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Determination of Flavonoids, Tannins and Ellagic Acid in Leaves from *Rubus* L. Species

Jan Gudej[1,2] and Michal Tomczyk[1]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Bialystok, ul. Mickiewcza 2a, 15-230 Bialystok, Poland and [2]Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Łódz, ul. Muszyñskiego 1, 90-151 Łódz, Poland

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This paper describes the quantitative determination of flavonoids, tannins and ellagic acid in the leaves from wild and cultivated variations of *Rubus* L. species (Rosaceae): raspberry (2 wild and 13 cultivars) and blackberry (3 wild and 3 cultivars). The content of flavonoids was analyzed using spectrophotometric (the Christ-Müllers method) and HPLC analysis after acid hydrolysis. The content of tannins was determined by the weight method, with hide powder, described by German Pharmacopoeia 10 (DAB 10). Ellagic acid content was examined using the HPLC method after acid hydrolysis. Flavonoid content, determined using the Christ-Muller's method was higher for the blackberry leaves than for the raspberry leaves and varied between 0.46% and 1.05%. Quercetin and kaempferol were predominant in all samples analyzed using the HPLC method. The highest flavonoid content was found in the leaves of *R. nessensis* (1.06%); with results in all of the examined samples varying between 0.27% and 1.06%. The concentration of ellagic acid in all species was determined after acid hydrolysis and ranged from 2.06% to 6.89%. The leaves of raspberries are characterized by greater amounts of tannins (varying between 2.62% and 6.87%) than the leaves of other species. The results from this study indicate that the analyzed species are a rich source of flavonoids, ellagic acid and tannins, which may be used for the quality assessment of *Rubus* L. species leaves.

Key words: *Rubus* L. species, Flavonoids, Tannins, Ellagic acid, HPLC determination, Spectrophotometric determination

INTRODUCTION

The genus *Rubus* (raspberry, blackberry) comprises around 700 species, naturally occurring in temperate climates (Alice and Campbell, 1999; Heslop-Harrison, 1968). Some are cultivated in numerous varieties as industrial plants for the quality of nutritious and tasty fruits. Of the many varieties of *Rubus* species, only ten are used commercially. Currently, in Poland, ten varieties of raspberry and two of blackberry were introduced to official Register (Gwozdecki, 1996). Monography of *R. fruticosus* leaf was introduced to Polish Pharmacopoeia (2002).

Rubus species have been used in traditional medicine for their many medicinal properties (Patel *et al.*, 2004). Blackberry leaves have been used for their astringent, antidiarrhoeic, hypoglycemic activities and as an anti-inflammatory agent for the mucous membrane of the oral cavity and throat (Borkowski *et al.*, 1994; Ozarowski and Jaroniewski, 1989). Raspberry leaves (*R. idaeus* L.) have been commonly used to treat a variety of ailments including diseases of the alimentary canal, air-passage, heart and the cardiovascular system. However, they are best known for their health benefits in treating fever, influenza, diabetes, menstrual pain, diarrhea and colic pain. The leaves of raspberry may also be applied externally as antibacterial, anti-inflammatory, sudorific, diuretic and choleretic agents (Czygan, 1995; Ozarowski and Jaroniewski, 1989). Raspberry leaf extract has been reported to have relaxant effect, particularly on uterine muscles (Burn and Withell, 1941; Robbers and Tyler, 1999; Rojas-Vera *et al.*, 2002). Beneficial effects of using raspberry leaves during pregnancy and labor have been noticed (Simpson *et al.*, 2001). Previous phytochemical

Correspondence to: Jan Gudej, Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Bialystok, ul. Mickiewicza 2a, 15-230 Bialystok, Poland
Tel: 48-85-748-5692, Fax: 48-85-748-5416
E-mail: fgnozja@amb.edu.pl

investigations, which were carried out on different plant parts taken from *Rubus* species, have proved the presence of structurally and biogenetically diverse secondary metabolites. Both *R. idaeus* and *R. fruticosus* leaves are especially rich in tannins (Marczal, 1963; Okuda *et al.,* 1992). They contain a notable amount of flavonoid compounds, represented by derivatives of kaempferol and quercetin, phenolic acids, triterpenes, mineral salts as well as vitamin C (Gudej and Rychlinska, 1996; Krzaczek, 1984; Wojcik, 1989). Chemical composition of epicuticular waxes from the flowers and leaves of *R. idaeus* has already been described (Shepherd *et al.,* 1999). Recently, derivatives of quercetin and kaempferol, ellagic acid and methyl gallate have been isolated from raspberry leaves (Gudej, 2003). As a part of our overall interest in the *Rubus* species leaves, we have characterized the polyphenolic composition in leaves of *R. saxatilis*. The chromatographic analysis confirmed the presence of phenolic acids and further studies revealed the presence of quercetin, kaempferol, tiliroside, ellagic acid and methyl brevifolincarboxylate. *R. saxatilis* is the first plant from *Rubus* species reported to contain this latter compound (Gudej *et al.,* 1998).

Taking leaves from different wild and cultivated varieties of raspberry and blackberry we have determined the content of flavonoids by the spectrophotometric method (Christ and Müller, 1960; Polish Pharmacopoeia, 2002) and the HPLC method after acid hydrolysis (Sticher, 1993; Olszewska *et al.,* 2001). The tannins content was established by using DAB 10 (1998) and ellagic acid by the HPLC method after acid hydrolysis. These polyphenolics are widely distributed in the plant kingdom and it is claimed that they have beneficial effects on health as antioxidant, antimicrobial, anti-inflammatory, antiviral and as anticarcinogen agents (Havsteen, 2002; Thiem and Goœliñska, 2004; Priyadarsini *et al.,* 2002; Barch *et al.,* 1996). The standardization of plant material requires a direct quantification of the naturally occurring active principles. This has encouraged us to describe the main polyphenolics (e.g. flavonoids, tannins) in *Rubus* leaves which appear to be correlated with their biological activities, and to determine the polyphenolic compounds by using different analytical methods. Further, we compared the content of flavonoids, ellagic acid and tannins in the leaves of the wild species with those of the cultivated species.

MATERIALS AND METHODS

Plant material

Leaves of *Rubus* (raspberry, blackberry) from cultivated and wild-growing varieties are listed in Table I. The leaves from cultivars were harvested from different experimental areas in The Research Institute of Pomology and Floriculture in Skierniewice, Poland in June 2001. The leaves from wild species of the plants were collected during June-July 2001 from Puszcza Knyszynska, near Bialystok, Poland. Plant material was authenticated by Dr. J. Gwozdecki (The Research Institute of Pomology and Floriculture in Skierniewice, Poland) and Prof. J. Gudej (Medical University of Bialystok, Poland). Voucher specimens have been deposited in the Herbarium of the Department of Pharmacognosy, Medical University of Bia^{3}ystok, Poland.

Chemicals and solvents

Quercetin, kaempferol and ellagic acid were purchased from ROTH (Karlsruhe, Germany). All the solvents, both of analytical and HPLC grade were purchased from POCH (Gliwice, Poland). Milli-Q Plus (Millipore, MA, USA) treated water (18.2 MΩ cm) was used throughout the analysis.

HPLC analysis for determination of flavonoids and ellagic acid

Samples were analyzed on the Waters HPLC system (Milford, MA, USA) equipped with Waters 600E pump, a 600 Controller and a 996 PDA detector scanning between 190 and 400 nm. The data were collected and analyzed with the Millenium^{32} Chromatography Manager V4.0 Software. Separation was carried out using Symmetry C_{18}, column (5 μm; 3.9×150 mm, Waters Corp., USA). The flavonoids and ellagic acid were detected at 370 nm and 254 nm, respectively, corresponding to the λ_{max} of analyzed compounds in methanol solution. The mobile phase consisted of solvent A [methanol] and solvent B [0.5% (v/v) *ortho*-phosphoric acid in water]. The elution profile was as follows: 0 min. 40% A in B, 0-0.5 min 40% to 60% A in B, 0.5-2.5 min 65% A in B, 2.5-6.0 min 65% to 45% A, 6.0-8.0 min 40% A in B. All gradients were linear. The flow rate was 1.0 mL/min. For each sample, three replicate assays were performed. The retention times for ellagic acid, quercetin and kaempferol were 6.30, 7.52 and 8.70 min, respectively. The calibration curves were defined for each compound in the range of sample quantity 0.02-0.5 μg.

Sample preparation for HPLC analysis

The dried and pulverized plant material (200 mg) was extracted with 6 mL of 25% hydrochloric acid and 20 mL methanol for 1 h. The obtained extract was filtered to a volumetric flask. The residue was then heated twice with 20 mL of methanol for 20 min. The combined extracts were diluted with methanol to 100 mL. A 5 mL portion of the solution was filtered through a Chromafil O-45/25 (PTFE 25 mm, 0.45 μm, Macherey-Nagel, Germany) and then were transferred to a volumetric flask and diluted with 10 mL of methanol. The filtrate (20 mL) was injected into the HPLC apparatus.

Determination of flavonoids according to the Christ-Müllers method

Total flavonoid content was measured according to the Christ-Müller's method (Christ and Müller, 1960; Polish Pharmacopoeia, 2002) using a SPECORD 40 Spectrophotometer (Analytik Jena, Germany). Content of flavonoids in all sources was calculated as quercetin and hyperoside and samples for the analysis were prepared according to the Polish Pharmacopoeia VI.

Determination of tannins

Total tannin content was determined by the weight method with hide powder according to the DAB 10 (1998).

RESULTS AND DISCUSSION

The content of flavonoids according to the Christ-Müllers method was calculated as quercetin and as hyperoside. Flavonoid content, based on the Christ-Mullers method was higher for the blackberry leaves than for the raspberry leaves and varied between 0.46% and 1.05%. The results obtained using the Christ-Mullers method (Table II) for hyperoside varied from 0.65% to 0.92% in the raspberry leaves collected from natural habitat, from 0.50% to 0.83% in the raspberry leaves from cultivars, from 0.46% to 1.05% in the blackberry leaves from wild species and from 0.50% to 0.82% in the blackberry leaves from cultivars. The results according to this method calculated for quercetin were much lower and varied for all sources from 0.35% to 0.73%. The extracts after acid hydrolysis were analyzed by RP-HPLC coupled with diode-array detector, using gradient elution of methanol and 0.50% *ortho*-phosphoric acid in water. The major phenolics were found to be flavonol such as quercetin and kaempferol and ellagic acid. The amount of flavonoids and ellagic acid was detected and quantified in the HPLC analysis in the same run (Fig. 1, 2). In the modified study of HPLC after acid hydrolysis (Sticher, 1993; Olszewska *et al.*, 2001) the values obtained for flavonoids total (Table II) were lower than those obtained using the Christ-Müllers method. The differences in flavonoid patterns observed between both methods showed a significant error in the spectrophotometric method and were caused by a possible reaction between aluminum chloride and non-flavonoid compounds present in the investigated samples (Tomczyk and Gudej, 2003). Quercetin and kaempferol were predominant in all investigated samples analyzed using HPLC method but there was no significant difference between quercetin and kaempferol content. The highest flavonoid content in the HPLC method was found in the leaves of *R. nessensis* (1.06%). Also, the highest flavonol content was observed in the leaves of wild raspberry, *R. saxatilis* (0.56%) as well as in cultivars of raspberry (*R. idaeus* Malling Promise) and blackberry (*R. fruticosus* Gazda) - 0.57% and 0.46%, respectively. The concentration of flavonoids in samples of berries had been previously investigated (Häkkinen *et*

Table I. Selected wild and cultivated raspberries and blackberries

Wild / Cultivar	Species
Raspberry from wild species	*R. saxatilis*
	R. idaeus
Raspberry from cultivars	*R. idaeus* Beskid
	R. idaeus Canby
	R. idaeus Malling Seeding
	R. idaeus Norna
	R. idaeus Veten
	R. idaeus Willamette
	R. idaeus Autumn Bliss
	R. idaeus Polana
	R. idaeus Malling Promise"
	R. idaeus Tulameen
	R. idaeus Glen Ample
	R. idaeus Zenith
	R. occidentalis Bristol
Blackberry from wild species	*R. fruticosus*
	R. caesius
	R. nessensis
Blackberry from cultivars	*R. odoratus*
	R. fruticosus Gazda
	R. fruticosus Thornfree

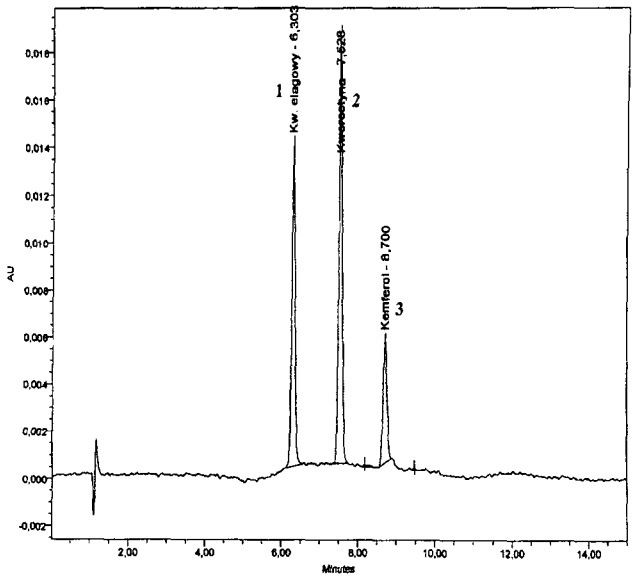

Fig. 1. HPLC chromatogram of standards: 1 - ellagic acid (t_R= 6.30 min.); 2-quercetin (t_R = 7.52 min.); 3 - kaempferol (t_R = 8.70 min.).

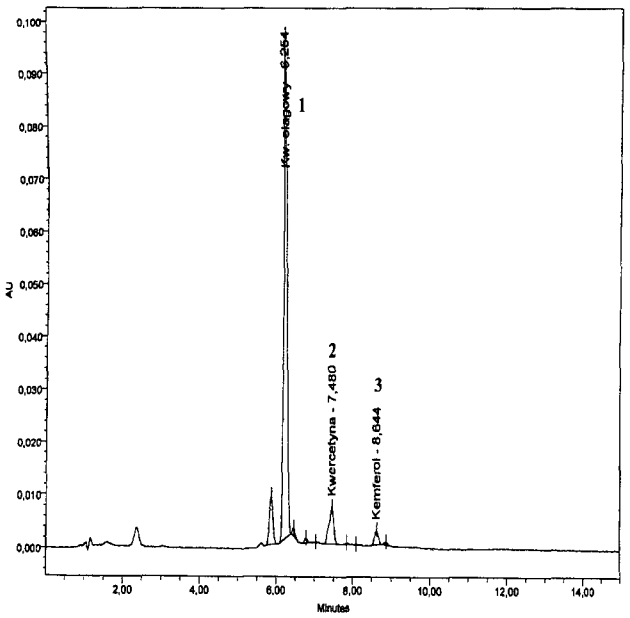

Fig. 2. Representative HPLC chromatogram of extract from one of *Rubus* L. species (*R. nessensis* L.) after acid hydrolysis. 1-ellagic acid; 2-quercetin; 3 kaempferol.

al., 1999; Mullen *et al.,* 2002), but the contents of flavonoids in samples of wild and cultivars *Rubus* L. leaves have been reported for the first time in this study. The ellagic acid content of the leaves from wild and cultivated varieties of raspberry and blackberry using the HPLC method was also determined. Quantitative results for all samples are shown in Table III. Ellagic acid derivatives are polyphenolics, which seem to be the main compounds present in different parts of many species belonging to *Rubus* genus. Ellagic acid may occur in plants in the free form but is very often released through hydrolysis of ellagitannins. The determination of ellagic acid as a main polyphenol in fresh, dry and processed fruits of *Rubus* species, had been reported earlier (Daniel *et al.,* 1989; Rommel and Wrolstad, 1993; Häkkinen *et al.,* 2000; Amakura *et al.,* 2000). In present analysis, the concentration of ellagic acid in all species was determined after acid hydrolysis and ranged between 2.06% and 6.89%. The leaves of *R. nessensis* showed the highest amount of this compound (6.89%). The results obtained from determination of ellagic acid by the HPLC analysis are similar to those obtained for cloudberrys (*R. chamaemorus* L.)

Table II. Results of the quantitative determination of flavonoids in leaves of *Rubus* L. species using spectrophotometric (Christ-Müllers method) and HPLC methods after acid hydrolysis

Sample of leaves	Spectrophotometric method		HPLC method		
	Total content of flavonoids [% of dry weight]		Content of flavonoids [% of dry weight]		
	Quercetin	Hyperoside	Quercetin	Kaempferol	Sum of aglycones
R. saxatilis	0.65 (± 0.3)	0.92 (± 0.7)	0.12 (± 3.5)	0.44 (± 2.5)	0.56
R. idaeus	0.45 (± 3.4)	0.65 (± 2.4)	0.21 (± 2.6)	0.18 (± 0.6)	0.39
R. idaeus Beskid	0.43 (± 2.1)	0.63 (± 1.9)	0.24 (± 0.8)	0.28 (± 1.3)	0.52
R. idaeus Canby	0.43 (± 1.6)	0.63 (± 1.5)	0.18 (± 1.3)	0.31 (± 0.9)	0.49
R. idaeus Malling Seeding	0.57 (± 1.9)	0.83 (± 2.2)	0.13 (± 0.4)	0.27 (± 2.0)	0.40
R. idaeus Norna	0.50 (± 1.1)	0.73 (± 1.6)	0.28 (± 1.2)	0.19 (± 1.4)	0.47
R. idaeus Veten	0.42 (± 0.7)	0.60 (± 0.8)	0.21 (± 2.9)	0.18 (± 3.8)	0.39
R. idaeus Willamette	0.45 (± 0.9)	0.65 (± 2.1)	0.19 (± 0.8)	0.19 (± 2.1)	0.38
R. idaeus Autumn Bliss	0.36 (± 2.5)	0.52 (± 2.0)	0.10 (± 1.7)	0.22 (± 1.9)	0.32
R. idaeus Polana	0.43 (± 3.2)	0.62 (± 1.8)	0.24 (± 2.6)	0.17 (± 0.6)	0.41
R. idaeus Malling Promise"	0.53 (± 3.9)	0.76 (± 2.6)	0.32 (± 1.8)	0.25 (± 1.4)	0.57
R. idaeus Tulameen	0.54 (± 2.1)	0.77 (± 1.4)	0.20 (± 0.8)	0.29 (± 0.9)	0.49
R. idaeus Glen Ample	0.56 (± 4.1)	0.80 (± 3.5)	0.25 (± 0.5)	0.18 (± 1.5)	0.43
R. idaeus Zenith	0.35 (± 0.7)	0.50 (± 0.9)	0.12 (± 1.7)	0.17 (± 3.2)	0.29
R. occidentalis Bristol	0.43 (± 1.3)	0.62 (± 2.6)	0.23 (± 1.5)	0.33 (± 2.5)	0.56
R. fruticosus	0.49 (± 1.6)	0.70 (± 2.2)	0.14 (± 0.5)	0.20 (± 2.8)	0.34
R. caesius	0.32 (± 2.6)	0.46 (± 3.7)	0.10 (± 0.9)	0.25 (± 0.8)	0.35
R. nessensis	0.73 (± 3.2)	1.05 (± 2.9)	0.64 (± 1.4)	0.42 (± 3.4)	1.06
R. odoratus	0.42 (± 2.3)	0.60 (± 1.5)	0.22 (± 2.3)	0.18 (± 2.3)	0.40
R. fruticosus Gazda	0.57 (± 1.8)	0.82 (± 1.3)	0.31 (± 1.1)	0.15 (± 0.4)	0.46
R. fruticosus Thornfree	0.35 (± 2.5)	0.50 (± 1.8)	0.16 (± 2.6)	0.11 (± 2.1)	0.27

Values in parentheses are relative standard deviations RSD (%) (n=3)

Table III. Results of the quantitative determination of tannins using the weight method and ellagic acid using the HPLC method after acid hydrolysis in leaves of *Rubus* L. species

Sample of leaves	Weight method	HPLC method
	Content of tannins [% of dry weight]	Content of ellagic acid [% of dry weight]
R. saxatilis	5.37 (± 1.4)	3.01 (± 0.9)
R. idaeus	3.25 (± 0.7)	2.54 (± 0.4)
R. idaeus Beskid	6.25 (± 4.5)	3.36 (± 1.7)
R. idaeus Canby	4.50 (± 3.6)	2.98 (± 2.1)
R. idaeus Malling Seeding	6.25 (± 1.5)	4.00 (± 3.2)
R. idaeus Norna	2.63 (± 0.7)	2.36 (± 1,8)
R. idaeus Veten	4.87 (± 3.8)	3.10 (± 1.4)
R. idaeus Willamette	6.50 (± 4.1)	4.11 (± 2.7)
R. idaeus Autumn Bliss	4.25 (± 0.8)	3.86 (± 1.6)
R. idaeus Polana	2.62 (± 1.3)	2.07 (± 0.7)
R. idaeus Malling Promise"	3.56 (± 2.5)	2.85 (± 3.1)
R. idaeus Tulameen	6.87 (± 3.1)	4.23 (± 2.6)
R. idaeus Glen Ample	4.25 (± 2.0)	2.89 (± 1.1)
R. idaeus Zenith	3.87 (± 1.6)	2.88 (± 2.0)
R. occidentalis Bristol	2.75 (± 1.8)	2.06 (± 1.5)
R. fruticosus	6.50 (± 2.3)	4.32 (± 2.4)
R. caesius	4.25 (± 1.9)	4.15 (± 0.8)
R. nessensis	5.25 (± 2.2)	6.89 (± 2.9)
R. odoratus	5.12 (± 1.9)	3.76 (± 2.3)
R. fruticosus Gazda	4.12 (± 3.4)	2.93 (± 1.7)
R. fruticosus Thornfree	5.25 (± 0.6)	4.21 (± 2.5)

Values in parentheses are relative standard deviations RSD (%) (n=3)

leaves (Krawczyk *et al.,* 2003). In addition, the total content of tannins using the weight method with hide powder recommended by DAB 10 (1998) was determined. The leaves of raspberries are characterized by greater amounts of tannins than the leaves of other species (Table III) and vary between 2.62% for leaves of *R. idaeus* Polana and 6.87% for leaves of *R. idaeus* Tulameen.

CONCLUSION

The results from this study indicate that analyzed species are a rich source of flavonoids, ellagic acid and tannins which may be used for the quality assessment of *Rubus* L. species leaves and may suggest that some leaves from different *Rubus* species (both wild species and cultivated varieties), could be of equal value to those which have been characterized as having medicinal properties.

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