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Antioxidant capacity of wild-growing orange mullein (*Verbascum phlomoides* L.)

Radenka Kolarov[1], Dejan Prvulović[1], Sonja Gvozdenac[2]

[1]*University of Novi Sad, Faculty of Agriculture, Novi Sad, Serbia*
[2] *Institute of Field and Vegetable Crops Novi Sad, Serbia*

Abstract

Orange mullein is a biennial plant belonging to the figwort (*Scrophulariaceae*) family. The vivid yellow flowers are arranged in spikes located on the top of the stem. It is a drought and cold-tolerant plant requiring much sunlight that grows on pastures, roadsides, and in dry weed associations. The aim of this study was to assess the effect of different solvents (water, 70% acetone, 70% methanol, and 70% ethanol) on the assessment of antioxidant capacity of leaves and flowers of orange mullein. Total phenolics are higher in the leaves and reach a value of up to 15.70 mg of gallic acid per one g of dry leaf weight, while flavonoids are more dominant in flowers and reach a value of 5.82 mg of quercetin per one g of dry flower. Correlation was performed between total phenolics, total tannins, total flavonoids, and antioxidant tests. It is the flavonoids that are mainly responsible a the high antioxidant activity establishing a correlation with all the tests performed, including FRAP, ABTS, DPPH, total antioxidant activity, total reduction capacity, and NBT reduction test.

Key words: orange mullein, polyphenols, antioxidant capacity, Kopaonik

Introduction

Plant species of the genus *Verbascum* L. are rich in biologically active compounds (Drobot′ko et. al, 1958; Markosyan et. al, 1976), widely used in folk medicine and homeopathic practice (Sepetchyan et. al, 1953) in many countries,

and are interesting as a raw plant source for pharmaceutical industry. *Verbascum phlomoides* L., orange mullein, is an evergreen species in the *Scrophulariaceae* family, native to Europe and Asia Minor and it is known as a pollinator attracting plant. *V. phlomoides* is biennial, but mulleins can be annual, biennial, or perennial (Turker & Gurel, 2005). Its height can reach up to 1.8 m, and it has dense yellow flowers from early to late summer. Phenolic compounds, including stress-linked phytochemicals, have been related to favourable impacts, which are resulting from the consumption of fruit and vegetables, particularly due to their antioxidant activity (Lin et al., 2016). Balasundram et al. (2006) reviewed the antioxidant activity, occurrence, and latent uses of phenolic compounds in plants and agri-industrial by-products. Under those reports, fruit, vegetables, and beverages are the principal sources of phenolic compounds in the human diet. Plant polyphenols as dietary antioxidants in human health and disease might protect against oxidative damage. As natural antioxidants, phenolic compounds are found abundantly in plant food and beverages, which play vital parts in pabulum and healthcare.

Orange mullein has many nutritional values and it is known as a medicinal plant with a strong antioxidant capacity. In particular, in Georgia it is used as a cure for many diseases (Shreter, 1972). Flowers are highly valued herbal drugs used in the treatment of inflammation, asthma, spasmodic coughs, and other respiratory tract diseases. Furthermore, it has diuretic, analgesic, expectorant, and antiseptic properties. Active compounds, such as flavonoids, phenylethanoid and neolignan glycosides, saponins, iridoid, and monoterpene glycosides are described as the most abundant classes of secondary metabolites in the investigated plant (Armatu et al., 2011). The aim of present study was to evaluate phenolic content and antioxidant capacity of leaves and flowers of *V. phlomoides* from the Kopaonik mountain extracted with four different solvents.

Material and methods

The orange mullein from the Kopaonik mountain, Serbia, was analysed. Harvesting of the plants was conducted in July 2020 during the flowering period using only the flowering tops in full blossom and young leaves. The collected plant material was dried in a dark place to a constant weight, and then ground into a fine powder using a laboratory mill. One gram of plant material was extracted with either 50 ml of 70% acetone, 70% ethanol, 70% methanol, or distilled water overnight. The extract was filtered and refrigerated at -20^{o}C until analysis.

The content of total phenols (TP) and total tannins (TT) was determined using a Folin-Ciocalteu reagent (Nagavani & Raghava Rao, 2010). To determine the content of total phenols reaction mixture contained 3.4 ml of distilled water, 200 µl of Folin-Ciocalteu solution, and 20 µl of the sample. After five minutes 400 µl of Na_2CO_3 solution was added. All measurements were performed in three

replications. Gallic acid was used as a standard both for TP and TT content. The extracts were expressed as mg of gallic acid per one g of dry weight (DW) of plant material (mg of gallic acid/ g DW). A reaction mixture consisting of 0.1 g of polyvinylpolypyrrolidone (PVPP), 1 ml of H_2O, and 1 ml of extract was used for determination of TT. After precipitation and filtration, this reaction mixture was used as an extract. The procedure was the same as for the TP content measurement. The amount of total tannins is expressed as mg of gallic acid per one g of dry weight of plant material (mg of GA/ g DW).

The method for determining the content of total flavonoids (TF) is based on the method by Markham (1989). Reaction mixture contained 0.5 ml of water, 1.5 ml of $AlCl_3$, and 200 µl of extract. Quercetin was used as a standard and TF content is expressed as mg quercetin per one g of dry weight of plant material (mg Q/g DW).

The FRAP method was applied as previously reported by Benzie and Strain (1996). 2 ml of FRAP reagent and 10 µl of extracts were added to the reaction mixture. The calibration curve was constructed using a series of dilutions of trolox solution. The FRAP total reducing capability is expressed in mg trolox equivalents per one g of dry weight of plant material (mg TE/g DW).

Spectrophotometric determination of the scavenging activity of the studied extracts is based on monitoring the transformation of $DPPH^{\cdot}$ radicals (2,2-diphenyl-1-picrylhydrazyl radicals) into reduced (DPPH-H) form (Przybylski et al., 1998). 3 ml of DPPH reagent and 10 µl of extract were used. Trolox was used as a standard, and $DPPH^{\cdot}$ radical scavenging activity is expressed in mg trolox equivalents per one g of dry weight of plant material (mg TE/g DW).

The ability of extracts of the tested plant species to neutralize the radical cation 2.2′-azino-bis- (3-ethylbenzothiazoline-6-sulfonic acid), $ABTS^{\cdot+}$, was measured according to the method by Re et al. (1999). 3 ml of 70% $ABTS^{\cdot}$ + reagent and 20 µl of sample were added. The calibration curve was constructed using a series of dilutions of trolox. $ABTS^{\cdot+}$ radical scavenging activity is expressed in mg trolox equivalents per one g of dry weight of plant material (mg TE/g DW).

The total antioxidant activity (TA) of plant extracts was evaluated by the phosphomolybdenum method (Kalaskar et. al, 2014). 100 µl of extract solutions were combined with 1 ml of reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The standard curve for total antioxidant capacity was plotted using trolox as a standard solution. An antioxidant capacity was expressed as mg trolox equivalents per one g of dry weight of plant material (mg TE/g DW).

A reducing power assay by Saha et al. (2013) (total reduction capacity-TRC) was performed as follows: 2.0 ml of plant extract and 2.5 ml of 1% Potassium Ferricyanide $[K_3Fe(CN)_6]$ solution were added into the test tubes. The tubes were incubated for 10 minutes at 50°C to complete the reaction. 2.5 ml of 10% trichloroacetic acid solution was added into the test tubes. 2.5 ml experimental

solution was withdrawn from the mixture and mixed with 2.5 ml of distilled water and finally 0.5 ml of 0.1% ferric chloride solution was added. The standard curve for total antioxidant activity was plotted using trolox solution. Total reduction capacity was expressed as mg of trolox equivalents per one g of dry weight (mg TE/g DW).

The superoxide free radical scavenging activity was carried out by using the NBT (nitroblue tetrazolium) test (Kalaskar et. al 2014). 200µl of EDTA, 100 µl of NBT, 50 µl of riboflavin, 2.5 ml of phosphate buffer pH 8.0, and 200 µl of extracts were mixed in the test tubes. The results were expressed as number of International Units (IU) of superoxide dismutase (SOD) equivalents per one gram of dry plant material (IU SOD/g).

Data of all measurements obtained in triplicate are expressed as the mean ± standard deviation. Multiple regression analyses were used to determine correlation among variables, using STATISTICA 13.5 (Dell, USA). The significance of differences in measured and calculated parameters between the samples was determined by Duncan's multiple range tests with the confidence of $p \leq 0.05$.

Results and discussion

There is considerable interest in identifying natural antioxidants from plants that protect against free radical damage as an alternative to synthetic medicines (Spiridon et al., 2011). Phenolic compounds especially flavonoids are widely distributed in almost all plants. Phenolics exert antioxidant, anticancer, antidiabetic, cardiovascular effect, anti-inflammatory, protective effects in neurodegenerative disorders, and many others therapeutic effects (Pandey & Rizvi, 2009). Flavonoids possess a wide range of pharmacological effects including anticancer, antioxidant, antidiabetic, immunological, anti-inflammatory, antipyretic, antibacterial, antifungal, antiviral, antiulcer, antiosteoporotic, endocrine, hepatoprotective, vasorelaxant, antiatherosclerotic, antithrombogenic, cardioprotective, anxiolytic, and many other effects (Al-Snafi, 2020). Content of phenolic compounds and antioxidant capacity of extracts from flowers and leaves of orange mullein extracted with four different solvent systems are presented in Table 1. It can be seen that TP contents in orange mullein leaves range from 5.50 to 15.70 mg of GA/DW, being the lowest in the aqueous, both for leaves and flower, and the highest in the methanol extract. However, the samples in ethanol and acetone belong to the same homogeneous group. TP in flowers are much lower than in leaves, with the maximum of 9.80 mg of GA/DW in the acetone extract. The TP values for the flower by the author Marian et al. (2018) were almost ten times higher, but the TF data matched in an aqueous solution. TT content is the highest in methanol extracts, both in flower and leaves samples, while the leaves extracts have twice the amount of the TT in leaves. The TF, both from flowers and leaves, are best extracted in acetone, then ethanol, methanol, and least in aqueous solution, with

approximately similar values. The content of polyphenols and antioxidant capacity depend on the solvent used for extraction. The results obtained in this work showed that the acetone extract of orange mullein possesses the strongest antioxidant activity, followed by the methanol and ethanol extracts. Antioxidant tests indicate that both the leaves and flowers of the investigated plant have the same distribution of antioxidant activity. The leaves have higher antioxidant activity in the FRAP, DPPH, and NBT reduction test. FRAP reaches a value of up to 48.18 mg TE/g DW, while DPPH 24.99 mg TE/g DW, both in the methanol sample. Flowers have higher antioxidant activity compared to leaves in the ABTS test, TRC, and TAC. In two tests out of three, they are better extracted by acetone. In the total antioxidant activity test, the highest obtained value is 279.11 mg TE/g DW.

Tab. 1. Contents of polyphenolics and antioxidant activity in *V. phlomoides* flowers and leaves extracted with different solvent systems

	Total phenolic[1]	Total tannins[1]	Total flavonoids[2]	FRAP[3]	DPPH[3]	ABTS[3]	Total reduction capacity (TRC)[3]	Total Antioxidant Capacity (TAC) assay by phosphomolybdenum method[3]	NBT reduction test[4]
Verbascum phlomoides leaf									
Water	$5.50^{d} \pm 1.09$	$3.79^{d} \pm 0.51$	$0.02^{f} \pm 0.01$	$16.78^{d} \pm 1.36$	$6.99^{e} \pm 0.97$	$20.39^{e} \pm 3.21$	$12.56^{e} \pm 2.21$	$98.75^{e} \pm 25.98$	$2.42^{d} \pm 0.23$
Methanol	$15.70^{a} \pm 0.68$	$8.58^{a} \pm 0.63$	$3.99^{d} \pm 0.070$	$48.18^{a} \pm 3.66$	$24.99^{a} \pm 0.23$	$53.13^{b} \pm 3.04$	$50.42^{cd} \pm 4.58$	$191.29^{c} \pm 6.08$	$4.18^{a} \pm 0.04$
Ethanol	$15.23^{a} \pm 0.99$	$8.45^{a} \pm 0.72$	$4.36^{cd} \pm 0.63$	$48.15^{a} \pm 4.18$	$24.36^{ab} \pm 1.74$	$51.97^{b} \pm 4.56$	$49.10^{cd} \pm 14.67$	$192.25^{c} \pm 15.54$	$3.84^{b} \pm 0.087$
Acetone	$15.22^{a} \pm 0.22$	$5.11^{b} \pm 0.38$	$4.91^{b} \pm 0.05$	$41.00^{b} \pm 1.77$	$22.96^{b} \pm 0.38$	$53.21^{b} \pm 0.81$	$59.43^{bc} \pm 8.62$	$223.10^{b} \pm 5.96$	$3.96^{ab} \pm 0.018$
Verbascum phlomoides flower									
Water	$4.96^{d} \pm 0.11$	$2.24^{e} \pm 0.14$	$0.52^{e} \pm 0.08$	$9.02^{e} \pm 1.18$	$7.08^{e} \pm 0.44$	$18.81^{e} \pm 2.81$	$32.79^{de} \pm 8.8$	$113.84^{e} \pm 8.77$	$2.58^{d} \pm 0.54$
Methanol	$8.95^{bc} \pm 0.31$	$4.71^{bc} \pm 0.27$	$4.21^{cd} \pm 0.04$	$25.36^{c} \pm 0.57$	$15.51^{cd} \pm 0.34$	$37.65^{c} \pm 3.60$	$77.56^{ab} \pm 12.51$	$169.16^{cd} \pm 4.68$	$2.94^{c} \pm 0.06$
Ethanol	$8.61^{c} \pm 0.22$	$4.19^{cd} \pm 0.44$	$4.59^{bc} \pm 0.14$	$23.88^{c} \pm 1.86$	$14.85^{d} \pm 0.51$	$30.76^{d} \pm 2.89$	$88.53^{a} \pm 24.1$	$166.80^{d} \pm 9.60$	$2.50^{d} \pm 0.17$
Acetone	$9.80^{b} \pm 0.10$	$2.79^{e} \pm 0.07$	$5.82^{a} \pm 0.20$	$25.07^{c} \pm 0.39$	$16.70^{c} \pm 0.37$	$72.52^{a} \pm 5.18$	$58.50^{bc} \pm 0.68$	$279.11^{a} \pm 20.36$	$3.23^{c} \pm 0.09$

The data are mean values ± standard error

a, b, c, d, e, f the values without the same superscript within each column differ significantly ($p \leq 0.05$)

[1] Expressed as mg of gallic acid equivalents /g of dry weight
[2] Expressed as mg of quercetin /100 g of dry weight
[3] Expressed as mg of trolox equivalents/g of dry weight
[4] Expressed as IU SOD/g of dry weight

Polyphenols may exert antioxidant activity in different ways. To gain more valuable information on the antioxidant potential of the extracts of *V. phlomoides* flowers and leaves, correlation between TP, TT and TF, and the results of different antioxidant capacity tests (FRAP, DPPH, ABTS, TRC, TAC, and NBT)

were made (Table 2). Good correlation of TP with FRAP, DPPH, ABTS, TAC, and NBT was demonstrated (r = 0.599 and 0.971, respectively). Results showed no correlation with total reduction capacity test. Total tannins, unlike TP, show a weak correlation with antioxidant tests. Correlation with FRAP, DPPH, and NBT reduction test, ranged from r = 0.736 to 0.886. Correlations between total flavonoids and antioxidant tests were strong for all performed assays.

Tab. 2. Correlations between polyphenolic compounds and antioxidant tests in *V. phlomoides* flowers and leaves

	FRAP	DPPH	ABTS	Total reduction capacity (TRC)	Total Antioxidant Capacity (TAC) assay by phosphomolybdenum method	NBT reduction test
Total phenolic						
Correlation coefficient (r)	0.967*	0.971*	0.689*	0.247	0.599*	0.921*
Coefficient of determination (r^2)	0.934	0.942	0.475	0.061	0.359	0.848
p	0.000	0.000	0.000	0.244	0.002	0.000
Total tannins						
Correlation coefficient (r)	0.886*	0.805*	0.347	0.057	0.182	0.736*
Coefficient of determination (r^2)	0.785	0.648	0.120	0.003	0.033	0.541
p	0.000	0.000	0.097	0.793	0.396	0.000
Total flavonoids						
Correlation coefficient (r)	0.595*	0.751*	0.830*	0.696*	0.890*	0.547*
Coefficient of determination (r^2)	0.354	0.563	0.690	0.485	0.792	0.299
p	0.002	0.000	0.000	0.000	0.000	0.006

*Values marked with asterix are statistically significant $p \geq 0.05$

Conclusion

The results on the investigated *V. phlomoides* extracts showed that the contents of phenolic compounds and antioxidant activity of extracts are strongly affected by the solvent used in the extraction process. Out of four solvents evaluated in this study for the extraction of the polyphenolic compounds, the use of 70% acetone scored the best for the flowers and 70% methanol for the leaves. The highest antioxidant activity is also noticeable in 70% acetone for most of the assays (four out of six). It can be concluded that total flavonoids, which have a strong correlation with all antioxidant tests, contribute most to the antioxidant activity of *V. phlomoides*.

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Антиоксидативни капацитет дивље дивизме (*Verbascum phlomoides* L.)

Раденка Коларов[1], Дејан Првуловић[1], Соња Гвозденац[2]

[1]Универзитет у Новом Саду, Пољопривредни факултет, Нови Сад, Србија
[2] Институт за ратарство и повртарство Нови Сад, Србија

Сажетак

Дивизма је двогодишња биљка која припада породици смокава (*Scrophulariaceae*). Цвјетови су распоређени у класове који се налазе на врху стабљике, жуте боје. У питању је биљка отпорна на сушу и хладноћу, којој је потребно много сунчеве свјетлости и која расте на пашњацима, поред путева, најчешће у коровским заједницама. Циљ овог рада био је да се процијени утицај различитих растварача (вода, 70% ацетон, 70% метанол и 70% етанол) на процјену антиоксидативног капацитета листова и цвјетова дивизме. Укупни феноли су већи у листовима и достижу вриједност до 15,70 mg галне киселине по g суве масе листа, док су флавоноиди доминантнији у цвјетовима и достижу вриједност од 5,82 mg кверцетина по g сувог цвијета. Урађена је корелација између тестова укупних фенола, укупних танина, укупних флавоноида и антиоксидантних тестова. Флавоноиди су ти који су одговорни за високу антиоксидативну активност успостављајући корелацију са свим спроведеним тестовима, укључујући FRAP, ABTS, DPPH, укупну антиоксидативну активност, укупни редукциони капацитет и NBT тест редукције.

Кључне ријечи: дивизма, полифеноли, антиоксидантни капацитет, Копаоник

Corresponding author: Radenka Kolarov
E–mail: kolar.rada@gmail.com

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