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Antioxidant tannins from Rosaceae plant roots

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Abstract

Polyphenols were analyzed by HPLC after thioacidolysis of proanthocyanidins polymers and acid hydrolysis of phenolic acid esters. The predominant constitutive units of the procyanidins of *Aruncus Silvester* and *Potentilla alba* roots were (−)epicatechin, and *Geum rivale* and *Waldsteinia geoides* roots (+)catechin. The highest proanthocyanidin concentrations were found in *Potentilla alba* roots (close to 80 g/kg) and *W. geoides* (64 g/kg). Ellagic acid was present at high concentration in *G. rivale* (2.68 g/kg) and *W. geoides* (2.75 g/kg) in dried roots. The antioxidant activity, measured by the DPPH method, ranged from 0.72 (*Filipendula vulgaris*) to 4.40 (*W. geoides*) mM trolox equivalents/kg dried roots and, measured by the ABTS method, from 1.50 to 6.60 mM trolox equivalents per kg of dried roots.

Keywords: Proanthocyanidins; Ellagic acid; ABTS; DPPH; *Filipendula vulgaris* L.; *Geum rivale* L.; *Aruncus Silvester*; *Waldsteinia geoides*; *Potentilla alba* L.

1. Introduction

Tannins are secondary plant metabolites subdivided into condensed and hydrolyzable compounds. Condensed tannins are also known as proanthocyanidins (PAs), the oligomeric and polymeric flavan-3-ols. The size of PA molecules can be described by their degree of polymerization (DP). Proanthocyanidins are of great interest in nutrition and medicine because of their potent antioxidant capacity and possible protective effects on human health (Santos-Buelga & Scalbert, 2000). It has recently been hypothesized that the free radical scavenging properties of PAs may reduce the risk of cardiovascular diseases, cancer (Bagchi et al., 2000) and blood clotting and certain types of trimeric PAs may protect against urinary tract infections (Santos-Buelga & Scalbert, 2000). Hydrolyzable tannins contain a central core of a polyhydric moiety, such as glucose and hydroxyl groups, which are esterified, either partially or wholly, by gallic acid (gallotannins) or ellagic acid (ellagotannins). Ellagic acid has been found to have antimutagenic, antiviral, antitumor and whitening of skin activities (Khanduja, Gandhi, Pathania, & Syal, 1999). Some Rosaceae species exhibit high tannin contents, essentially *Filipendula vulgaris* L., *Geum rivale* L. (Lamaison, Carnat, & Petitjean-Freytet, 1990; Ming, Jiang, But, Towers, & Yu, 2002; Panizzi, Catalano, Mirelli, Cioni, & Campeol, 2000), and *Potentilla alba* L. (Gonzalez-Hernandez, Karchesy, & Starkey, 2003).

Tannins make a major contribution to antioxidant activity of Rosaceae species (Sun, Chu, Wu, & Liu, 2002; Zheng & Wang, 2001). Previous studies found that there was a direct relationship between antioxidant activity and total phenolic content in selected herbs, vegetables and fruits.

Several methods have been developed to assay free radical-scavenging capacity and total antioxidant activity of plant extracts. The most common and reliable method involves the determination of the disappearance of free radicals, using a spectrophotometer, e.g., 2,2-azinobis (3-thylbenzothiazoline-6-sulfonic) acid radical ($ABTS^{\cdot+}$) and 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\cdot}$). The improved $ABTS^{\cdot+}$ method, established by Re et al. (1999) was successfully used in this study to systematically assess the total antioxidant capacity of the medicinal herb extracts on a large

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scale, being simple, fast, reliable, inexpensive and also very adaptable to both hydrophilic and lipophilic antioxidant/ systems. This effective and efficient method can be used for systematic screening of medicinal herbs and dietary plants for their relative antioxidant contents.

The purpose of the present study was to evaluate proanthocyanidins, phenolic acids and antioxidant activity of chosen Rosaceae medical plant roots.

2. Materials and methods

2.1. Chemicals

All chemicals, standards and reagents were of analytical or HPLC grade and were purchased from Sigma Chemical Co. (St Louis, MQ, USA), Aldrich Chemical Co. (Steineheim, Germany) and Merck (Darmstadt, Germany).

2.2. Plant materials

Plants of *F. vulgaris* L., *G. rivale* L., *Aruncus Silvester, W. geoides, P. alba* L, were grown from authenticated seeds obtained from the Garden of Medicinal Plants herbarium at the Medical University in Wroclaw, Poland, by cultivation in the University's experimental field. About 1 kg of roots was collected in the middle of October, 2004, then washed in distilled water and lyophilised. The dried roots (200 g) were then crushed using a laboratory mill and stored at −20 °C until analysed.

2.3. Phenolic acid analysis

The pulverized freeze-dried roots (0.25 g) was hydrolyzed with 2 ml of 2 M HCl in a boiling water bath for 1 h. After cooling, 2 ml of 2 M NaOH and then 6 ml methanol were added to the vial. The slurry was sonicated for 20 min with occasional shaking. Further, the slurry was centrifuged at 19,000*g* and the supernatant was used for HPLC analysis.

The HPLC apparatus consisted of an Hitachi L-7455 diode array detector and a quaternary pump L-7100, equipped with D-7000 HSM Multisolvent Delivery System (Merck–Hitachi), was employed.

Twenty microlitres of the clear supernatant after centrifugation (5 min., 19,000*g*) were injected. Separation was performed on a LiChroCART® 125–3 Purospher® RP-18 (5 μm) MerckLabs column thermostatted at 30 °C. The mobile phase was composed of solvent A (4.5% formic acid) and solvent B (80% acetonitrile and 20% of solvent A). The programme began with isocratic elution with 95% A and 5% B (0–1 min); then a linear gradient was used until 16 min, lowering A to 20% (increasing B to 80%), from 17 to 24 min 100% B and finally, from 25 to 35 min, 100% A. The flow rate was 1 ml min^{-1}, and the runs were monitored at the following wavelengths: gallic acid and *p*-coumaric acid at 280 nm, caffeic acid at 320 nm, ellagic acid at 360 nm. Scanning was performed between 200 and 600 nm. Retention times and spectra were compared with those of pure standards within 200–600 nm. Each sample analysis was repeated three times.

2.4. Proanthocyanidins analysis

Direct thiolysis on freeze-dried root powders was performed as described by Guyot, Marnet, Sanoner, and Drilleau (2001). Powders were precisely weighed (30–50 mg) in 1.5 ml Eppendorf vials. Acidic methanol (3.3%, (v/v), 400 μl) and toluene α-thiol (5% in methanol 800 μl) were added. Vials were closed and incubated at 40 °C for 30 min with agitation (vortex) every 10 min. Then, the vials were cooled in ice water and centrifuged (5 min, 19,000*g*) immediately at 4 °C in a refrigerated centrifuge during 10 min. Samples were stored at 4 °C prior to RP-HPLC analysis. All incubations were repeated three times.

Thiolysis products were separated on a Merck Purospher RP 18 end-capped column, 250 × 4 mm, 5 μm (Merck, Darmstadt, Germany). The HPLC apparatus was a Waters (Milford, MA) system (DAD and scanning fluorescence detectors). The solvent system was a gradient of solvent A (aqueous acetic acid, 2.5%, v/v) and solvent B (acetonitrile) and the following gradient was applied: initial 3% B, 0–5 min, 9% B linear, 5–15 min, 16% B linear, and 15–45 min, 50% B linear, followed by washing and reconditioning the column. The flow rate was 1 ml min^{-1}, temp. 30 °C. The analyses were monitored at 280 nm. Compounds, for which reference standards were available, were identified on chromatograms according to their retention times and UV–Vis spectra. Fluorescence detection, also, was recorded simultaneously at excitation wavelength 278 nm and emission wavelength 360 nm for all compounds. Calibration curves (based on peak area at 280 nm) were established using flavan-3-ol and benzylthioether standards. The average degree of polymerization was measured by calculating the molar ratio of all the flavan-3-ol units (thioether adducts + terminal units) to (−)epicatechin and (+)-catechin corresponding to terminal units.

2.5. Scavenging effect on DPPH and ABTS radicals

DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) and potassium sufate (di-potassium peroxdisulfate) were obtained from Sigma–Aldrich Co. USA. The effect of Rosaceae plant root extracts on DPPH radical was determined according to the method of Yen and Chen (1995). A 1 g of freeze-dried sample was homogenized in 20 ml of methanol. The slurry was filtered ands filtrate was diluted in methanol. A 1 ml aliquot of diluted extract was added to 3 ml of absolute methanol and 1 ml of DPPH (0.012 g DPPH/100 ml). The mixture was shaken and left at room temperature for 10 min; the absorbance was measured spectrophotometrically at 517 nm.

The effect of root extracts on ABTS radical was measured according to Re et al. (1999). ABTS stock solution

(7 mM concentration) and 2.45 mM potassium persulfate were left at room temperature for 16 h to produce ABTS radical cation $ABTS^{\cdot+}$. A 1 g of sample was homogenized in 50 ml of absolute methanol and filtered. $ABTS^{\cdot+}$ solution was diluted with distilled water to an absorbance of 0.700 (±0.02) at 734 nm. A 1 ml of $ABTS^{\cdot+}$ solution was added to 10 μl of root extract; the absorbance was monitored at 734 nm after 6 min. A concentration–response curve, for the absorbance at 734 nm after 6 min for $ABTS^{\cdot+}$ as a function of different trolox concentrations was prepared. The decrease in absorption at 734 nm 6 min after addition of the compound was used for calculating the TEAC (trolox equivalent antioxidant capacity).

All determinations were performed in triplicate.

2.6. Statistical analysis

Results were given as means ± standard deviation of three independent determinations. One way analysis of variance (ANOVA) was used to compare the means. Differences were considered to be significant at $P < 0.05$. All statistical analyses were performed with Statistica 6.0.

3. Results and discussion

The results, presented in Table 1, indicate variability in quantitative and qualitative composition of proanthocyanidins in chosen Rosaceae family plant roots. The highest proanthocyanidin concentrations were found in *P. alba* roots (close to 80 g/kg) and *W. geoides* (63.5 g/kg). This is much higher than, in the same Rosaceae family, chokeberry fruits (52 g/kg dried weight), which are known as a very rich source of proanthocyanidins (Oszmianski & Wojdylo, 2005). Variability with respect to tannins composition can exist between species of the same family.

G. rivale roots contain the lowest quantity of those compounds (10 g/kg). The main constitutive unit of the procyanidins of *A. Silvester* and *P. alba* roots was (−)epicatechin and, of the procyanidins of *F. vulgaris*, *G. rivale* and *W. geoides* roots, (+)catechin. The chromatograms (HPLC) corresponding to the analysis of *A. Silvester* (A) and *W. geoides* (B) have the thiolytic degradation products shown in Fig. 1. The detection of peaks indicated the presence of chain extension units (peaks 3 and 4) and chain terminating units (peaks 1 and 2); in *A. Silvester* these were (−)epicatechin and, in *W. geoides*, (+)catechin. Moreover, thiolysis allows distinction between extension and terminal units of proanthocyanidins to assess the average degree of polymerization (DP). *A. Silvester* roots showed a DP of 13 (Table 1) with small extension peak (−)epicatechin and high (−)epicatechin-4-benzylthioether (Fig. 1) and *W. geoides* a DP of 3 with high (+)catechin and (+)catechin-4-benzylthioether. The DPs of 2–3, which correspond to oligomeric procyanidins, are present in *F. vulgaris*, *G. rivale* and *W. geoides* roots whereas highly polymerized procyanidins, with a DP 6–13, are present in *P. alba* and *A. Silvester* roots. The DP and the nature of the constitutive units are important structural features that are related to the bitterness and astringency of taste and biological activity of proanthocyanidins (Lea & Arnold, 1978; Noble, 1998).

Hydrolyzable tannins, constituted of phenolic acid derivatives, are another important class of phenolic compounds, which was analysed in Rosaceae family root tissues as phenolic acids after hydrolysis (Table 1, Fig. 2). Ellagic acid, as a constituent of ellagotannins, was present at high concentration in *G. rivale* (2.68 g/kg) and *W. geoides* (2.75 g/kg) dried roots. Gallic acid (gallotannins constituent) was found also in *G. rivale* (0.33 g/kg) and *W. geoides* (0.08 g/kg) roots but in smaller quantity than ellagic acid. Levels of condensed tannins are often higher than those of hydrolyzable tannins (Bos et al., 1996). However, various environmental factors determine the extent to which genetic potentialities are achieved. Ecology, drought, soil type/structure, disease, herbivore damage, and farming

Table 1
Polymeric proanthocyanidins and phenolic acids distribution [g/kg] and antioxidant activity of some Rosaceae plant roots[a]

	Filipendula vulgaris	*Geum rivale*	*Aruncus silvester*	*Waldsteinia geoides*	*Potentilla alba*
Thiolytic degradation of proanthocyanidins					
(−)Epicatechin 4-benzyl thioether	0.20 ± 0.01c	0.30 ± 0.03c	14.2 ± 0.06b	0.35 ± 0.15c	64.3 ± 0.07a
(−)Epicatechin	0.35 ± 0.15bc	0.04 ± 0.07d	0.50 ± 0.04b	0.54 ± 0.65b	13.2 ± 0.10a
(+)Catechin 4-benzyl thioether	7.46 ± 0.10b	6.33 ± 0.08b	0.28 ± 0.09d	42.8 ± 1.12a	1.40 ± 0.34c
(+)Catechin	7.69 ± 0.07b	3.84 ± 0.02b	0.70 ± 1.11c	19.8 ± 1.00a	0.86 ± 0.02c
Degree polymerization (DP)	2	3	13	3	6
Total proanthocyanidins	15.7	10.5	15.7	63.5	79.8
Phenolic acids after hydrolytic degradation:					
Ellagic acid	0.03 ± 0.09b	2.70 ± 0.10a	0	2.75 ± 0.99a	0.08 ± 0.18b
Gallic acid	0	0.33 ± 0.00b	0	0.08 ± 0.06b	0
p-Coumaric acid	0	0	0.18 ± 0.19a	0	0.03 ± 0.10b
Caffeic acid	0	0	1.61 ± 0.48a	0	0
Total phenolic acids	0.03 ± 0.04c	3.00 ± 0.02a	1.79 ± 0.57b	2.83 ± 0.67a	0.11 ± 0.19c
Antioxidant activity [μM trolox/100 g dried roots]					
DPPH radicals	0.72 ± 0.00c	2.95 ± 0.05b	0.98 ± 0.24c	4.39 ± 0.76a	3.94 ± 0.09a
ABTS radicals	1.86 ± 0.13c	4.96 ± 0.23ab	1.50 ± 1.00c	3.79 ± 0.10b	6.60 ± 0.37a

[a] Values are means ± SD, $n = 3$;mean values within a column with different letters are significantly different at $P < 0.05$.

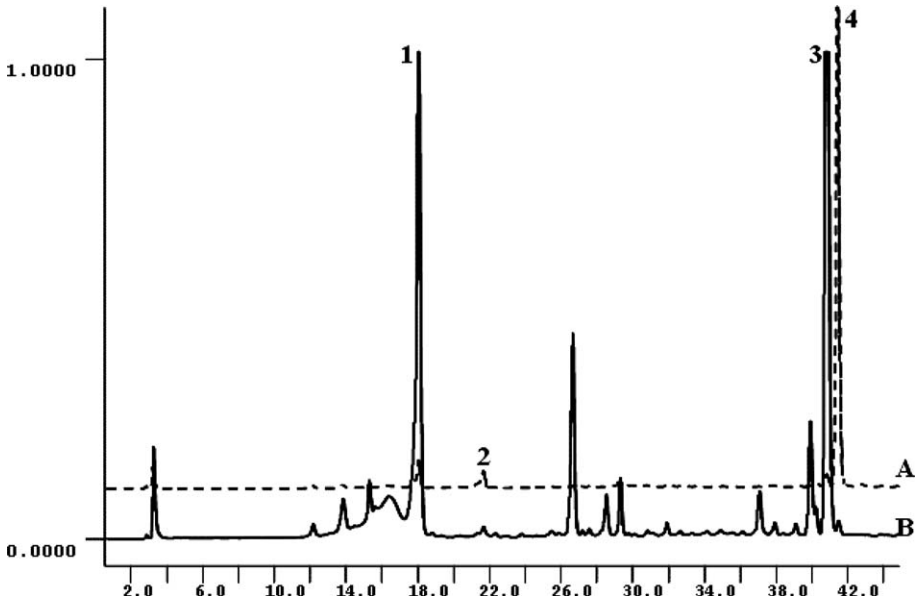

Fig. 1. Chromatograms by HPLC-FD (fluorescence detector) of thiolytic degradation of *Aruncus silvestris* (A); *Waldsteinia geoides* (B) dried roots. 1, (+)Catechin; 2, (−)epicatechin; 3, (+)catechin-4-benzyl thioether; 4, (−)epicatechin-4-benzyl thioether.

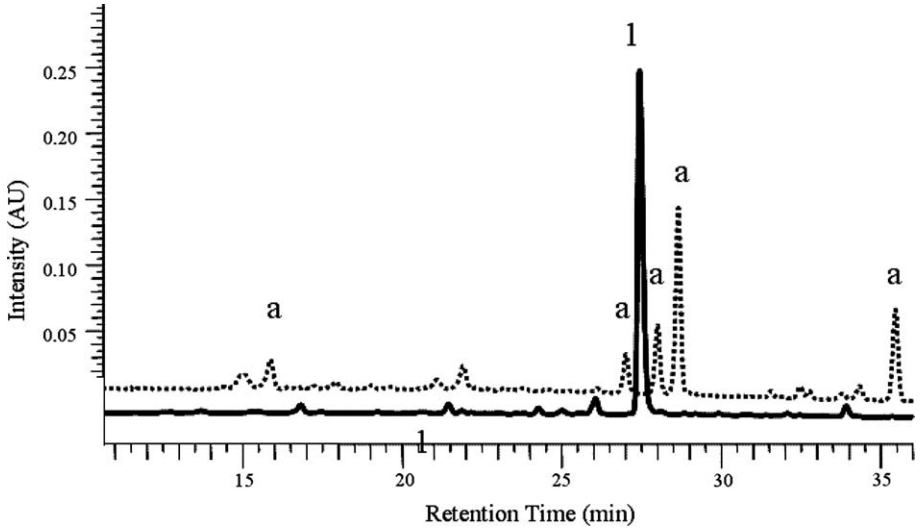

Fig. 2. Chromatogram by HPLC (360 nm) of *Geum rivale* extract before (dotted line) and after hydrolysis (continuous line). a, Ellagic acid derivatives; 1, ellagic acid.

practices (e.g., pruning on application of pesticides) have an influence on secondary plant metabolism.

Caffeic acid derivatives were only detected in the root tissues of *A. Silvester* (1.61 g/kg), and *p*-coumaric acid only in *A. Silvester* (0.18 g/kg) and *P. alba* (0.03 g/kg).

There were large inter-species variations in antioxidant activities, which ranged from 0.72 (*Filipendula ulmaria*) to 4.39 (*W. geoides*) per mM trolox equivalents/kg dried roots for DPPH radicals and from 1.50 (*A. Silvester*) to 6.60 (*P. alba*) mM troloxequivalents/kg dried roots for ABTS radical (Table 1). The antioxidant potentials of *P. alba*, *G. rivale* and *W. geoides* samples of roots were two to three times higher than those of *F. vulgaris* and *A. Silvester*. High TEAC values of *P. alba*, *W. geoides* and *G. rivale* corresponded, to a great extent, to high phenolics content as well (Table 1). This plant contains a number of powerful radical-scavenging compounds: (+)catechin, (−)epicatechin, ellagic and gallic acid derivatives. Only *A. Silvester* was very poor in the phenolic acid derivatives (ellagic and gallic) and flavan-3-ols. The lower antioxidant activity is probably because not all polyphenolic compounds possess $ABTS^{\cdot+}$ or synthetic radical DPPH quenching activities (Ivanova, Gerova, Chervenkov, & Yankova, 2005). On the other hand, antioxidant activity of phenolic compounds was correlated with their chemical structures and degrees of polymerisation (Lu & Foo, 2000). Structure–activity relationships of some phenolic compounds (e.g., flavonoids, phenolic acid, tannins) has very often been

studied (Rice-Evans, Miller, & Pagana, 1997; Schofield, Mbugua, & Pell, 2001).

We found very high antioxidant activities in the roots of Rosaceae plant, comparable to the values reported for traditional Chinese medicinal plants associated with anticancer activity (Cai, Luo, Sun, & Corke, 2004). They might thus be potential sources of potent natural antioxidants. Moreover, Chen, Plumb, Bennet, and Bao (2005) and Miliauskas et al. (2004) show that roots *Potentilla fryniana* not only have high antioxidant activities, but also antiviral properties. Ivanova et al. (2005) show that several Bulgarian herbs are good, and may even be better antioxidants than ascorbic acid, BHT and BHA. Medicinal plants demonstrated much stronger antioxidant activity and contained significantly more phenolics than common vegetables and fruits (Cai et al., 2004).

4. Conclusions

The present study demonstrates the high condensed tannin content and antioxidant potential of some of Rosaceae medical plants. The results obtained are promising and therefore further investigations should be targeted on such important issues as activity in real food systems relative to commercially used antioxidant extracts, stability, toxicology, processing (highest possible activity and absence of smell, taste and colour) and economic feasibility of practical applications.

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