Department of Pharmacy[1], Faculty of Medicine, University of Niš; Faculty of Pharmacy[2], University of Belgrade, Serbia

Arbutin content and antioxidant activity of some Ericaceae species

R. D. Pavlović[1], B. Lakušić[2], Z. Došlov-Kokoruš[2], N. Kovačević[2]

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Pavlović R. Dragana, Department of Pharmacy, Faculty of Medicine, University of Niš, Bulevar Zorana Đinđića 81, 18000 Niš, Serbia
anagard@medfak.ni.ac.rs

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Quantitative analyses and investigation of antioxidant activity of herb and dry ethanolic extracts of five species from Ericaceae family (*Arbutus unedo* L., *Bruckentalia spiculifolia* Rchb., *Calluna vulgaris* Salisb., *Erica arborea* L. and *Erica carnea* L.) were performed. Total polyphenols, tannins and flavonoids were determined spectrophotometrically and arbutin content was measured both spectrophotometrically and by HPLC coupled with DAD detection. Antioxidative properites of the ethanolic extracts were tested by means of FRAP (total antioxidant capacity), lipid peroxidation and DPPH free radical scavenging activity. A significant amount of arbutin was detected only in *Arbutus unedo*. All samples investigated showed excellent antioxidant activity. The best inhibition of lipid peroxidation has been shown by *Bruckentalia spiculifolia* herb extract (62.5 μg/ml; more than 95%), which contained the highest amount of flavonoids (11.79%). The highest scavenging activity was obtained with leave extract of *Arbutus unedo* ($IC_{50} = 7.14$ μg/ml). The leaves of *A. unedo* contained a small amount of flavonoids but high content of non-tannins polyphenols.

1. Introduction

Arctostaphylos uva-ursi, the famous member of the Ericaceae family, is an ancient astringent and urinary antiseptic. The antimicrobial effect of *Uvae ursi folium* is associated with the aglycone hydroquinone released from arbutin or arbutin waste products in the alkaline urine (Heinrich et al. 2004).

In traditional medicine, other Ericaceae species are also used for the treatment of urinary tract infections (Tucakov 1997; Tasić et al. 2004; Maleš et al. 2006). Our research involved five species from Ericaceae family that grow wild in Serbia and Montenegro in addition to *Arctostaphylos uva-ursi*. The species *Arbutus unedo* L., *Bruckentalia spiculifolia* (Salisb.) Reichenb., *Calluna vulgaris* Salisb., *Erica arborea* L. and *Erica carnea* L. are widespread throughout Europe. Despite the fact that these species are often used in popular medicine in their region of origin, it was only in the last decade that a few studies have focused on their pharmacological effects.

Recently, investigations have shown that *Arbutus unedo*, traditionally used for the treatment of diabetes and hypertension (Bnouham et al. 2007; Ziyyat and Boussairi 1998), possesses significant antiaggregant, vasorelaxant, diuretic, anti-inflammatory and antioxidant properties (Pabuçcuoğlu et al. 2003; Legssyer et al. 2004; ElHaouari et al. 2007; Afkir et al. 2008; Mariotto et al. 2008).

Calluna vulgaris is often used for the treatment of various inflammatory ailments, mainly due to considerable *in vivo* anti-inflammatory and antinociceptive activities (Najid et al. 1992; Orhan et al. 2007).

Erica species have been traditionally used as antirheumatic, diuretic, as astringent agents and in the treatment of urinary infections. Akkol et al. (2008) stated that *Erica arborea* displayed remarkable anti-inflammatory and antinociceptive activities. Antioxidant activity of *Erica arborea* has been also demonstrated (Ay et al. 2007).

As far as our literature survey could ascertain, there is no information concerning the pharmacologic effects of *Erica carnea* and *Bruckenthalia spiculifolia*.

2. Investigations, results and discussion

2.1. Hydroquinone derivates and arbutin content

Uvae ursi folium is an official drug acording to many pharmacopoeias. The specific quality of this drug is defined by the content of total hydroquinone derivates calculated as arbutin (hydroquinone-β-*O*-glucopyranoside), but the requirements that pharmacopoeias set as a minimum content of arbutin are different. Our first approach was to study arbutin and hydroquinone content in investigated species (*Arbutus unedo*, *Bruckentalia spiculifolia*, *Calluna vulgaris*, *Erica arborea* and *Erica carnea*) and their ethanolic extracts (AuE, BsE, CvE, EaE and EcE, respectively). This is the first report of arbutin content in these five wild-growing Ericaceae species from Serbia and Montenegro.

Wide ranges of hydroquinone derivates were observed within all investigated Ericaceae species (Table 1); consequently the genus means were significantly different. Our results demonstrate that among the samples investigated only *Arbutus unedo* leaves and AuE possess a relatively high content of total hydroquinone derivatives (1.76% and 2.65%, respectively). The content of total hydroquinone derivatives in other tested species is less than 1%.

Table 1: Content of the phenolic constituents in the investigated species from Ericaceae family

Plant species		Flavonoids %	Polyphenols %		
			Total polyphenols	Nontannins	Tannins
Arbutus	Plant material	1.92 ± 0.07	11.08 ± 0.28	3.05 ± 0.10	8.03 ± 0.10
unedo	Dry extract	3.05 ± 0.01	20.48 ± 0.30	8.16 ± 0.15	12.32 ± 0.15
Bruckentalia	Plant material	3.71 ± 0.05	4.67 ± 0.08	0.86 ± 0.06	3.80 ± 0.06
spiculifolia	Dry extract	11.79 ± 0.25	19.22 ± 0.48	3.99 ± 0.07	15.23 ± 0.07
Calluna	Plant material	3.70 ± 0.04	4.22 ± 0.12	1.27 ± 0.15	2.95 ± 0.15
vulgaris	Dry extract	1.56 ± 0.12	18.60 ± 0.19	5.63 ± 0.28	12.97 ± 0.28
Erica	Plant material	1.93 ± 03	1.46 ± 0.07	0.52 ± 0.09	0.94 ± 0.09
arborea	Dry extract	6.95 ± 0.08	28.00 ± 0.25	6.14 ± 0.09	21.86 ± 0.09
Erica	Plant material	0.85 ± 0.04	3.34 ± 0.20	1.74 ± 0.21	1.59 ± 0.21
carnea	Dry extract	1.39 ± 0.00	25.95 ± 0.21	6.99 ± 0.05	18.96 ± 0.07

Considering the special warning (ESCOP, 1997) that the amount of free hydroquinone in bearberry leaf preparations should be controlled, because of its toxic effects, we determined both arbutin and hydroquinone in dry plant materials. Quantitative HPLC analyses showed that beside *Vaccinium vitis-idea* and comercial drug *Uvae ursi folium*, only *Arbutus unedo* contained detectable levels of arbutin (1.21 ± 0.03% of dry plant material). Hydroquinone was absent in all samples which proves its compliance with the ESCOP directive. A chromatogram of *Arbutus unedo* is presented the Fig. Thus, according to HPLC analysis, only the leaves of *Arbutus unedo* and *Vaccinium vitis-idea* could be rightfully treated as substitutes for officinal bearberry leaf (*Arctostaphylos uva-ursi*). In other tested samples neither arbutin nor hydroquinone were detectable (possibly present in traces).

2.2. Polyphenols content

Regarding other constituents of investigated samples, the levels of total flavonoids and polyphenols (including tannins) were high (Table 2). Considerable amounts of phenolic compounds were found in plant materials and dry extracts; the tannin fraction represent more than a half of the total polyphenolic compounds (from 60.55% in AuE to 79.24% in BsE). The leaves of *Arbutus unedo* had the highest levels of total polyphenols and tannins, while among the tested extracts, EaE had the highest total polyphenols and tannins values (28.00±0.25 and 21.86±0.09 %, respectively).

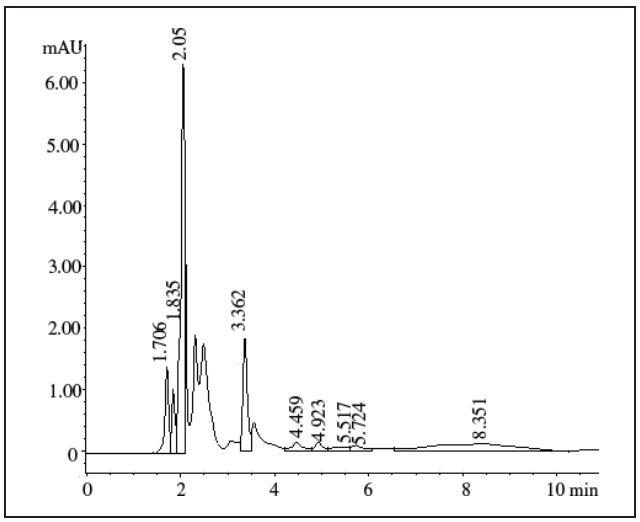

Fig: HPLC UV chromatogramm of *Arbutus unedo* (retention time of arbutin 3.362 min)

Flavonoids are represented in a range from 0.85 ± 0.04 and 1.39 ± 0.00% (*Erica carnea* and EcE) to 3.71 ± 0.05 and 11.79 ± 0.25% (*Bruckentalia spiculifolia* and BsE).

2.3. Antioxidative properties

As our phytochemical study showed that investigated samples contained significant amounts of polyphenolic compounds, tannins and flavonoids, it was considered reasonable to provide data about their *in vitro* antioxidant and radical-scavenging potential. AuE, BsE, CvE, EaE and EcE were subjected to screening for possible antioxidant activity. According to our knowledge, so far, there have been little data in the literature on antioxidant properties of Ericaceae species.

Antioxidant activity was determined through three complementary test systems, namely total antioxidant capacity (FRAP), DPPH free radical scavenging and inhibition of the lipid peroxidation (LP). All results are presented in Table 3.

According to FRAP value (from 3.11 ± 0.21 μmol Fe^{2+}/g BsE to 5.11 ± 0.09 μmol Fe^{2+}/g AuE) our samples possess notable antioxidative activities.

All extracts showed remarkable anti-radical activity with IC_{50} range from 13.44 ± 0.87 μg/ml (EcE) to 7.17 ± 0.46 μg/ml (AuE), compared to the synthetic antioxidant agent BHT (IC_{50} = 13.03 ± 0.16 μg/ml).

The highest DPPH radical scavenging activity was obtained with the ethanolic extract of *Arbutus unedo*. The herb of this plant contained a small amount of flavonoids (3.05%), but high content of non-tannins polyphenols (8.16%).

All extracts also showed considerable activity against lipid peroxidation. The addition of crude extracts at a level of 62.5 μg/ml decreased the formation of MDA by more than 65%. CvE, EaE and EcE reached maximum of LP inhibition at concentration of 62.5 μg/ml (90.26, 78.97 and 65.97%, respectively); in higher concentrations, inhibitory effect of these extracts on LP was less pronounced. AuE and BsE provided dose-dependent inhibition: in concentration of 125.0 μg/ml AuE and BsE reached 74.31% and 96.32% versus 66.16% and 95.33% respectively, when 62.5 μg/ml was applied. However, BsE was a statistically more active inhibitor of lipid peroxidation than the other investigated extracts except for CvE. This activity can be connected to the highest quantity of herbs flavonoids (11.79%) in BsE.

According to the results of our study, the content of arbutin in all investigated species is minimal according to the well-known herbal drug *Uvae ursi folium*. Although only

Table 2: Content of hydroquinone derivates, arbutin and hydroquinone in the investigated species from Ericaceae family

Plant species		Hydroquinone derivates %	Arbutin %	Hydroquinone %
Arbutus	Plant material	1.76 ± 0.12	1.21 ± 0.03	n.d.
unedo	Dry extract	2.65 ± 0.13	/	/
Bruckentalia	Plant material	0.50 ± 0.01	n.d.	n.d.
spiculifolia	Dry extract	0.65 ± 0.07	/	/
Calluna	Plant material	0.53 ± 0.02	n.d.	n.d.
vulgaris	Dry extract	0.80 ± 0.05	/	/
Erica	Plant material	0.46 ± 0.01	n.d.	n.d.
arborea	Dry extract	0.93 ± 0.03	/	/
Erica	Plant material	0.46 ± 0.00	n.d.	n.d.
carnea	Dry extract	0.76 ± 0.02	/	/
Arctostaphylos uva ursi	Plant material	7.29 ± 0.11	4.71 ± 0.06	n.d.
Vaccinium myrtillus	Plant material	1.06 ± 0.01	/	n.d.
Vaccinium vitis-idea	Plant material	8.47 ± 0.18	3.54 ± 0.02	n.d.

/ not determined; n.d. – not detected

Table 3: Antioxidant activity of ethanolic extracts of investigated species from Ericaceae family

Extract	Inhibition (%) of lipid peroxidation					Radical scavenging activity IC_{50} (μg/ml)	FRAP value (μmol Fe^{2+}/g)
	6.25 μg/ml	12.5 μg/ml	25.0 μg/ml	62.5 μg/ml	125.0 μg/ml		
AuE	12.85	23.71	36.73	66.16	74.31	7.17 ± 0.46	5.11 ± 0.09
BsE	50.32	70.70	83.44	95.33	96.32	10.22 ± 0.74	3.11 ± 0.21
CvE	22.04	34.06	85.97	90.26	88.25	12.00 ± 0.64	3.32 ± 0.08
EaE	−5.63	14.79	22.63	78.97	67.44	13.40 ± 0.67	3.55 ± 0.11
EcE	−0.95	3.61	15.50	65.97	29.54	13.44 ± 0.87	3.49 ± 0.04

Arbutus unedo could be treated as adequate substitute for *Uvae ursi folium*, due to arbutin content, other investigated species could provide positive health benefits, due to high content of valuable plant phenols and notable antioxidant activities.

3. Experimental

3.1. Plant material

Plant materials were collected from wild growing species of Ericaceae family: *Arbutus unedo* L. (Luštica, Montenegro); *Bruckentalia spiculifolia* Rchb. (Kopaonik – Pančićev vrh, Serbia); *Calluna vulgaris* (L.) Hull (Loznica – Gučevo, Serbia); *Erica arborea* L. (Luštica, Montenegro); *Erica carnea* L. (syn.: *Erica herbacea* L., *Erica saxatilis* Salisb.) (Mokra Gora, Serbia).

Authenticated voucher herbarium specimens have been deposited in the Herbarium collection of the Faculty of Pharmacy, University of Belgrade: *Arbutus unedo* HFF No. 1173; *Bruckentalia spiculifolia* HFF No. 1217; *Calluna vulgaris* HFF No. 1272; *Erica arborea* HFF No. 1430 and *Erica carnea* HFF No. 1431.

3.2. Extraction procedure

Plant material was reduced to a fine powder and extracted with ethanol (70%, v/v) by percolation (Ph. Eur. 3, 1997). Ethanolic extracts of *Arbutus unedo* (AuE), *Bruckentalia spiculifolia* (BsE), *Calluna vulgaris* (CvE), *Erica arborea* (EaE) and *Erica carnea* (EcE) were obtained after evaporation to the dryness in vacuo under 40 °C and extraction yields were 45.05, 32.35, 33.84, 38.98 and 38.61% (w/w), respectively.

3.3. Chemicals and instrumentation

All reagents and solvents used in this investigation were of analytical grade. Spectrophotometric measurements were performed using the apparatus Specol 11 UV – vis spectrophotometer (Carl Zeiss, Jena, Germany). HPLC analyses were performed on Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with binary pump G1312A, column Zorbax Eclipse XDB-C18 (4.6 × 250 mm) and DAD detector G1315B.

3.4. Determination of constituents

3.4.1. Determination of total hydroquinone derivatives

The content of total hydroquinone derivatives calculated as anhydrous arbutin was determined spectrophotometrically in plant materials and dry extracts according to the method in Ph. Eur. 3 (Ph. Eur. 3, 1997).

3.4.2. Determination of arbutin and hydroquinone

HPLC/DAD qualitative analysis of arbutin and hydroquinone in dry leaves of our samples were performed according to the monograph of *Uvae ursi folium* in European Pharmacopoeia 5.0 (Ph. Eur. 5, 2005). The results were calculated with reference to the dried drug and expressed as a pecent of dry sample.

In order to compare potential use of investigated species as substituents for *Uvae ursi folim*, determination of total hydroquinone derivatives, arbutin and hydroquinone was done also in the comercial drug *Uvae ursi folium* and in two *Vaccinium* species: *Vaccinium myrtillus* (collected in July 2006, Mountain Stara Planina, Serbia) and *Vaccinium vitis-idaea* (collected in July 2006, Mountain Stara Planina, Serbia).

3.4.3. Determination of total flavonoids

The content of total flavonoids was determined spectrophotometrically in dry extracts and plant materials according to DAB 10 monograph *Crataegi folium et flores* (DAB 10, 1994). Glycosides and aglycones were determined together in $AlCl_3$-complex form of purified ethyl acetate phase obtained after acid hydrolysis. The results were expressed as total flavonoids in % (w/w) of dry matter.

3.4.4. Determination of total polyphenols

The total phenolic content of extracts and plant materials was estimated using the Folin-Ciocalteu colorimetric procedure (Hagerman et al. 2000; Maksimović et al. 2005). Quantification was done on the basis of a standard curve with (+)-catechin. Results were expressed as % (w/w) of sample (dry plant material or crude ethanolic extract).

3.4.5. Determination of total tannins

Total tannins content was determined by the same Folin-Ciocalteu procedure after removal of tannins by their adsorption on insoluble matrix (Ha-

german et al. 2000; Maksimović et al. 2005). Assay was carried out with clear supernatant and results were expressed as % (w/w) of sample.

3.5. Determination of antioxidant capacity

3.5.1. FRAP assay

Ferric reducing antioxidant power (FRAP) assay was used to measure the concentration of total antioxidants. An intense blue colour with absorption maximum at 593 nm appears when the TPTZ-Fe^{3+} complex reduces to the TPTZ-Fe^{2+} form in the presence of antioxidants. The reduction occurred rapidly with all reductants with half-reaction reduction potentials above that of Fe^{3+}/Fe^{2+} (Pellegrini et al. 2003). Results, FRAP values, are expressed as μmol ferric iron reduced per g of sample (μmol Fe^{2+}/g of extract).

3.5.2. DPPH assay

The free radical scavenging activities were determined using a stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the method described by Cuendet et al. (1997). Inhibition of DPPH free radical in percent was calculated according to:

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

where A_b is the absorbance of the control reaction (containing ethanol instead of test solution), and A_s is the absorbance of the sample.
Dose response curves were constructed and DPPH results calculated as the concentration of sample required to scavenge 50% of the free radical (IC_{50}). Concentrations are expressed in μg/mL. BHT was used as reference compound.

3.5.3. Inhibition of lipid peroxidation

Antioxidative effects of extracts were also examined by inhibition of LP in liposomes induced by Fe^{2+}/ascorbate system and quantified spectrophotometrically by the TBA-test (Štajner 1990; Simić et al. 2003). LP was evidenced by the formation of malondialdehyde (MDA) – the last product of lipid breakdown caused by oxidative stress. Inhibition of lipid peroxidation was expressed in percents.

3.6. Statistical analysis

The results are presented as mean values ± standard error of mean (± SEM). Statistical comparisons between groups were performed with Student's t-test and $p < 0.05$ was accepted for statistical significance.

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