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PHYTOCHEMICAL AND *IN VITRO* ANTIOXIDANT STUDIES OF *PRIMULA VERIS* (L.) GROWING WILD IN KOSOVO

Ibrahim Rudhani, Florentina Raci, Hamide Ibrahimi, Arben Mehmeti, Ariana Kameri, Fatmir Faiku, Majlinda Daci, Sevdije Govori, Arben Haziri
University of Prishtina (Kosovo)

Abstract. The antioxidant capacities, total phenolic and flavonoid content of five different extracts (Et_2O, $CHCl_3$, EtOAc, n-BuOH and H_2O) of *Primula veris* (L.) growing wild in Kosovo were analyzed. Total phenolic and flavonoid content were quantitatively determined to the *Primula veris* (L.) growing wild in North-Western part of Kosovo in two different altitude levels (670 m and 1200 m). The amount of total phenolics in *Primula veris* (L.) extracts ranged from 5.10 mg GAE/g d.e. in altitude 1200 m to 17.30 mg GAE/g d.e. in altitude 670 m. The amount of total flavonoids in *Primula veris* (L.) extracts ranged from 12.15 mg RE/g d.e. in altitude 670 m to 31.43 mg GAE/g d.e. in altitude 1200 m. Free radical scavenging capacity (RSC) was evaluated by measuring the scavenging capacity of extracts on DPPH, NO as well as on hydrogen peroxide (H_2O_2). EtOAc, n-BuOH and H_2O extracts of *Primula veris* (L.) expressed very strong scavenger activity. On the other hand, Et_2O and $CHCl_3$ extracts showed much weaker effect in the neutralization of DPPH, NO and H_2O_2. The observed differences in antioxidant activity could be partially explained by the levels of phenolics and flavonoids in extracts of *Primula veris* (L.). Hence, this plant can be used to discover bioactive natural products that may serve as leads in the development of new pharmaceuticals.
Keywords: *Primula veris* L.; extracts; phytochemical; antioxidant

Introduction

Oxidative compounds lead to the damage of biomolecules like DNA and RNA (Dastmalchi et al., 2007), which can further initiate the development of diseases such as ageing, atherosclerosis, lipofuscinosis, oxygen toxicity, cancer and liver injury (Iyer & Devi, 2009; Smerq & Sharma, 2011). Antioxidants are compounds which inhibit oxidation, and therefore are potential quenchers of free radicals or reactive oxygen species. A recent investigation shows that plant secondary metabo-

lites are as potential antioxidants against various diseases, induced by free radicals due to presence of these phytochemicals (Hou et al., 2003). These antioxidants in plants also react with other organisms in the environment, inhibiting bacterial or fungal growth hence responsible for the antimicrobial activity of plants (Bruneton, 1995). These substances are considered as basis for developing new antimicrobial drugs as they inhibit pathogens and have little toxicity to host cells.

In the last years, interest in medicinal plants is more and more increasing, particularly using them as antibacterial agents. The reason for that is the growth of antibiotic resistance of bacteria against synthetic drugs (Gruenwald et al., 2000).

There are about 400 species included in the plant genus Primula. Phenolic glycosides and saponins are characteristic compounds for the genus Primula (Müller et al., 2006). Ten lipophilic flavones were isolated from *Primula veris* L. *in vitro* cultures (Budzianowski et al., 2005). Flavonoids have important effects in plant biochemistry and physiology, acting as antioxidants, enzyme inhibitors, precursors of toxic substances and have long been recognized to possess anti-allergic, anti-inflammatory, antiviral, anti-proliferative and anti-carcinogenic activities as well as to affect some aspects of mammalian metabolism (Huck et al., 2000). Two new flavonol glycosides have been identified and isolated from Italian Primula species (Fico et al., 2007). Otherwise *Primula veris* L. has a potential anxiolytic activity (Sufka et al., 2001). Primula species can also contain allergens (Paulsen et al., 2006) and some species are used traditionally to treat epilepsy and convulsions (Jäger et al., 2006). Another Primula species has flavonoids that possessed strong cytostatic properties against HL 60 cells even at low concentrations (Tokalov et al., 2004).

Our research group was interested to analyze the chemical profile and biological activities of different medicinal plants, which are growing wild in the region of Kosovo and Albania (Haziri et al., 2009; 2010; 2013; 2017; Faiku & Haziri, 2013; 2015; Faiku et al., 2012; 2015; 2016). The aim of this research was to determine the quantity of phenols, flavonoids and antioxidant activity of the different extracts from *Primula veris* (L.) growing wild in North-Western part of Kosovo.

Material and methods

Plant materials

The aerial parts of *Primus veris* (L.), growing in North-Western part of Kosovo, in two different altitudes (670 m and 1200 m) were collected on May 2016. Voucher specimens were deposited in the herbarium of the Department of Plant protection, University of Prishtina. The plants were dried at room temperature.

Preparation of plant organic extracts

A portion of the finely powdered material (200 g) was extracted three times with 70% methanol (methanol, 4 L) during a 24-h period. After removal of methanol under reduced pressure, the aqueous phase was successively extracted with four

solvents of increasing polarity, namely diethyl ether, chloroform, ethyl acetate and n-butanol. The extraction was carried out until a colorless extract was obtained. The residue was the aqueous extract. All five extracts (ethyl acetate, diethyl ether, water, n- butanol and chloroform) were evaporated to dryness and then dissolved in 50% ethanol to make 10% (w/v) solutions. These solutions, either as such or in diluted state, were used in subsequent experiments.

Determination of total phenolic and flavonoid content

The amount of total phenolic contents in the extracts was determined spectrophotometrically with the Folin Ciocalteu (FC) reagent using the method of Fukumoto & Mazza (2000) with small modifications (Bozin et al., 2008). The reaction mixture contained 1.0% dilution of examined extracts (100 μL), freshly prepared 0.2 mol/L FC reagent (2.5 mL) and 10% sodium carbonate solution (2 mL). The mixture was incubated in the dark at room temperature for 1 hour to complete the reaction. The absorbance of the resulting solution was measured at 760 nm on a UV/VIS spectrophotometer using distilled water as the blank. The concentration of total phenolic contents was expressed in mg gallic acid equivalents (GAE) per g dried extract (d.e.), using a standard curve of gallic acid (0.1-2.0 μg/mL). All measurements were replicated five times. Total flavonoid content in the extracts was determined spectrophotometrically according to Zhishen et al. (1999), using a method based on the formation of a flavonoid-aluminium complex with an absorbance maximum at 430 nm. The examined extracts (1 mL) were mixed with 2% $AlCl_3 \times 6H_2O$ (0.5 mL). After incubation at room temperature for 30 min, the absorbance of the reaction mixtures was measured. The blank sample was a 1:1 mixture of the examined extracts and distilled water. Flavonoid content was expressed in μg rutin equivalent (RE) per g dried extract by using a standard curve of rutin (concentration range 0.5 – 6.0 μg/mL). All measurements were replicated five times.

Antioxidant activity- DPPH assay

The DPPH assay was performed as described previously (Blois, 1958; Sanchez-Moreno et al., 1998), following the transformation of the DPPH radical to its reduced, neutral form (DPPH-H). The samples of all extracts of *Primula veris* L. (from 2.50 to 50.00 μg/mL) were mixed with 90 μM DPPH• solution (1 mL) and made up with 95% methanol to a final volume of 4 mL. The absorbance of the resulting solutions was recorded spectrophotometrically at 515 nm after 1 h at room temperature, against the blank (with the same chemicals, except for the sample). The same procedure was repeated with tertbutylhydroxytoluene (BHT) and tert-butyl-4- hydroxyanisole (BHA) as a positive control. For each sample five replicates were recorded.

Production of NO radicals was determined spectrophotometrically. NO radical generated from sodium-nitropruside (SNP) reacts with oxygen in water solution

at a physiological pH to give nitrite ions. Concentration of nitrite anions was determined using the Griess reagent (Green et al., 1982; Babu et al., 2001). At room temperature nitrite ions react with the Griess reagent and form a purple complex. The samples of *Primula veris* L. extracts were investigated in concentrations of 2.50–50.00 µg/mL. The intensity of color, which is the function of the nitrite concentrations, was measured spectrophotometrically (λ = 546 nm). The absorbance of the resulting solutions and the blank (with the same chemicals, except for the sample) were recorded. For each sample, five replicates were recorded.

Scavenging activity on H_2O_2 was carried out according to the method of Ruch et al. (1989). A solution of H_2O_2 (40 mmol/L) was freshly prepared in 0.05 mol/L KH_2PO_4–K_2HPO_4 phosphate buffer (pH 7.4). The samples (from 2.50 to 50.00 µg/mL) were mixed with phosphate buffer (3.4 mL) and 40 mmol/L H_2O_2 (0.6 mL). The absorbance of the resulting solutions and the blank (4.0 mL phosphate buffer) was detected spectrophotometrically at 230 nm. The percentage of RSC for each radical and H_2O_2 was calculated using the following equation:

$$RSC (\%) = 100 \times (A_{blank} - A_{sample} / A_{blank})$$

From the obtained RSC values, the IC_{50} values, which represented the concentrations of the examined extracts that caused 50% neutralization, were determined by linear regression analysis.

Results and discussion

Results of the amount of total phenolic contents and content of total flavonoids in *Primula veris* (L.) extracts are given in Table 1. The amount of total phenolics in *Primula veris* (L.) extracts ranged from 5.10 mg GAE/g d.e. (diethyl ether extract to 1200 m altitude) to 17.30 mg GAE/g d.e. (ethyl acetate extract to 670 m altitude). A significant amount of these compounds has also been observed in the H_2O extracts 12.23 mg GAE/g d.e. in altitude 670 m and 11.92 mg GAE/g d.e. in altitude 1200 m (Table 1).

Table 1. The amount of total phenolic contents and content of total flavonoids in *Primula veris* (L.) extracts

Altitude (m)		Et_2O		$CHCl_3$	EtOAc	n-BuOH	H_2O
670	Total phenolic content	5.13		5.16	17.30	9.01	12.23
1200	Total phenolic content	5.10		5.93	11.63	6.44	11.92
670	Total flavonoids content	12.15		15.30	30.42	14.50	27.73
1200	Total flavonoids content	14.16		17.82	31.43	15.91	28.20

Total phenolic content is expressed in mg GAE/g d.e.; Content of total flavonoids is expressed in µg RE/g d.e.

Furthermore, considerable total flavonoids content was determined in the water and ethyl acetate extracts of *Primula veris* (L.). Little less total flavonoids was determined in the n-butanol and in diethyl ether extracts. The amount of phenols and flavonoids of *Primula veris* (L.) growing wild in Kosovo is smaller comparing with the amount of phenols and flavonoids found in *Primula veris* (L.) growing wild in Serbia (Stojkovic, 2014). The amount of total phenolic contents and content of total flavonoids in *Primula veris* (L.) extracts were given in Fig. 1.

The antioxidant activity of *Primula veris* (L.) extracts has been evaluated in a series of in vitro tests. The DPPH radical is one of the most commonly used substrates for fast evaluation of antioxidant activity because of its stability (in radical form) and the simplicity of the assay. In the DPPH assay, the ability of the investigated extracts to act as donors of hydrogen atoms or electrons in transformation of DPPH into its reduced form DPPH-H was investigated (Table 2). All of the assessed extracts of *Primula veris* (L.) were able to reduce the stable, purple colored radical DPPH to the yellow-colored DPPHH form with IC50 (50% of reduction) values as follows: 9.45 µg/mL in altitude 670 m and 9.30 in altitude 1200 m for water, 12.10 µg/mL in altitude 670 m and 12.00 µg/mL in altitude 1200 m for ethyl acetate, 11.93 µg/mL in altitude 670 m and 14.24 µg/mL for n-butanol, 22.16 µg/mL in altitude 670 m and 23.62 µg/mL in altitude 1200 m for chloroform, and 30.14 µg/mL in altitude 670 m and 28.71 µg/mL in altitude 1200 m for diethyl ether extract. Comparison of the DPPH scavenging activity of the investigated *Primula veris* (L.) extracts with those expressed by BHT (11.17 µg/mL) showed that only the water and ethyl acetate extracts expressed stronger antioxidant effects. Comparing the DPPH activity of *Primula veris* (L.) extracts with the activity exhibited by BHA (9.79 µg/mL) and BHT (11.17 µg/mL), we found that only the water extract showed stronger antioxidant activity than BHA and BHT, but neither of the extracts showed better antioxidant properties than BHA and BHT. Comparing with the DPPH test results of total flavonoids content in the extracts (Table 2), it could be concluded that only in case of the ethyl acetate and water extracts of *Primula veris* (L.) there is some correlation between the DPPH scavenger activity and content of flavonoids.

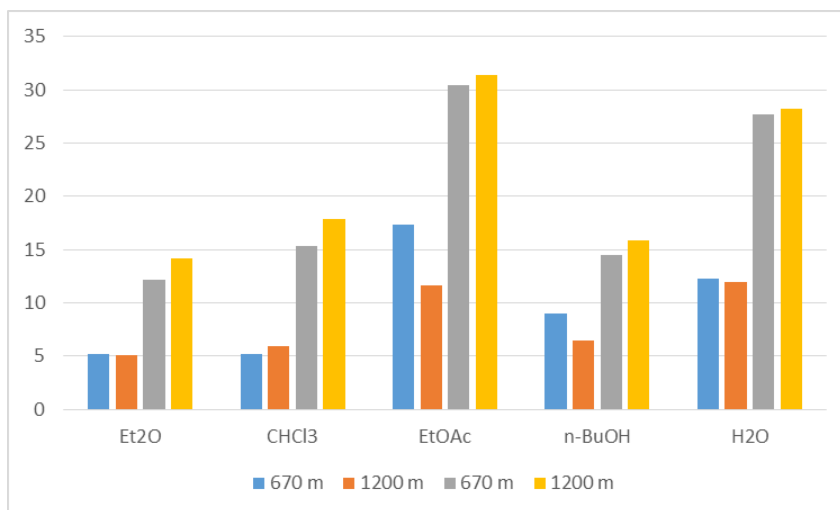

Figure 1. The amount of total phenolic contents and content of total flavonoids in *Primula veris* (L.) extracts

An extremely strong neutralization of NO radicals is expressed by a number of the tested extracts of *Primula veris* (L.), such as water (IC_{50} = 7.86 µg/mL in altitude 1200 m) and ethyl acetate (IC_{50} = 10.11 µg/mL in altitude 670 m (Table 3). Only water extract exhibited stronger antioxidant effects than BHT. The effect of the water extract was closely to the action of BHA (7.16 µg/mL). The lowest antioxidant activity was expressed by the chloroform (IC_{50} = 23.16 µg/mL in altitude 670 m) and diethylether (IC_{50} = 22.12 µg/mL in altitude 670 m). The IC_{50} values (µg/mL) of the neutralization of NO radical with *Primula veris* (L.) extracts is given in Fig. 3.

Table 2. IC_{50} values (µg/mL) of the neutralization of DPPH radical with *Primula veris* (L.) extracts

Altitude (m)	Et_2O	$CHCl_3$	EtOAc	n-BuOH	H_2O	BHT	BHA
670	30.14	22.16	12.10	11.93	9.45	11.17	9.79
1200	28.71	23.62	12.00	14.24	9.30	11.17	9.79

The IC_{50} values (µg/mL) of the neutralization of DPPH radical with *Primula veris* (L.) extracts were given in Fig. 2.

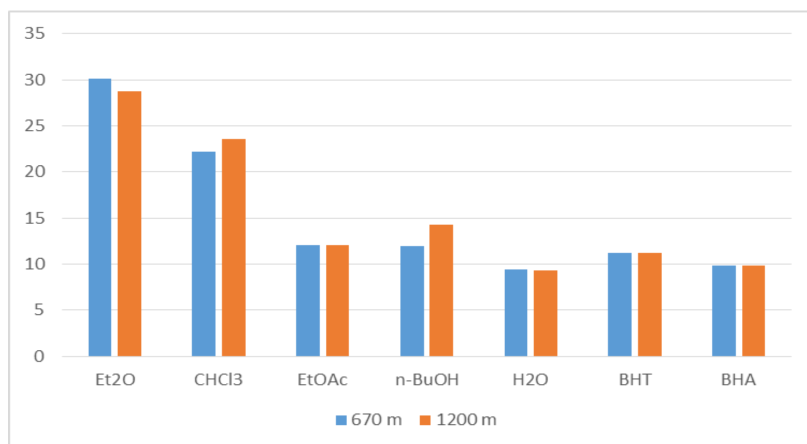

Figure 2. The IC_{50} values (µg/mL) of the neutralization of DPPH radical with *Primula veris* (L.) extracts

Table 3. IC_{50} values (µg/mL) of the neutralization of NO radical with *Primula veris* (L.) extracts

Altitude (m)	Et_2O	$CHCl_3$	EtOAc	n-BuOH	H_2O	BHT	BHA
670	22.12	23.16	10.11	16.37	8.10	8.76	7.16
1200	20.18	20.17	10.16	15.40	7.86	8.76	7.16

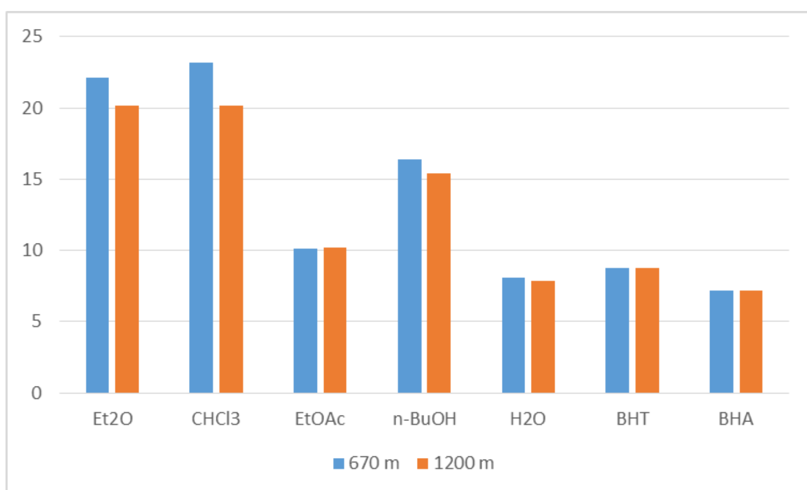

Figure 3. The IC_{50} values (µg/mL) of the neutralization of NO radical with *Primula veris* (L.) extracts

H_2O_2 can cross membranes and may slowly oxidize a number of cell compounds. Thus, the elimination of H_2O_2, as well as the OH radical is very important for protection of pharmaceutical and food systems (Zhishen et al., 1999). The neutralization of H_2O_2 by the examined extracts was measured spectrophotometrically (Table 4).

Table 4. IC_{50} values (μg/mL) of the neutralization of H_2O_2 radical with *Primula veris* (L.) extracts

Altitude (m)	Et_2O	$CHCl_3$	EtOAc	n-BuOH	H_2O	BHT	BHA
670	73.17	73.89	46.15	63.62	30.77	19.20	18.90
1200	81.27	72.40	30.18	40.51	18.80	19.20	18.90

Based on the results of the spectrophotometric measurement of *Primula veris* (L.) the H_2O_2, in reference to reducing its concentration in solution, we can see that all the examined extracts showed the ability to remove H_2O_2. Extremely high RSC, as well as in the case of neutralization of DPPH and NO radicals, was found in the water extract (IC_{50} = 18.80 μg/mL in altitude 1200 m). If we compare the values, we can see that all extracts showed smaller antiradical activity. A small antioxidant activity shows diethyl ether extract (IC_{50} = 81.27 μg/mL in altitude 1200 m). The IC_{50} values (μg/mL) of the neutralization of H_2O_2 radical with *Primula veris* (L.) extracts is given in Figure 4. Antioxidant activity of the extracts of *Primula veris* (L.) growing wild in Kosovo are almost same with the antioxidant activity of *Primula veris* (L.) growing wild in Serbia (Stojkovic, 2014).

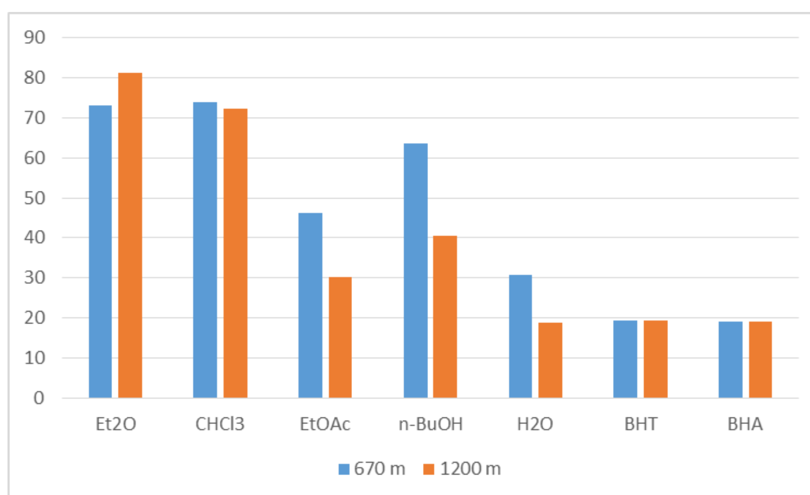

Figure 4 The IC_{50} values (μg/mL) of the neutralization of H_2O_2 radical with *Primula veris* (L.) extracts

Conclusion

The aim of this research was to determine phenols, flavonoids and antioxidant activities of different extracts from *Primula veris* (L.) growing wild in North-Western part of Kosovo. To conduct this type of research, we took two samples of the plant *Primula veris* (L.) at different altitudes 670 m and 1200 m. The amount of total phenols in organic and aqueous extracts of *Primula veris* (L.) were in the region of 5.10 mg GAE/ g de (diethylether extract to 1200 m altitude) to 17.30 mg GAE/ g de (extract of ethyl acetate at 670 m altitude). Also, great amount of flavonoids was found in aqueous extract and ethyl acetate. Small amounts of flavonoids were found in the n-butanol extract until very small amount we found in the diethyl ether extracts. Simultaneously we have investigated the antioxidant activity of five different extracts (diethyl ether, chloroform, ethyl acetate, n-butanol and water) from the plant *Primula veris* (L.) growing in two different heights above sea level (670 m and 1200 m). Neutralization of free radicals (RSC) is estimated by making measurement of neutralizing capacity of various extracts on DPPH, NO and H_2O_2. In general, ethyl acetate and water extracts have a strong antioxidant activity. In most tests (DPPH reduction, NO radicals and neutralizing H_2O_2), H_2O extract of *Primula veris* (L.) growing wild at 1200 m of altitude expresses stronger antioxidant activity. Results obtained from water extracts of *Primula veris* (L.) is very logical, since we found that this extract contains the largest amount of total phenolic and flavonoid content.

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✉ **Dr. Arben Haziri (corresponding author)**
Department of Chemistry
Faculty of Natural Sciences
University of Pristina
George Bush St., p.n.
10000 Pristina, Kosovo
E-mail: arben.haziri@uni-pr.edu