Industrial Crops and Products 87 (2016) 304–314

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Phenolics composition of leaf extracts of raspberry and blackberry cultivars grown in Serbia

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ARTICLE INFO

Article history:
Received 18 January 2016
Received in revised form 14 March 2016
Accepted 20 April 2016
Available online 2 May 2016

Keywords:
Phenolic profile
Antioxidant potential
Leaves
Raspberry
Blackberry
LC–MS/MS

ABSTRACT

The polyphenolic profiles of leaves from three raspberry (*Rubus idaeus* L.) cultivars ('Willamette', 'Tulameen', 'Meeker') and one blackberry (*Rubus fruticosus* L.) cultivar, ('Čačanska Bestrna') grown in West Serbia were evaluated. To characterise the extracts, two hyphenated techniques were employed. Ultra-high performance liquid chromatography (UHPLC) coupled with an LTQ OrbiTrap mass spectrometer was used to identify polyphenolics. A rapid and accurate procedure in negative ion mode using a gradient elution within 15 min was established. This allowed us to identify 83 compounds, mostly hydroxycinnamic acid esters, flavonol glycosides, flavan-3-ols and ellagic acid derivatives. Hydroxybenzoic acid derivatives were identified in all investigated samples. Quantification was achieved with a time selected reaction monitoring (tSRM) method using UHPLC coupled with a diode array detector (DAD) and connected to a triple-quadrupole mass spectrometer. Among 23 compounds that were quantitated using available standards, ellagic acid was found to be the main phenolic acid in the *Rubus* leaves [ranging from 1574 to 2875 mg kg^{-1} dry weight (DW)]. The raspberry leaves had higher contents of catechin, epigallocatechin gallate, rutin, ferulic acid, chlorogenic acid and *p*-hydroxybenzoic acid than the blackberry leaf sample. On the other hand, there was more caffeic acid and aesculin in the blackberry leaf sample than in raspberry leaves. All leaves were characterized by a high total phenolic content (84.64–144.20 mg GAE g^{-1} DW), favourable radical scavenging capacity (38.05–83.77%), adequate ferric ion reducing power (59.74–92.80 μmol L^{-1} of an ascorbic acid solution), good superoxide anion radical scavenging activity (28.43–39.87%), but low metal chelating ability (1.55–8.86%). To our best knowledge, this is the first report describing the phenolic profile of 'Čačanska Bestrna' leaves.

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1. Introduction

Due to favourable agro-ecological conditions, Serbia is one of the leading producers and exporters of small berries belonging to the *Rosaceae* family, especially raspberries (*Rubus idaeus* L.) and blackberries (*Rubus sp.* L.). Western Serbia is the major raspberry growing region and the North American 'Willamette' cultivar predominates among the raspberries cultivated in Serbia (Nikolić et al., 2008), while the 'Meeker' cultivar is less cultivated. The highest quality raspberry fruits are given by 'Tulameen' cv., which is primarily, consumed fresh. Among blackberries, the local 'Čačanska Bestrna' cultivar has been widely planted in Serbia and it displays excellent performance regarding cropping, fruit quality and resistance to diseases (Milošević et al., 2012).

Various medicinal properties have been ascribed not only to the fruit, but also to the leaves and roots (Nile and Park, 2014; Ryan et al., 2001; Venskutonis et al., 2007). Both raspberry and blackberry leaves have a long tradition as suitable herbal medicines for treating certain digestive disorders and other maladies. Infusions from raspberry leaves are traditionally used for easing childbirth-related muscle spasms, morning sickness, colds, sore throats, diarrhea, wounds, colic pain, and as a uterine relaxant (Četojević-Simin et al., 2015). Blackberry leaves are widely used as a tea substituent, and are present in herbal mixtures used for curing nervous disorders, atherosclerosis, hypertension and radiation diseases (Melkadze

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http://dx.doi.org/10.1016/j.indcrop.2016.04.052
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et al., 2008; Wang and Lin, 2000). Blackberry leaf extract was found to be useful against *Helicobacter pylori*, the bacterial species associated with stomach ulcers (Martini et al., 2009). Also, it is very effective for relieving acute diarrhea, and for treating mucous membrane inflammation of the mouth and throat. According to several publications (Oszmiański et al., 2015; Wang and Lin, 2000) young blackberry leaves have high levels of antioxidants and considerable antioxidant capacity.

Raspberry leaves are mainly characterized by hydrolysable tannins (2.6–6.9%) including gallotannins (Durgo et al., 2012; Gudej and Tomczyk, 2004), and ellagitannins (Haslam et al., 1989). Other polyphenolic compounds are mainly flavonoids such as kaempferol, quercetin, and their respective glycosides, representing 0.46–1.05% of dried leaf mass (Gudej, 2003; Gudej and Tomczyk, 2004), while phenolic acids, such as caffeic and chlorogenic acid, are found in small amounts (Durgo et al., 2012). Many studies have demonstrated that blackberry leaves contain various components which are believed to be responsible for their beneficial effects on human health (Martini et al., 2009; Tavares et al., 2012). High levels of various phenolic acids together with ellagic acid and flavan-3-ols were reported (Buřičová et al., 2011). Recently, the study of twenty-six different wild blackberry leaf samples showed that ellagic acid derivatives were the predominant compounds found in extracts (Oszmiański et al., 2015).

Previously, we have published data on polyphenols in the berries of four different raspberry cultivars, 'Willamette', 'Tulameen', 'Meeker' and 'Yellow Meeker', and the blackberry, 'Čačanska Bestrna', grown in Serbia (Pavlović et al., 2013). The fruits of 'Willamette' and 'Meeker' grown in Serbia were evaluated for their phenolic content and biological activity (Četojević-Simin et al., 2015). Also, studies on the physical and chemical properties of the fruits of 'Čačanska Bestrna' blackberry cultivar were reported (Ivanović et al., 2014; Milivojević et al., 2013; Stanisavljević, 1999).

However, a literature survey led us to conclude that comprehensive studies on the polyphenolic content and antioxidant activity of raspberry and blackberry leaves are rather scarce. Also, according to our knowledge, there are no published data concerning the antioxidant potential and phenolic content of leaf of the Serbian 'Čačanska Bestrna' blackberry cultivar. Therefore, the aim of this work was to investigate polyphenols in the leaves of the three raspberry cultivars that predominate in Serbia ('Willamette', 'Tulameen', and 'Meeker') and of the blackberry cultivar, 'Čačanska Bestrna'. To this end, UHPLC-LTQ OrbiTrap MS/MS and UHPLC-DAD MS/MS were used to identify and quantitate characteristic flavonoids and phenolic acids, respectively. Coupling of liquid chromatography with mass spectrometry provided us with important structural information by resolving each metabolite in both chromatographic (retention time) and mass spectrometric (m/z) dimensions, as discussed in the results. Also, the antioxidant potential of the leaf extracts was evaluated using four assays, DPPH·-scavenging activity (DPPH-SA), superoxide anion radical scavenging activity (SAS), reducing power (RP), and metal chelating ability (MCA). Finally, the total phenolic content (TPC) and polyphenolic profiles characteristic for each investigated cultivar were compared, manifesting certain differences in flavonoid and phenolic acid contents among *Rubus* leaves.

2. Experimental

2.1. Chemicals and materials

All MS grade solvents (methanol, acetonitrile, and formic acid), ethyl acetate, methanol, sodium hydroxide, monobasic potassium phosphate, hydrochloric acid and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), iron(II) chloride, 2,2 diphenyl-1-picrylhydrazyl radical (DPPH·), ferrozine, potassium hexacyanoferrate(III), iron(III) chloride, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), Tris-HCl buffer, nicotinamide adenine dinucleotide reduced dipotassium salt (NADH), Nitroblue Tetrazolium Chloride (NBT), phenazine methosulphate (PMS), and phenolic compound standards used for the LC–MS analysis were purchased from Sigma-Aldrich (Steinheim, Germany). All other reagents were of analytical grade. Syringe membrane filters (13 mm, PTFE, 0.45 μm) were purchased from Supelco (Bellefonte, PA, USA).

Table 1
Plant material examined in this study. Total phenolic content (TPC) and antioxidant activity (DPPH-SA, MCA, RP, and SAS)[a].

Sample	RW	RM	RT	BC
Cultivar	Willamette	Meeker	Tulameen	'Čačanska Bestrna'
Species	*Rubus idaeus* L.	*Rubus idaeus* L.	*Rubus idaeus* L.	*Rubus fruticosus* L.
Harvest time	June, 2012	July, 2012	July, 2012	July, 2012
TPC[b]	143.38 ± 4.68^a	144.20 ± 1.58^a	84.64 ± 2.05^b	132.90 ± 3.33^c
DPPH-SA[c]	66.78 ± 1.98^c	72.55 ± 1.13^b	38.05 ± 2.51^d	83.77 ± 0.91^a
MCA[c]	4.08 ± 0.26^b	2.76 ± 0.09^c	1.55 ± 0.11^d	8.86 ± 0.32^a
RP[d]	59.74 ± 10.80^b	92.80 ± 1.87^a	77.14 ± 6.73^{ab}	71.62 ± 11.68^b
SAS[c]	39.87 ± 4.02^a	39.15 ± 2.65^a	28.43 ± 3.05^b	36.22 ± 1.98^a

Different letters in the same column denote a significant difference according to Tukey's test, $p < 0.05$.

[a] The values shown are the mean ± standard deviation of three replications.

[b] Total phenolic contents are expressed as mg GAE g^{-1} dry weight.

[c] DPPH· scavenging activity (DPPH-SA), metal chelating ability (MCA), and superoxide anion radical scavenging activity (SAS) are expressed as percent.

[d] The reducing substances were expressed as the concentration (μmol L^{-1}) of an ascorbic acid solution, used as standard.

2.2. Plant material

Raspberry leaf ('Willamette', 'Meeker', and 'Tulameen' cultivars) and blackberry leaf ('Čačanska Bestrna' cultivar) samples were collected in Arilje, West Serbia (Table 1) during the full ripening stage of the fruits (June–July in 2012). A total of 20 leaves were collected from five individual plants of each cultivar. All leaves were harvested from healthy and non-diseased plants and were at the same developmental stage. All the herbs were authenticated in the field by the authors, and plant material was deposited at herbarium of the Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia.

2.3. Sample preparation

After harvesting, the leaves were rinsed with water to remove dust and air-dried at room temperature in the dark. After removal of the mid-vein, the dry material was pooled and ground into a fine powder using a mortar and pestle, and subsequently stored at room temperature for further analysis.

2.4. Preparation of the extracts

Polyphenols were extracted from raspberry and blackberry leaves using the previously described method (Pavlović et al., 2013). Briefly, the ground leaves (2 g) were mixed with 100 mL of a mixture of methanol/water (70:30, v/v) and extracted in an ultrasonic bath for 1 h at room temperature. After 24 h in the dark at 4° C, the extraction step was repeated twice. After collecting together all the supernatants and evaporating the solvents, the residue was dissolved in a mixture of methanol/water (60:40, v/v) and filtered through a 0.45 μm membrane filter (Syringe Filter, PTFE, Supelco) before analysis. All the extractions were performed in triplicate.

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