

Quality control, phenolic content and *in vitro* antioxidant potential of orange mullein flower and leaf

Original Article

Abstract:

Verbascum species are traditionally used in the treatment of respiratory disorders. Orange mullein (*Verbascum phlomoides* L.) is one of three species that are the official source of drug mullein flower (*Verbasci flos*). Our study aimed to determine the overall quality of the *V. phlomoides flos* and *V. phlomoides folium*, the contents of polyphenols, tannins, flavonoids, and phenylpropanoid derivatives alongside the antioxidant capacity of their ethanolic extracts. Quality control tests were conducted according to monographs of Ph. Eur. 10.0, secondary plant metabolites were quantified spectrophotometrically while the antioxidant potential was estimated by two complementary methods (DPPH free radical scavenging and β -carotene bleaching assay). The higher contents of examined compounds were determined in flower extract although the leaf extract showed a stronger antioxidant capacity. We can conclude that *Verbascum phlomoides* is a rich source of biologically valuable phenolics, it possesses considerable antioxidant capacity and investigated herbal drugs fulfill pharmacopeia's quality requirements.

Key words:

Verbascum phlomoides, overall quality, polyphenols, flavonoids, antioxidant activity

Apstrakt:

Ispitivanje opšteg kvaliteta, sadržaj fenola i *in vitro* antioksidativni potencijal cveta i lista krupnocvetne divizma

Vrste roda *Verbascum* se tradicionalno koriste u lečenju respiratornih poremećaja. Krupnocvetna divizma (*Verbascum phlomoides* L.) je jedna od tri vrste koje se koriste za dobijanje biljne droge cvet divizme (*Verbasci flos*). Cilj naše studije bio je da se utvrdi opšti kvalitet droge *V. phlomoides flos* i *V. phlomoides folium*, kao i sadržaj polifenola, tanina, flavonoida i derivata fenilpropanoida i ispita antioksidativna aktivnost etanolnih ekstrakata. Opšti kvalitet droge ispitan je u skladu sa Ph. Eur. 10.0, sekundarni biljni metaboliti su određeni spektrofotometrijski, dok je antioksidativna aktivnost ispitivana pomoću dve metode (DPPH metoda i test inhibicije obezbojavanja β -karotena). Veći sadržaji ispitivanih jedinjenja utvrđeni su u ekstraktu cveta, iako je ekstrakt lista pokazao jači antioksidativni kapacitet. Možemo zaključiti da je *Verbascum phlomoides* bogat izvor biološki vrednih fenola, da poseduje značajan antioksidativni kapacitet i da ispitani biljni lekovi ispunjavaju zahteve farmakopeje u pogledu kvaliteta.

Ključne reči:

Verbascum phlomoides, ispitivanje opšteg kvaliteta, polifenoli, flavonoidi, antioksidativna aktivnost

Introduction

Orange mullein (*Verbascum phlomoides* L.) belongs to the genus *Verbascum* from the family Scrophulariaceae (Georgiev et al., 2011). This genus comprises about 360 species around the world. They are mostly represented in Europe (95 species, 23 of which are also found in Serbia), Asia, and North America (Georgiev et al., 2011; Mihailović et al., 2016). Plant species of this genus are known

as mullein (Georgiev et al., 2011). Mulleins are flowering biennial plants (rarely perennial or annual), which form a rosette of leaves in the first year and flowering stems in the second year (Georgiev et al., 2011; Marian et al., 2018). They reach a height of 0.3-2 m (Georgiev et al., 2011). Yellow flowers are grouped under bracts. They bloom from June to August, sometimes in September (Marian et al., 2018).

Traditionally, *Verbascum* species are used in

Maja Grigorov

Department of Pharmacy, Faculty of Medicine, University of Niš, Bulevar dr Zorana Đinđića 81, 18000 Niš, Serbia
maja.grigorov@gmail.com (corresponding author)

Dragana Pavlović

Department of Pharmacy, Faculty of Medicine, University of Niš, Bulevar dr Zorana Đinđića 81, 18000 Niš, Serbia

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the treatment of respiratory disorders, such as dry cough, bronchitis, tuberculosis, asthma (Georgiev et al., 2011; Selseleh et al., 2019). Yet, these species show beneficial effects in hemorrhoids, wounds, rheumatism, diarrhea, eczema, and other types of inflammatory conditions (Luca et al., 2019), burns and, according to some literature data, are also effective against headache (Mihailović et al., 2016). A mild sedative, as well as mild diuretic and soothing effects on the ureter, have been noticed. *Verbascum* species are sometimes used to reduce urinary inflammation (Süntar et al., 2010; Selseleh et al., 2019), and as a tea to reduce abdominal pain (Süntar et al., 2010).

Mulleins have been used since ancient times and in recent years their popularity is growing (Georgiev et al., 2011). Nowadays, herbal products for tea preparation from flowers of *V. thapsus*, *V. densiflorum* and *V. phlomoides* can be found in American and European health stores (Mihailović et al., 2016). According to data from 2010, *Verbascum* spp. is used in six European Union countries as traditional herbal supplements or herbal medicinal products (Mihailović et al., 2016).

Verbascum phlomoides is one of three species of the genus *Verbascum* (the other two are: *V. densiflorum* Bartol, and, rarely used due to some reported deficiencies, *V. thapsus* L. (Georgiev et al., 2011)) that are the source of the drug mullein flower (*Verbasci flos*) (EMA, 2007). It is a biennial plant, known as a very rich phytochemical source (Paun et al., 2016). The most common compounds of the drug *Verbasci flos* are iridoid glycosides (aucubin, catalpol, 6-xylosilaukubin, and 6-xylosylcatalpol), flavonoids (tamarixetine 7-rutinoside, tamarixetin 7-glucoside, apigenin, drimolphein and luteolin), phenylethanoid glycosides (verbascoside), triterpene saponins (verbascosaponin), polyphenols, polysaccharides, phytosterols, oleic acid (EMA, 2007). Due to such complex composition, *Verbascum* species show many beneficial effects on human health. Numerous studies have confirmed that the fractions of saponins, iridoids, and phenylethanoids are responsible for their anti-inflammatory effect (Mihailović et al., 2016).

In numerous developing countries, traditional medicines are preferred over conventional ones. The interest in herbal products is in constant increase in developed countries too. Consequently, the quality, safety and efficacy of herbal products must be at the highest level (He et al., 2015; van Wyk & Prinsloo, 2020). That is why a comprehensive specification must be developed for each herbal substance even if the starting material for the manufacture of the finished product is a herbal preparation. The specification should be established based on

recent scientific data and the monographs of the European Pharmacopoeia (Sarfaraz, 2007) and other Pharmacopoeias (like German pharmacopoeia, Swiss pharmacopoeia, British Herbal Pharmacopoeia).

Considering the mentioned pharmacological potential of *Verbascum* species, our study aimed to determine the overall quality of the *Verbasci flos* and *Verbasci folium* as well as the content of polyphenols, tannins, flavonoids, and phenylpropanoid derivatives in their ethanolic extracts. Estimation of antioxidant activity of these extracts was added into the study since this activity could be linked to many beneficial health outcomes.

Materials and Methods

Plant material and extracts

The plant material (flowers and leaves) was collected in the vicinity of Bosilegrad during August 2019. Botanical identification was performed by prof. Bojan Zlatković (Faculty of Science and Mathematics, University of Niš). The voucher specimens are stored in the herbarium of the Faculty of Science and Mathematics, University of Niš (Herbarium code: 14506). After drying at room temperature, in a dark place with a constant flow of air, the plant material was ground using a laboratory mill and stored in glass bottles until the examination. Extracts were prepared by percolation with 50% ethanol (VPF – *V. phlomoides* flower extract and VPL – *V. phlomoides* leaf extract) according to European Pharmacopoeia 10.0 (2019) (European Pharmacopoeia, 2019), followed by total removal of the solvent in a rotary vacuum evaporator at 40 °C. Yields after evaporation are expressed in % of dry plant material used and as DER (drug:extract ratio specifies the initial amount of drug used for the preparation of a certain amount of extract). The dry extracts were preserved for one month in the refrigerator until examinations. The determinations were performed in extracts dissolved in 50% alcohol.

Chemical reagent and instrumentation

All reagents used in this study were of analytical grade. Rutin and gallic acid were purchased from Sigma-Aldrich Co. (Sigma-Aldrich Co., St Louis, MO), and catechin from Extrasynthese (Lyon, France). Spectrophotometric measurements were performed on an Evolution 60 Thermo scientific spectrophotometer (Fisher Scientific, Loughborough, U.K.) and Multiskan Ascent No354 (Thermo Labsystems, Finland) ELISA microplate reader. For incubation Incuterm Raypa® trade (Catalonia, Spain) was used.

Quality control

The overall quality examinations of herbal drugs included a swelling index, loss on drying, total ash, ash insoluble in hydrochloric acid following the regulations of Ph. Eur. 10.0 (2019) and by the monograph of the examined herbal drug *Verbasci flos* (European Pharmacopeia, 2019). These parameters are listed in the part *Tests* of the monograph.

Table 1. Results of the overall quality testing of orange mullein flower and leaf

	Swelling index	Loss on drying (%)	Total ash (%)	Ash insoluble in hydrochloric acid (%)	% of dry plant material used	DER*
	Ph. Eur.10	Ph. Eur.10	Ph. Eur.10	Ph. Eur.10		
<i>Verbasci flos</i>	9.20±1.32 >9	9.06±0.47 <12	3.5±0.92 <6	0.97±0.12 <2	40.82%	2.45:1
<i>Verbasci folium</i>	14.75±1.86 >9	7.28±0.31 <12	6.05±0.06 <6	1.76±0.23 <2	12.93%	7.73:1

* Drug/extract ratio

Total polyphenols and total tannins

The content of total polyphenols was determined by Folin–Ciocalteu colorimetric method (Makkar et al., 2000). The absorbances of the samples and standards were measured at 725 nm, and the results were expressed as gallic acid equivalents per g of extract.

The total tannin content was determined by the same Folin-Ciocalteu procedure (Makkar et al., 2000) after removal of tannins using insoluble binder (polyvinylpyrrolidone, PVPP). The test was performed with a clear supernatant, and the results were also expressed as gallic acid equivalents per g of extract.

Phenylpropanoid derivatives

The content of total phenylpropanoid derivatives was determined spectrophotometrically, according to the Arnou method (Arnou, 1937). This method is based on the reaction of derivatives of o-dihydroxycinnamic acid with sodium molybdate. Absorbance was measured at 525 nm, and the phenylpropanoid content was expressed as the content of the corresponding hydroxycinnamic acid derivative.

Total flavonoids

The total flavonoid content was determined spectrophotometrically by the AlCl₃ method at 430 nm (Lamaison & Carnat, 1990). Results were expressed as a microgram of rutin per milligram of the sample (mg RU/mg).

Antioxidant activity

Radical scavenging activity was determined using stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). The determination was done according to the method described by Chang et al (2010) (Chang et al., 2010) with certain modifications. DPPH free radical inhibition in percent was calculated according to:

$$\% \text{ DPPH} = (A_b - A_s / A_b) \times 100,$$

where *A_b* presents the absorbance of the control reaction (containing ethanol instead of the test solution) and *A_s* is the absorbance of the sample. A regression curve was constructed, and the obtained results were presented as the IC₅₀ value, which indicates the sample concentration required to remove 50% of DPPH free radicals. Rutin was used as a reference compound. Concentrations are expressed in µg/ml.

β-Carotene bleaching test (BCBT) was done according to the method described by Koleva et al. (2002) with slight modifications. The absorbance was measured using an ELISA reader at a wavelength

of 450 nm after shaking the plate on a microplate shaker. The initial absorbance was measured, the plate was placed in an incubator at 47 °C for 120 minutes, afterward, the absorbance was measured again. The ability to inhibit β -carotene decolorization is expressed as a percentage of inhibition according to the formula:

$$\% \text{ inhibition} = (A_{120} / A_0) \times 100,$$

where A_{120} is the absorbance measured at $t=120$ min and A_0 is the absorbance measured at $t=0$ min. After the regression curve was constructed, the IC_{50} value (concentration required to inhibit β -carotene bleaching to 50%) was calculated and expresses as the average value of three independent experiments in $\mu\text{g/ml}$.

Statistical analysis

All tests and experiments were performed in

an appreciable amount of mucilage, pectin or hemicellulose) (WHO, 1998). The swelling index of *V. phlomoides* flower and leaf define mucilage content and present a valuable parameter of drug quality. In our study, *Verbasci folium* showed a higher swelling index than *Verbasci flos*, although both parameters are in accordance with pharmacopoeial demand (>9).

Polyphenols show numerous biological effects, such as anti-inflammatory, antioxidant, immunomodulatory, anticancer, cardioprotective effects (Yahfoufi et al., 2018). Their content was determined by the colorimetric Folin–Ciocalteu method, which is based on the reducing ability of the hydroxyl groups of polyphenolic compounds. The polyphenolic compounds are oxidized, and the resulting phenoxide anion reduces the Folin–Ciocalteu reagent to a blue-colored ion. Based on the results, shown in **Tab. 2**, the plant species *V.*

Table 2. Contents of total polyphenols, total tannins, phenylpropanoid derivatives and total flavonoids in ethanolic extracts of orange mullein flower and leaf and IC_{50} values of DPPH test and BCBT

	Total polyphenols (mg GA ¹ /g extract)	Total tannins (mg GA ¹ /g extract)	Phenylpropanoid derivates (HA ² /g extract)	Total flavonoids (mg R ³ /g extract)	IC_{50} in DPPH test ($\mu\text{g/ml}$)	IC_{50} in β -carotene bleaching test ($\mu\text{g/ml}$)
VPF	49.15±2.70	25.19±4.17	6.32±0.19	16.71±0.91	257.61±2.62	164.24±7.11
VPL	39.70±4.12	16.56±1.82	7.10±0.01	12.81±0.49	157.04±3.86	132.59±5.08

¹GA – gallic acid, ²HA – chlorogenic acid, ³R – rutin

triplicates, and the experimental results were presented as the mean of three consecutive measurements \pm standard deviation.

Results

Results of the overall quality testing of dry flowers and leaves alongside pharmacopoeial demands are presented in **Tab. 1**. **Tab. 2** shows the contents of total polyphenols, total tannins, phenylpropanoid derivatives, and total flavonoids and the results of *in vitro* antioxidant activities of ethanolic extracts VPF and VPL.

Discussion

The obtained results of the overall quality testing of the herbal drug are in accordance with the recommendations of the Ph. Eur. 10.0 (European Pharmacopeia, 2019). The extract yields were 40.82% and 12.93% for flower and leaf extract, respectively. Many herbal drugs are of specific therapeutic or pharmaceutical utility because of their swelling properties (e.g., gums and those containing

phlomoides is rich in polyphenols and their higher content was determined in the VPF compared to the VPL. These results are close to the results of the determination of polyphenols obtained by Mihailović et al. (2016) in the aqueous extract (51.3±0.3 mg gallic acid equivalents (GAE)/g extract), while content in the methanol extract in the mentioned study was slightly higher (88.2±1.6 mg GAE/g extract) (Mihailović et al., 2016). The total polyphenol content expressed as a percentage of gallic acid is 4.91, a bit higher than the 4.18%, which is the content of polyphenols given by EMA (EMA, 2007). Our results are also similar to the results of determining polyphenols in the butanol extract of *V. phlomoides* (Grigore et al., 2013).

Numerous epidemiological data indicate beneficial effects of tannins in the treatment of inflammation and skin injuries, as well as in preventing the development of chronic diseases (Badal McCreath & Delgoda, 2017). Flavonoids are a group of polyphenols, with a lot of beneficial effects on human health. Their antioxidant effect is most often discussed, but their anti-inflammatory,

anticancer, antimutagenic effects are equally important (Panche et al., 2016). The results of the determination of tannins and flavonoids show that the VPF is richer in these compounds than the VPL. This could be one of the explanations why is the mullein flowers rather than leaves used in folk medicine. Our results of total flavonoids determination are close to the results obtained by Mihailović et al. (2016) for the aqueous extract but lower than their results for the methanol extract (Mihailović et al., 2016). Armatu et al. (2011) determined the chemical compositions of two extracts of *V. phlomoides* collected in the southeastern part of Romania. Their results for the total flavonoid content are close to ours, with the butanol extract being richer in flavonoids, compared to the ethanol (Armatu et al., 2011). Paun et al. (2016) in their work determined the content of polyphenols and flavonoids in aqueous and ethanol extract of *V. phlomoides* and reported a higher content of polyphenols and flavonoids compared to ours (Paun et al., 2016). The content of polyphenols and flavonoids, as well as other compounds in plant species, is influenced by numerous factors, such as the region in which the species grows, the conditions in which it grows, the time in which plant material is collected, storage methods and many others (Elmastaş et al., 2017). Additionally, the determination of phenylpropanoids was included in this study since this class of secondary metabolites besides anti-inflammatory, antimicrobial, cytoprotective and wound healing effects could be beneficial for skin regeneration (Pavlović et al., 2013). The total phenylpropanoid derivatives content in VPL was slightly higher than in VPF (Tab. 2).

There are several different methods for assessing the antioxidant capacity of natural products. However, given the range of different types of free radicals that could be generated under *in vivo* conditions and the different reaction mechanisms involved in the antioxidant capacity of antioxidants, there is still no universal method to determine antioxidant capacity accurately and qualitatively (Mihailović et al., 2016). In our work, we used the DPPH and the BCBT tests. The obtained results of the DPPH test show that the VPF has a lower antioxidant capacity ($IC_{50}=257.61\pm 2.62$ µg/ml) compared to the VPL ($IC_{50}=157.04\pm 3.86$ µg/ml).

The antioxidant activity tested in the BCBT test was also higher for VPL extracts compared to VPF extracts, although the difference in IC_{50} values is not so pronounced (132.59 ± 5.08 µg/ml and 164.24 ± 7.11 for VPL and VPF, respectively). It is important to underline that the antioxidant capacity was dose-dependent (higher doses of extract demonstrated higher effects which was the base for the construction

of regression curves). Since plant extracts are a mixture of different compounds, more different antioxidant tests are needed for a full understanding of the properties of one particular extract (Pavlović et al., 2013). As expected, antioxidant activities of rutin, used as reference substance (positive control), were much lower (IC_{50} values in DPPH and BCBT: 2.68 ± 0.25 µg/ml and 33.29 ± 4.06 µg/ml, respectively) compared with antioxidant activities of tested extracts.

Conclusions

Based on the presented results, we can conclude that the plant species *Verbascum phlomoides* contains significant amounts of polyphenols, tannins, and flavonoids, and due to the well-known positive effects of these compounds on human health, this species deserves to remain in the focus of our study. Also, the contents of these compounds were higher in flowers compared to leaves. This could be one of the explanations why is the mullein flowers rather than leaves used in folk medicine for topical applications. Yet, the leaf extract showed a stronger antioxidant capacity in applied tests. Further investigations and detailed chemical profiling are needed to assess the relevance of ethnopharmacological application and define the potential health benefits of orange mullein.

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