et al, 2000), with rutin as a standard. Shortly, equal volumes of extracts and reagent are 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 for the correction of sample absorbance. Final results are expressed as mg rutin equivalents (RE) per gram of dry sample.

#### **Determination of total phenolic acids – *Određivanje ukupnih fenolnih kiselina***

Arnow reagent was used for the quantification of total phenolic acids as described by GAWLIC-DZIKI, (2012). Diluted sample (1 mL) was mixed with distilled water (5 mL), HCl (1 mL, 0.5 M), Arnow reagent (1 mL) and NaOH (1 mL, 1 M). A calibration curve was established with caffeic acid as a standard. 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 – *Određivanje ukupnih proantocijanidina***

Butanol-HCl assay was used for the determination of proanthocyanidins as described by HAGERMAN (2000). Diluted extract (0.5 mL) was mixed with butanol-HCl reagent (3.0 mL, butanol-HCl 95:5 v/v) and ferric reagent (0.1 mL, 2% ferric ammonium sulphate 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 activity – *Određivanje antioksidacijske aktivnosti DPPH method - DPPH metoda***

Methods of BRAND-WILLIAMS et al (1995) and THAIPONG et al (2006) were used for determination of antioxidant activity of the extracts. Dilution of the extracts was done with methanol. DPPH stock solution (0.094 M) was prepared in methanol on a daily basis and diluted to absorbance of  $1.1 \pm 0.02$  at 515 nm before use. Aliquots of extracts (0.1 mL) were mixed with 1.9 mL of DPPH solution and left at room temperature in the dark for 30 minutes. Standard solutions of Trolox were used for preparation of a calibration curve. Final results are expressed as  $\mu\text{mol}$  of Trolox equivalents (TE) per gram of dry sample.

#### **ABTS method - ABTS metoda**

The method of RE et al (1999) modified by THAIPONG et al. (2006) was used in ABTS assay. Stock solutions of ABTS (7 mM) and potassium persulfate (2.45 mM) were mixed in equal volumes and left to stand in the dark for 12-16 hours prior use. Freshly prepared  $\text{ABTS}^+$  solution was diluted with methanol to absorbance of  $1.1 \pm 0.02$  units at 734 nm. Plant extracts (0.1 mL) were allowed to react with 1.9 mL of working  $\text{ABTS}^+$  solution for 6 minutes after that the reduction in absorbance was measured. Standard solutions of Trolox were used to prepare a calibration curve, and the results are expressed as  $\mu\text{mol}$  of Trolox (TE) per gram of dry sample.

#### **FRAP method – FRAP metoda**

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  $\text{FeCl}_3$  in the ratio 10:1:1. Obtained solution was heated at  $37^\circ\text{C}$  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  $\mu\text{mol}$  of Trolox (TE) per gram of dry sample.

### **RESULTS AND DISCUSSION- *Rezultati i diskusija***

In this work quantitative content of investigated bioactive compounds in leaves and twigs of mistletoe samples hosted by *Crataegus monogyna* (VAC), *Malus domestica* (VAM), *Prunus cerasifera* (VAPR), and *Populus x canadensis* (VAPO) trees are investigated and the results are presented in Table 1. Total phenols were in range 7.32-11.70 mg GAE/g DW, total flavonoids 3.576-4.468 mg RE/g DW, total phenolic acids 0.811-1.789 mg CAE/g DW, and total proanthocyanidins 0.71-1.42 mg LCE/g DW in leaves. In twigs, total phenols were in range 7.16-9.92 mg GAE/g DW, total flavonoids 2.115-2.707 mg RE/g DW, total phenolic acids 1.045-1.138 mg CAE/g DW, and total proanthocyanidins 1.31-2.62 mg LCE/g DW.

Table 1. Total phenols (TP), total flavonoids (TF), total phenolic acids (TPA), total proanthocyanidins (TPC) in leaves (l) and twigs (t) of mistletoe

Tabela 1. Ukupni fenoli (TP), ukupni flavonoidi (TF), ukupne fenolne kiseline (TPA), ukupni proantocijanidini (TPC) u listovima (l) i grančicama (t) imele

Samples	TP (mgGAE/g±SD)	TF (mgRE/g±SD)	TPA (mgCAE/g±SD)	TPC (mgLCE/g±SD)
Leaves				
<b>VAC(l)</b>	11.70±0.01	4.668±0.022	1.789±0.006	1.39±0.03
<b>VAM(l)</b>	9.98±0.01	4.460±0.014	1.166±0.001	1.12±0.01
<b>VAPR(l)</b>	9.27±0.02	3.722±0.021	1.069±0.001	1.42±0.02
<b>VAPO(l)</b>	7.32±0.01	3.576±0.012	0.811±0.002	0.71±0.07
Twigs				
<b>VAC(t)</b>	8.38±0.02	2.707±0.009	1.138±0.006	1.83±0.02
<b>VAM(t)</b>	9.92±0.01	2.327±0.009	1.119±0.006	1.31±0.03
<b>VAPR(t)</b>	7.16±0.01	2.115±0.005	1.046±0.011	2.62±0.05
<b>VAPO(t)</b>	7.43±0.02	2.559±0.003	1.045±0.003	2.48±0.07

SD – standard deviation

Mistletoe collected from VAC-*Crataegus monogyna*; VAM-*Malus domestica*; VAPR-*Prunus cerasifera*; VAPO-*Populus x canadensis*

SD – standardna devijacija

Imela prikupljena sa VAC-*Crataegus monogyna*; VAM-*Malus domestica*; VAPR-*Prunus cerasifera*; VAPO-*Populus x canadensis*

Generally, higher content of phenols and flavonoids was found for leaves extracts compared to twigs for all samples. This is similar to the results of VICAŞ et al. (2011a) who found that leaves extracts are richer in the content of phenolic compounds than the twigs extracts. Mistletoe leaves hosted by VAC, VAM and VAPR had higher content of phenols, flavonoids and phenolic acids than the twigs. However, twigs of mistletoe from VAPO were richer in the content of total phenolic acids than their leaves. The highest content of total phenols (11.70 mg GAE/g DW), total flavonoids (4.668 mg RE/g DW) and total phenolic acids (1.789 mg CAE/g DW) was found in mistletoe leaves from VAC, while the lowest levels of phenols (7.32 mg GAE/g DW), flavonoids (3.576 mg RE/g DW) and phenolic acids (0.811 mg CAE/g DW) was determined in mistletoe leaves from VAPO. The highest content of proanthocyanidins was found in mistletoe leaves from VAPR (1.42 LCE/g DW) while the lowest content was in mistletoe leaves from VAPO (0.71 LCE/g DW). The highest content of flavonoids (2.707 mg RE/g DW) and phenolic acids (1.138 mg CAE/g DW) had the mistletoe twigs extracts hosted by *C. monogyna* (VAC), content of proanthocyanidins (2.62 LCE/g DW) was the highest with mistletoe from VAPR and total phenols (9.92 mg GAE/g DW) were the highest for mistletoe from VAM. These results are lower than the results obtained for mistletoe collected from *Robinia pseudoacacia* obtained in our previous investigation (TAHIROVIĆ and BAŠIĆ, 2017). Also, *V. album* from *R. pseudoacacia* L. had higher content of flavonoids than other investigated hosts (ORHAN et al 2014). It is also found that mistletoe from *Malus domestica* (17.48 mg %) is richer in phenolic acids content than *Populus nigra* (12.34

mg %) (LUCZKIEWIEZ et al 2001), which is in agreement with the results in this study. The lowest content of phenols (7.16 mg GAE/g DW) and flavonoids (2.115 mg RE/g DW) was found in mistletoe twigs collected from VAPR. In mistletoe twigs collected from VAPO content of phenolic acid was 1.045 mg CAE/g DW and for mistletoe from VAM had the lowest content of proanthocyanidins (1.31 LCE/g DW). Influence of the host tree may have a key role in the phenolic composition of mistletoe leaves or twigs (VICAŞ et al 2011A; ORHAN et al 2014). As it was observed by VICAŞ et al (2011B) 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 twigs. (VICAŞ et al, 2011B).

#### **Antioxidant capacity- antioksidacijski kapacitet**

Antioxidant capacity of mistletoe leaves and twigs extracts was investigated by DPPH, ABTS and FRAP methods. The results are presented in Table 2. In mistletoe leaves, antioxidant capacity varied from 25.57 to 50.86 µmol Trolox/g DW for DPPH, from 39.98 to 57.67 µmol Trolox/g DW for ABTS, and from 40.28 to 65.93 µmol Trolox/g DW for FRAP method. Generally, antioxidant capacity decreased in the order: FRAP>ABTS>DPPH.

Table 2. Antioxidant capacity in mistletoe leaves (l) and twigs (t) determined with DPPH, ABTS and FRAP methods

*Tabela 2. Antioksidacijski kapacitet za listove (l) i grančice (t) imele određeni sa DPPH, ABTS i FRAP metodama*

	<b>DPPH</b> (µmolTE/g±SD)	<b>ABTS</b> (µmolTE/g±SD)	<b>FRAP</b> (µmolTE/g±SD)
Leaves			
<b>VAC (l)</b>	50.86±0.23	57.67±0.07	65.93±0.19
<b>VAM (l)</b>	43.56±0.29	52.67±0.39	62.24±0.06
<b>VAPR(l)</b>	35.54±0.81	47.52±0.18	52.64±0.04
<b>VAPO(l)</b>	25.77±0.14	39.98±0.10	40.28±0.02
Twigs			
<b>VAC (t)</b>	38.09±0.16	40.18±0.52	45.66±0.29
<b>VAM(t)</b>	44.63±0.19	50.62±0.41	57.13±0.04
<b>VAPR (t)</b>	37.14±0.15	39.79±0.36	43.33±0.01
<b>VAPO(t)</b>	37.54±0.15	40.43±0.43	44.14±0.14

SD- standard deviation

Mistletoe collected from VAC-*Crataegus monogyna*; VAM-*Malus domestica*; VAPR-*Prunus cerasifera*; VAPO- *Populus x canadensis*

SD-standarna devijacija

Imela prikupljena sa VAC-*Crataegus monogyna*; VAM-*Malus domestica*; VAPR- *runus cerasifera*; VAPO-*Populus x canadensis*

Antioxidant capacity of *V. album* has been investigated by different researches (ONAY-UCAR et al, 2006; OLUWASEUN and GANIYU, 2007; VICAŞ et al, 2008, 2009, 2011B; PAPUC et al, 2010; SHARMA and BHAT, 2010; ROMAN et al 2010). As it was presented by VICAŞ et al (2008, 2009, 2011B ) extracts of *V. album* harvested in autumn were richer in phenolics as antioxidant with ferric reducing ability. These differences were explained by different environmental factors. Antioxidant capacity of mistletoe leaves extracts from VAC was higher than the corresponding twigs extracts. Interestingly, antioxidant capacity of mistletoe leaves extracts collected from VAPO was lower than corresponding twigs extracts. This can be explained by higher contents of phenols and phenolic acids found in mistletoe twigs from VAPO than in leaves which may contribute to higher values of antioxidant capacity in twigs. Antioxidant capacity for leaves decreased in the following order: VAC>VAM>VAPR>VAPO for all three methods. The highest antioxidant capacity was determined in mistletoe leaves from VAC while the lowest values were found for mistletoe leaves from VAPO. These results are in agreement with other investigators who found that antioxidant capacity differs depending on the host trees and harvesting time (ONAY-UCAR et al, 2006; OLUWASEUN and GANIYU, 2007; VICAŞ et al, 2009).

Antioxidant capacity for twigs varied from 37.14 to 44.63 µmol Trolox/g DW for DPPH, from 39.79 to 50.62 µmol Trolox/g DW for ABTS and from 43.33 to 57.13 µmol Trolox/g DW for FRAP (Table 2). Antioxidant capacity for twigs decreased in the following order: VAM>VAC>VAPO>VAPR and for leaves in the order VAM>VAPO=VAC>VAPR for DPPH and FRAP methods. The highest values of antioxidant capacity were determined for mistletoe twigs from VAM and the lowest for twigs from VAPR. VICAŞ et al (2009B) found that methanol extracts of *V. album* from *M. domestica* had the highest antioxidant capacity (0.14 mg/g fw). Results obtained for antioxidant capacity in this study are lower compared to the results for mistletoe leaves and twigs extracts collected from *Robina pseudoacacia* (ONAY-UCAR et al, 2006; TAHIROVIĆ and BAŠIĆ, 2017).

Correlations between antioxidant capacity and bioactive compounds in leaves and twigs are given in Table 3. For leaves, correlation coefficients were in range  $r^2=0.9293-0.9818$  for phenols;  $r^2=0.8787-0.9117$  for flavonoids and for phenolic acids  $r^2=0.7302-0.8593$ . Moderate correlations were found between phenolic acids and antioxidant capacity ( $r^2=0.5082-0.5458$ ) which suggest that these compounds also contribute to the antioxidant capacity of the extracts but in some less extend.

Table 3. Correlations between antioxidant activity and phenolic compounds in mistletoe leaves and twigs

Tabela 3. Korelacije između antioksidacijskog kapaciteta i fenolnih jedinjenja u listovima i grančicama imele.

	DPPH	ABTS	FRAP
Leaves			
<b>TP</b>	0.9776	0.9818	0.9293
<b>TF</b>	0.9117	0.8907	0.8787
<b>TPA</b>	0.8593	0.8499	0.7302
<b>TPC</b>	0.5082	0.5458	0.5378
Twigs			
<b>TP</b>	0.8974	0.8348	0.9211
<b>TF</b>	0.0245	0.0473	0.0154
<b>TPA</b>	0.274	0.1948	0.3112
<b>TPC</b>	0.7738	0.6919	0.8075

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

TP- ukupni fenoli, TF-ukupni flavonoidi, TPA- ukupne fenolne kiseline, TPC-ukupni proantocijanidini

In case of twigs, correlations were in range of 0.8348-0.9211 for phenols and 0.6919-0.8075 for proanthocyanidins. The lowest correlation coefficients were found for flavonoids  $r^2=0.5918-0.7724$  in leaves and for twigs  $r^2=0.688-0.873$ . Other investigators found that phenolics and flavonoids may contribute mostly to antioxidant capacity of mistletoe extracts (PAPUC et al, 2010; ORHAN et al, 2014; PIETRZAK et al, 2014). No correlation was observed between other investigated compounds and antioxidant capacity.

### CONCLUSIONS – Zaključci

In all investigated samples, flavonoids are presented in higher amounts in the leaves than the twigs. Mistletoe from *Crataegus monogyna* was the richest in the content of flavonoids and phenolic acids. Mistletoe from *Populus x canadensis* had the lowest content of phenols, flavonoids and phenolic acids in leaves. In twigs, the lowest content of phenols and flavonoids was found for mistletoe from *Prunus cerasifera*, phenolic acids from *Populus x canadensis* and proanthocyanidins from *Malus domestica*.

Antioxidant capacity of *V.album* spp. *album* leaves and twigs generally were similar and decreased in the order: FRAP>ABTS>DPPH for leaves and twigs. The highest antioxidant capacity was found for mistletoe leaves from *Crataegus monogyna* and mistletoe twigs from *Malus domestica*. This indicates that host tree is important parameter in assessment of the mistletoe as a raw material in medical and pharmaceutical applications. Antioxidant capacity was highly correlated with content of total phenols and flavonoids for leaves and phenols and proanthocyanidins for twigs.



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## SAŽETAK

U ovoj studiji je izvršeno određivanje sadržaja fenolskih jedinjenja i antioksidacijskog kapaciteta ekstrakata listova i grančica imele (*Viscum album* ssp. *album* Beck.) spektrofotometrijskim metodama. Imela je prikupljena sa četiri domaćina: *Crataegus monogyna*, *Malus domestica*, *Prunus cerasifera* i *Populus x canadensis*. Određivanje ukupnih fenola, flavonoida, fenolskih kiselina i proantocijanidina vršeno je Folin–Ciocalteu metodom,  $AlCl_3$  metodom, Arnou reagensom i kiselinsko-butanolnom metodom. Antioksidacijski kapacitet ekstrakata ispitivan je DPPH, ABTS i FRAP metodama. Ukupni fenoli kretali su se u granicama 7,16-11,70 mg GAE/g s.u., flavonoidi 2,115-4,668 mg RE/ g s.u., fenolske kiseline 0,811-1.789 mg CAE/g s.u. i proantocijanidini 0,71-2,62 mg LCE/g s.u. Dobiveni sadržaj fenola i flavonoida veći je u listovima u odnosu na grančice. Listovi imele prikupljeni sa *C. monogyna* imali su najveći antioksidacijski kapacitet (67,28-81,72  $\mu$ mol TE/g s.u.), a grančice imele prikupljene sa *M. domestica* (44,63–57.13  $\mu$ mol TE/g s.u.). Uočena je visoka korelacija između sadržaja ukupnih fenola, fenolskih kiselina, proantocijanidina i antioksidacijske aktivnosti za listove, kao i ukupnih fenola i proantocijanidina za grančice imele.

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