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VARIATION IN THE PHENOLIC CONTENT AND *IN VITRO* ANTIOXIDANT ACTIVITY OF *SORBUS AUCUPARIA* LEAF EXTRACTS DURING VEGETATION

MONIKA A. OLSZEWSKA*

Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Łódź, Muszyńskiego 1, 90-151 Łódź, Poland

Abstract: Seasonal variation in the antioxidant activity and content of phenolic compounds was studied for the 70% methanol extracts of *Sorbus aucuparia* leaves harvested monthly over the full course of the growing season. The antioxidant potential of the extracts was evaluated using two complementary *in vitro* tests: the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging assay and the AAPH [2,2'-azobis-(2-amidinopropane)dihydrochloride]-induced linoleic acid (LA) peroxidation test. The radical-scavenging capacities of the extracts towards the DPPH radical were in the range of 0.40 to 0.57 millimolar Trolox equivalents/g dry weight of the leaves. They were significantly correlated ($r = -0.8480$, $p < 0.05$) with the results of the LA-peroxidation test, indicating the *S. aucuparia* leaf extracts to be universal antioxidants. Significant linear correlations were also found between the different antioxidant potentials and total phenolic contents as estimated by the Folin-Ciocalteu method and further verified by serial determinations of proanthocyanidins, chlorogenic acid isomers and flavonoids ($|r|$ in the range of 0.81-0.97, $p < 0.05$). As the best antioxidant capacities and the highest phenolic contents were found for the leaf samples harvested during the three summer months (June, July and August), this period could be considered to be optimal for cost-effective production of natural health products. For the leaf samples collected in July, the values of EC_{50} and IC_{50} for the two antioxidant tests were 2.02 and 93.45 μg phenolics/mL, respectively. These antioxidant capacities were found to be higher or comparable to those of synthetic and natural phenolic antioxidants, such as BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole), TBHQ (*tert*-butylhydroquinone), quercetin and Trolox.

Keywords: *Sorbus aucuparia* L., leaves, antioxidant activity, DPPH, lipid peroxidation, phenolic content

Oxidative stress induced by oxygen radicals is believed to be a primary factor in various degenerative diseases, such as cancer, atherosclerosis, coronary disease and age-related degenerative brain disorders (1). Epidemiological studies have revealed that diets rich in fruits, vegetables and other plant products are beneficial to human health and desease prevention (2–4), mostly owing to the ability of plant constituents to neutralize reactive oxygen species (ROS). Many natural compounds, especially plant phenolics, have indeed been identified as potent antioxidants (5) functioning as free radical scavengers, reducing agents (quenchers of ROS) and protecting living cells against lipid peroxidation *in vitro* and *in vivo* (6, 7). Interest in the antioxidant properties of plant phenolics also derives from their strong activity and low toxicity compared with those of synthetic phenolic antioxidants, such as BHA (butylated hydroxyanisole, 2-*tert*-butyl-4-hydroxyanisole and 3-*tert*-butyl-4-hydroxyanisole), BHT [butylated hydroxytoluene, 2,6-bis(1,1-dimethylethyl)-4-methylphenol] or TBHQ [*tert*-butylhydroquinone, 2-(1,1-dimethylethyl)-1,4-benzenediol], which are commonly used as antioxidants in processed foods (7). The search for antioxidants from natural sources has, therefore, become one of the most important research fields (7).

Sorbus aucuparia L. (rowan, European rowan or European mountain ash) is a small to medium-sized deciduous tree typically growing to 10 m tall and can be distinguished from all other *Sorbus* species by its blackish ovoid buds, non-leathery pinnate foliage with coarsely serrated leaflets papillose beneath, and orange-red fruits with densely white-tomentose free carpel apices (8). Depending on the assumed taxonomic classification, *S. aucuparia* is a model species for the genus *Sorbus* s.s. (8) and for the subgenus *Sorbus* of the genus *Sorbus* s.l. (9). *S. aucuparia* occurs from North Africa across Europe,

* Corresponding author: monika.olszewska@umed.lodz.pl, monolsz@op.pl; phone: +48 426779169; fax: +48 426788398

central and northern Asia to northern China, being the most widely distributed species in the whole *Sorbus* s.l. genus (8).

The edible fruits of *S. aucuparia* and the sweet variety *S. aucuparia edulis* Dieck have been traditionally used for ethnomedical properties, such as diuretic, anti-inflammatory, anti-diarrhoeal, vasoprotective, and vasorelaxant activities, and also as dietary and vitamin agents (10–12). The inflorescences of *S. aucuparia* are recommended in traditional Polish and Eastern European medicine to treat similar disorders as the fruits, especially as diuretic and anti-inflammatory agents (13). Most of these bioactivities can be explained by the presence of phenolic constituents and their antioxidant activities (10, 11).

In previous studies (14, 15), high antioxidant capacity strongly correlating with phenolic levels has been found for numerous species representing the genus *Sorbus* s.s., including *S. aucuparia*. The inflorescences of *Sorbus* and the leaves collected at the flowering period showed antioxidant activities that were especially high and greater than the activity of fruits (14). Moreover, the activity of the phenolic fractions existing in the leaves and inflorescences has been found comparable or higher than the activity of strong standard antioxidants, such as quercetin and Trolox (14, 15). The main phenolic constituents of *Sorbus* plants (proanthocyanidins, caffeoylquinic acids and flavonols) (14, 15) are considered to be safe in rational therapy (16–18). Potential safety issues could exist only if megadoses of these compounds are consumed daily (16, 17). Given the potential safety and also the harvest availability over the full growing season, the leaves of *Sorbus* species thus appeared to be promising sources for the cost-effective production of safe natural antioxidants. However, the percentage of components in plants may vary greatly with the vegetation period (19, 20), therefore, investigation of the effects of seasonal dynamics on antioxidant activity and phenolic content of *Sorbus* leaves is highly advisable to fully characterise the value of these plant materials as sources of natural antioxidants.

The aim of this research project was to study the variation in antioxidant activity and phenolic content of *S. aucuparia* leaves harvested monthly over the full course of the growing season. Activity of the leaf extracts was assayed using two complementary *in vitro* test systems: the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical-scavenging method and the AAPH [2,2'-azobis-(2-amidinopropane) dihydrochloride]-induced linoleic acid peroxidation test. The quantitative phenolic profiles of the plant materials were monitored by HPLC and by UV-photometric methods, and the relationship between antioxidant capacity and phenolic content was investigated.

MATERIALS AND METHODS

Plant material

Leaf samples of *S. aucuparia* were collected monthly between May and October 2009 on the 10th day of each month in the Arboretum (51°49'N, 19°53'E), Forestry Experimental Station of Warsaw University of Life Sciences (SGGW) in Rogów (Poland). Voucher specimen (KFG/HB/07001-SAUC) was authenticated by the author and was deposited at the Department of Pharmacognosy, Medical University of Łódź, Poland.

Chemicals and instrumentation

HPLC or GC grade purity reagents and standards, such as 2,2-diphenyl-1-picryl hydrazyl (DPPH); 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH); (±)-6-hydroxy-2,2,7,8-tetramethylchroman-2-carboxylic acid (Trolox); linoleic acid; chlorogenic acid hemihydrate; gallic acid monohydrate; and quercetin trihydrate were purchased from Sigma-Aldrich Inc. (Germany/USA). Analytical grade antioxidant standards, such as BHA BHT and TBHQ were obtained from the same supplier. HPLC grade solvents, MeCN and H_3PO_4 were used for HPLC analyses and were purchased from Merck (Germany). For other analyses, redistilled water and analytical grade chemicals and solvents (POCh S.A., Poland) were used.

Absorbance was measured using a Lambda 25 spectrophotometer (Perkin-Elmer, USA), in 10 mm quarz cuvettes. Samples for the tests were incubated in a constant temperature using a BD 23 incubator (Binder, Germany). HPLC analyses were carried out on a Waters 600E Multisolvent Delivery System (Waters Co., USA) with a PDA detector (W 996), a 20 µL sample injector (Rheodyne 7725i), and a LC workstation equipped with Waters Empower software for data collection and acquisition. A C18 Lichrosphere 100 column (5 µm, 250 mm × 4.6 mm, i.d.) (Merck) guarded by a C18 Hypersil ODS pre-column (5 µm, 4 × 4 mm, i.d.; Agilent Technologies, USA) was used. Constant temperature of the column was maintained using a Jetstream Plus 5480 termostat (Peltier, Austria). Before injection to HPLC system, all samples were filtered through a PTFE syringe filter (13 mm, 0.2 µm, Whatman, USA).

Preparation of plant extracts for testing antioxidant activity and phenolic profile

The samples of plant material were air-dried under normal conditions, powdered with an elec-

tric grinder and sieved through a 0.315 mm sieve. An accurately weighed mass (2.0–3.0 g for the LA-peroxidation test, and 100–250 mg for the other assays) was refluxed first for 30 min with 30 mL of 70% (v/v) aqueous methanol, and then twice for 15 min with 20 mL of the same solvent. The obtained extracts were combined, filtered and diluted with methanol to 100 mL to give the test extracts (TE).

DPPH free radical-scavenging test

The scavenging activity was determined based on the method of Brand-Williams, Cuvelier and Berset (21) with slight modifications. The DPPH working solution (35.5 mg/L, 90 mM) was prepared in methanol and equilibrated everyday to the absorbance of the negative control of 0.700 ± 0.030 at 517 nm. The negative control was prepared by mixing 2 mL of the DPPH working solution with 1 mL of methanol. Five different concentrations of all extracts and standards were prepared in 70% (v/v) aqueous methanol. An aliquot of 1 mL of the sample was added to 2 mL of the equilibrated DPPH working solution and vigorously shaken. After 60 min of incubation in screw-cap vials at room temperature in the dark, the decrease in the absorbance was measured at 517 nm. The mixtures of the sample (1 mL) and methanol (2 mL) were used as blanks. The concentration of the analyte (standard or plant material used for extract preparation) in the reaction medium (in μg/mL) was plotted against the percentage of remaining DPPH using the DPPH calibration curve, and the EC_{50} value was calculated. The standards of quercetin, BHA, BHT, TBHQ and Trolox were used as positive controls. The activity of the plant materials was then expressed in terms of Trolox (TEAA) equivalent antioxidant activity in mmol/g d.w.

Linoleic acid (LA) peroxidation test

Basic procedure

The ability of TE to inhibit AAPH-induced LA-peroxidation was assayed according to the method of Azuma et al. (22) with some modifications. An aliquot of 0.30 mL of TE was placed in a screw-cap vial and mixed with 1.40 mL of 1.3% (w/v) LA in methanol, 1.40 mL of 0.2 M phosphate buffer (pH 7.0), and 0.70 mL of water. The negative control was prepared using 0.30 mL of methanol instead of the sample. Peroxidation was initiated by the addition of 0.20 mL of 55.30 mM AAPH solution in phosphate buffer. The vial was incubated at 50.0 ± 0.1°C in the dark, sampling being carried out every hour for up to at least 5 h until the absorbance of the control reach the value of 0.550 ± 0.020. The degree of oxidation was measured according to the ferric thiocyanate method (23). The reaction mixture (0.10 mL) was diluted with 9.70 mL of 75% (v/v) methanol and mixed with 0.10 mL of 20 mM $FeCl_2$ solution in 3.5% (w/w) HCl and 0.10 mL of 10% (w/w) aqueous NH_4SCN solution. After precisely 3 min, the absorbance was measured at 500 nm *versus* 75% methanol. The inhibition ratio (I%) of the peroxidation process was calculated as follows: $I\% = 100 \times (1 - DA_{sample}/DA_{control})$, where DA is the difference between the absorbances measured at the end and the start of the test.

Determination of the IC_{50} value

The value of IC_{50} was determined for the leaf sample collected in July. The accurately weighed mass (3.0 g) was used for extraction as described above. The prepared extract was serially diluted with 70% (v/v) aqueous methanol, and the obtained solutions of six different concentrations were analyzed using the basic procedure. The concentration of the plant material used for extract preparation in the reaction medium (in μg/mL) was plotted against the inhibition ratio (I%) of the peroxidation process, and the IC_{50} value was read from the obtained regresion curve.

Determination of total phenolic content

The total phenolic content in TE was determined according to Folin-Ciocalteu (FC) method (14). An aliquot of 1 mL of TE was mixed with 5 mL of FC reagent, which was previously diluted with water in a volume ratio of 1:10. After 3 min, the reaction mixture was diluted to 10 mL with aqueous sodium carbonate solution ($Na_2CO_3 \times 10H_2O$, 202.5 g/L). After 120 min incubation in screwcap vials at room temperature in the dark, the absorbance was read at 760 nm. The solution without the FC reagent was used as the blank. Results were expressed as gallic acid (GAE) equivalents per dry weight of the plant material.

Determination of total proanthocyanidin content

The total proanthocyanidin content in TE was quantified by the modified acid/butanol assay (24). An aliquot of 0.5 mL of TE was placed in a screw-cap vial and mixed with 3 mL of *n*-BuOH-35% HCl (95:5, v/v) and 0.1 mL of 2% (w/v) $NH_4Fe(SO_4)_2 \times 12H_2O$ in 2 M HCl. After 45 min of incubation at 95.0 ± 0.2°C, the vial was cooled to 25°C and the absorbance was read at 550 nm *versus* the unheated sample used as the blank. The results were expressed as cyanidin chloride (CyE) equivalents per dry weight of the plant material.

Total flavonoid content

The content of flavonoids in TE was determined by RP-HPLC as the total content of flavonoid aglycones after acid hydrolysis, according to the method optimized and validated previously (25).

The content of chlorogenic acid isomers

The content of main caffeoylquinic acids in TE was estimated by RP-HPLC. The samples of TE were filtered through a syringe filter, and the filtrate was injected (20 µL) into the HPLC system. The elution system consisted of solvent A (0.5% water solution of orthophosphoric acid, w/v) and solvent B (MeCN) with the elution profile as follows: 0–1 min, 5% B (v/v); 1–4 min, 5–15% B; 4–10 min, 15% B; 10–11 min, 15–50% B; 11–15 min, 50% B; 15–16 min, 50–5% B; 16–22 min, 5% B (equilibration). All gradients were linear. The flow rate was 1.2 mL/min, the column was maintained at 40°C and the detection wavelength was set at 350 nm. Two main chlorogenic acid isomers in TE were identified by comparison with the standards of 5-*O*-caffeoylquinic acid (chlorogenic acid, ChA, commercial standard) and 3-*O*-caffeoylquinic acid (neochlorogenic acid, NChA, qualitative standard prepared by isomerization of ChA using the method of Nagels et al. (26)). The contents of the two isomers were expressed per dry weight of the plant material and were calculated from the calibration curve of ChA.

Statistical analysis

The statistics (calculation of standard deviation, analysis of variance) were performed using the software StatisticaPl for Windows (StatSoft Inc., Poland).

RESULTS AND DISCUSSION

The total phenolic content (TPC) of the *S. aucuparia* leaf extracts was determined by the Folin-Ciocalteu (FC) photometric assay. The FC reaction is commonly used to obtain a crude estimate of TPC as gallic acid equivalents (GAE), but it does not give a full picture of the real phenolic constituents of plant extracts (11, 27). Thus, for verification of the real phenolic levels in the leaves of *S. aucuparia*, further measurements of the main phenolic groups were performed by photometric and HPLC assays. The verified total phenolic content (TPh) was expressed as the sum of total proanthocyanidins (PRC, calculated as CyE, cyanidin chloride equivalents), total flavonoids (FL, sum of quercetin and kaempferol) and total chlorogenic acid isomers (CA, sum of chlorogenic and neochlorogenic acids). The leaf samples were harvested over the full course of

Table 1. Antioxidant activity and total phenolic content of *S. aucuparia* leaves harvested in different months [a].

Month of harvest/ standard	Antioxidant activity				Total phenolic content [f]
	DPPH EC_{50}			LA-peroxidation	
	(µg/mL) [b]	TEAA [c]	(µg/mL) [d]	(% inhibition) [e]	TPC (GAE, %)
May	32.25 ± 0.96 [A]	0.40	2.28	47.91 ± 0.83 [A]	7.07 ± 0.07 [A]
June	24.10 ± 0.29 [B, C]	0.54	1.98	58.69 ± 0.46 [B]	8.23 ± 0.07 [B]
July	22.74 ± 0.75 [B]	0.57	2.02	60.50 ± 0.54 [C]	8.90 ± 0.09 [C]
August	25.12 ± 0.23 [C]	0.50	2.17	58.21 ± 0.76 [B]	8.37 ± 0.03 [D]
September	29.65 ± 0.43 [D]	0.44	2.18	47.37 ± 0.55 [A]	7.34 ± 0.06 [A]
October	25.89 ± 0.86 [C]	0.52	2.07	51.85 ± 0.36 [D]	8.24 ± 0.03 [B]
Quercetin	1.44 ± 0.05	-	-	88.38 ± 1.15	-
Trolox®	3.27 ± 0.10	-	-	92.74 ± 1.10	-
TBHQ	2.73 ± 0.10	-	-	100.00 ± 2.70	-
BHA	2.35 ± 0.09	-	-	58.25 ± 2.30	-
BHT	6.54 ± 0.12	-	-	64.50 ± 2.54	-

[a] Results are the mean values of triplicate analyses ± SD. Different superscripts (capitals) in each column indicate significant differences in the mean values at $p < 0.05$. [b, c, d] Scavenging efficiency (amount of antioxidant needed to decrease the initial DPPH concentration by 50%) expressed as follows: [b] in µg of dry plant material or the standard/mL of DPPH solution; [c] in millimolar Trolox antioxidant equivalents (TEAA)/g of dry plant material; [d] in µg of phenolics/mL of DPPH solution (value obtained by converting the orginal EC_{50} value with the TPC level. [e] Inhibition ratio of LA-peroxidation after incubation with the final antioxidant concentration of 1.50 mg/mL for the plant materials or 0.20 mg/mL for the standards. [f] Total phenolic content expressed in GAE, gallic acid equivalents.

Table 2. Total content of proanthocyanidins, chlorogenic acid isomers and flavonoid aglycones in *S. aucuparia* leaves harvested in different months [a].

Month of harvest	Proanthocyanidins [b] (CyE, %)	Caffeoylquinic acids (CA, %) [c]		Flavonoid aglycones (FL, %) [d]	
		NChA	ChA	QU	KA
May	2.80 ± 0.09 [A]	0.69 ± 0.01 [A]	1.69 ± 0.03 [A]	0.813 ± 0.008 [A]	0.146 ± 0.005 [A]
June	3.60 ± 0.12 [B]	0.51 ± 0.01 [B]	1.90 ± 0.08 [B]	0.903 ± 0.023 [B]	0.157 ± 0.005 [A]
July	4.08 ± 0.05 [C]	0.41 ± 0.01 [C]	1.85 ± 0.02 [B]	0.817 ± 0.006 [A]	0.196 ± 0.003 [B]
August	3.73 ± 0.03 [B]	0.38 ± 0.01 [D]	1.73 ± 0.02 [A]	0.746 ± 0.030 [C]	0.193 ± 0.010 [B]
September	3.23 ± 0.04 [D]	0.34 ± 0.01 [E]	1.39 ± 0.01 [C]	0.642 ± 0.009 [D]	0.210 ± 0.006 [C]
October	3.92 ± 0.13 [C]	0.34 ± 0.01 [E]	1.56 ± 0.01 [D]	0.698 ± 0.007 [E]	0.244 ± 0.003 [D]

[a] Results are the mean values of triplicate analyses calculated per d.w. of the plant material ± SD. Different superscripts (capitals) in each column indicate significant differences in the mean values at $p < 0.05$. [b] Total proanthocyanidin content expressed in CyE, cyanidin chloride equivalents. [c] Content of chlorogenic acid isomers (CA) quantified by HPLC: NChA, neochlorogenic acid; ChA, chlorogenic acid. [d] Total content of flavonoid aglycones (FL) quantified by HPLC: QU, quercetin; KA, kaempferol.

Table 3. Correlation (r) and determination (R^2) coefficients between antioxidant capacity of *Sorbus* extracts and content of phenolic compounds.

r (R^2)	DPPH EC_{50} (μg/mL) [c]	LA peroxidation (% inhibition) [c]
TPC (GAE, %) [a]	-0.9727 (0.9461)*	0.9060 (0.8209)*
TPh (%) [b]	-0.8115 (0.6585)*	0.9548 (0.9116)*
Proanthocyanidins, PRC (CyE, %)	-0.9162 (0.8394)*	0.9095 (0.8272)*
Flavonoid aglycones, FL (QU + KA, %)	-0.5715 (0.3266)	0.7183 (0.5160)
Caffeoylquinic acids, CA (ChA + NChA, %)	-0.1729 (0.0299)	0.5788 (0.3351)

[a] TPC, total phenolic content determined by the FC method expressed in GAE, gallic acid equivalents. [b] TPh, total phenolic content expressed as the sum of proanthocyanidins (PRC), caffeoylquinic acids (CA) and flavonoid aglycones (FL). [c] Significance levels: *$p < 0.05$.

the growing season. As presented in Figure 1a, the TPC and TPh values were consistent, which is in accordance with the results from an earlier study of the leaves of 16 *Sorbus* species collected at the flowering period (14, 15). The observed correlation between the TPC and TPh levels was statistically significant at $p < 0.05$, and was characterized by high correlation and determination coefficients r (R^2) = 0.8030 (0.6448). Similar values and high correlation between both parameters are clear evidence that the listed groups of phenolic compounds are the main phenolic metabolites present in the leaves of *S. aucuparia*. Given the basic redox mechanism of the FC method (27), high correlation found between TPC and TPh levels proved that the mentioned three phenolic groups are also the most important phenolic determinants of redox activity in the leaf samples of *S. aucuparia*.

As shown in Table 1, the leaves collected in different months exhibited similar but statistically different ($p < 0.05$) levels of TPC ranging from 7.07 to 8.90% d.w. of GAE, with the average value of 8.03 ± 0.69% GAE. The highest content was found for the leaves harvested in July. As presented in Table 2, the predominant components of all the assayed leaf samples were proanthocyanidins, followed by caffeoylquinic acids and flavonoids. The same gradation was observed earlier (15) for the samples of leaves and inflorescences from 16 species representing the genus *Sorbus* s.s. collected at the flowering period (in June). The tested leaves showed a narrow range of total proanthocyanidin content (2.80-4.08% d.w. of CyE), sum of caffeoylquinic acid isomers (1.73-2.41% d.w.), and total content of flavonoid aglycones (0.85-1.01% d.w.). However, the differences in phenolic contents observed for leaf samples from different months were statistically significant at the significance level $p < 0.05$. Among caffeoylquinic acids (CA), chlorogenic acid was the predominant isomer constituting 71 to 82% of the total content. Among flavonoids, quercetin was the main aglycone with its levels constituting 74 to 85% of the total aglycone content (FL). Much like the leaves of other species from the

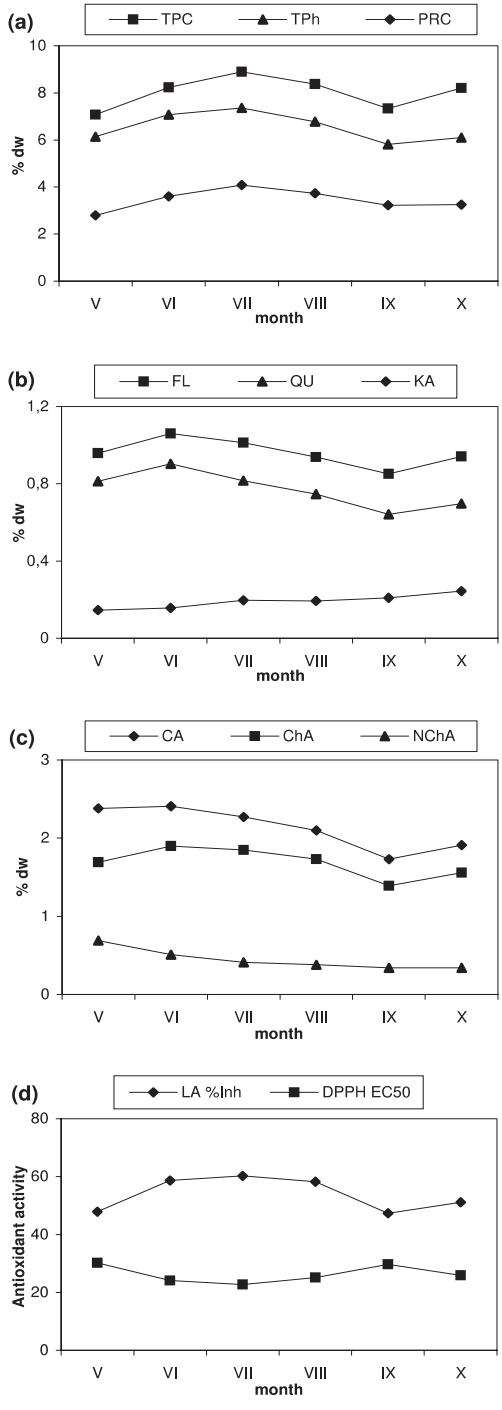

Figure 1. Variations in phenolic content and antioxidant activity of *S. aucuparia* leaves in different harvesting months of 2009: TPC, total phenols as GAE (gallic acid equivalents); PRC, total proanthocyanidins as CyE (cyanidin chloride equivalents); FL, total flavonoid aglycones as sum of quercetin (QU) and kaempferol (KA); CA, caffeoylquinic acids as sum of chlorogenic acid (ChA) and neochlorogenic acid (NChA); TPh, total phenolics as sum of PRC, FL and CA; LA %Inh, inhibition ratio in LA-peroxidation test; DPPH EC_{50}, effective concentration towards the DPPH radical. Each graph point represents the average value of triplicate analyses. For standard deviations, see Tables 1 and 2

genus *Sorbus* s.s. (15), the analyzed leaves of *S. aucuparia* contained only two flavonoid aglycones, quercetin and kaempferol.

The seasonal variation in the content of phenolics in the assayed leaf samples is illustrated in Figure 1a, 1b and 1c. The most important factor influencing the total phenolic level is the content of total proanthocyanidins (PRC), which exhibited seasonal dynamics similar to those of the TPC and TPh levels (Fig. 1a). The lowest PRC, TPC and TPh contents were observed in the spring, in May. Thereafter, they increased to their highest values in July, and then slightly decreased between August and October. However, the lowest autumn contents of TPC, TPh and PRC observed in September were higher than those found in May.

The seasonal dynamic of the total content of caffeoylquinic acids (CA) was primarily influenced by the levels of chlorogenic acid (ChA) (Fig. 1c). The levels of CA and ChA were maximal at the flow-

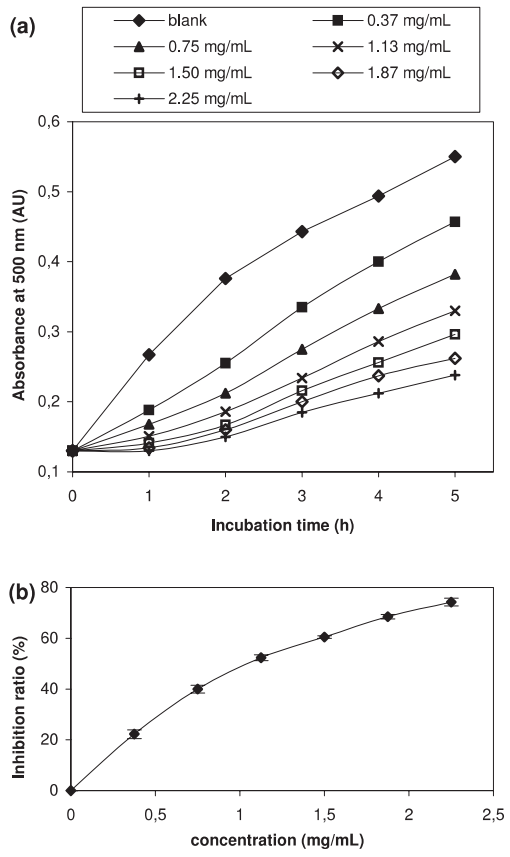

Figure 2. Inhibition of LA-peroxidation by *S. aucuparia* leaf sample harvested in July 2009 as measured by the FTC method. Each graph point represents the average value of three measurements, and the error bars are the respective standard deviations. The concentrations given in mg/mL are the concentrations of the plant material used for extract preparation in the final reaction medium

ering period, in June. In the next few months, the contents of CA and ChA decreased gradually to reach their minimal values in September. In October, the levels of CA and ChA increased slightly, but the achieved values were lower than those observed between May and August. The seasonal dynamic of the content of neochlorogenic acid (NChA) was different, and the levels of this metabolite decreased gradually from its highest value observed in May to its lowest in September and October.

The total content of flavonoids (FL) was influenced primarily by the content of quercetin (QU). The levels of FL and QU exhibited seasonal variations (Fig. 1b) similar to those observed for the levels of CA and ChA (Fig. 1c), with the highest contents found at the flowering period in June, and the lowest in September. The seasonal dynamic of the kaempferol level (KA) was different and the contents of this aglycone increased gradually to its highest value in October.

Given the differences in basic reaction mechanisms among the wide number of available test systems, an approach with at least two different assays is highly advisable to fully characterize the antioxidant properties of plant extracts (7, 27). On this basis, the *in vitro* antioxidant activity of the *S. aucuparia* leaves was assayed using two complementary tests: the DPPH free radical scavenging method (a widely used system involving a single electron transfer (SET) reaction) and inhibition of the AAPH-induced linoleic acid (LA) peroxidation test (involving a hydrogen atom transfer (HAT) mechanism, a more physiological and food-relevant system).

The antioxidant results are summarized in Table 1. A significant ($p < 0.05$) correlation was found between the results of the two methods. It was characterized by high coefficients of correlation ($r = -0.9209$) and determination ($R^2 = 0.8480$), confirming a previous report (11) that the *Sorbus* extracts are universal antioxidants utilizing both the HAT and SET reaction mechanisms. The analyzed leaf extracts exhibited a relatively narrow range of antioxidant capacities in both systems with a mean scavenging efficiency of the DPPH radical of $EC_{50} = 26.63 \pm 3.43$ μg/mL (TEAA = 0.49 ± 0.06 mmol/g) and a mean LA-inhibition in the peroxidation test of 54.09 ± 5.46%. There are limited data in the literature (28, 29) with which it would be possible to easily compare the obtained antioxidant results, given the differences in the assay protocols. According to these systematic screening studies, only about 20% of known plant materials have high or extremely high antioxidant activity as measured by the DPPH method, and exhibit TEAA values in the range of 0.25 to 1.00 mmol/g d.w. (14% of plants) or even higher (6%). As shown in Table 1, the analyzed leaf samples of *S. aucuparia* can thus be counted among the plant materials having high antioxidant activity. By converting the determined original values of EC_{50} for the DPPH test to μg phenolics/mL using the TPC values, we obtained the recalculated values of EC_{50} in the range of 1.98 to 2.28 μg phenolics/mL (Tab. 1), with an average value of 2.12 ± 0.11 μg phenolics/mL. The majority of the simultaneously assayed standards, such as Trolox ($EC_{50} = 3.27 \pm 0.10$ μg/mL), BHA ($EC_{50} = 2.35 \pm 0.09$ μg/mL), BHT ($EC_{50} = 6.54 \pm 0.12$ μg/mL) and TBHQ ($EC_{50} = 2.73 \pm 0.10$ μg/mL), exhibited significantly higher values of EC_{50} ($p < 0.05$), which means lower free radical-scavenging activity. Only quercetin had higher antioxidant capacity ($EC_{50} = 1.44 \pm 0.05$ μg/mL). The magnitude of the DPPH radical-scavenging efficiencies observed for the standards was consistent with earlier reports (30–32). This is convincing evidence of the potent free radical-scavenging activity of *S. aucuparia* leaves.

The results of both antioxidant tests were significantly ($p < 0.05$) correlated with the levels of TPC, TPh and PRC (Tab. 3). Consequently, the observed similarity in seasonal variations between the antioxidant capacities and the levels of TPC, TPh and PRC (Fig. 1a and 1d) is not suprising. The highest antioxidant activity was found for leaf samples most abundant in phenolics, the same samples harvested during the three summer months (June, July and August).

Low-density (LDL) peroxidation has been reported to contribute significantly to atherosclerosis development (33). Therefore, the prevention or delay of LDL peroxidation is a very important function of antioxidants. Figures 2a and 2b show the time and dose effects of the *S. aucuparia* leaf extract on the AAPH-induced inhibition of LA-peroxidation, as measured by the ferric thiocyanate method (FTC). The value of $IC_{50} = 1.05 \pm 0.05$ μg/mL was determined for the leaf sample collected in July, the same sample exhibiting the highest TPC and TPh values and also the highest antiradical activity towards the DPPH radical. The original value of IC_{50} was converted to the recalculated value of $IC_{50} = 93.45 \pm 2.45$ μg phenolics/mL using the TPC level of the sample. The values of IC_{50} determined simultaneously for the standards were 59.40 ± 1.76 μg/mL for Trolox and 100.60 ± 2.52 μg/mL for quercetin. The results indicate that *S. aucuparia* leaf extracts may act as significant inhibitors of LA-peroxidation ($p < 0.05$).

CONCLUSION

This is the first report on the seasonal variability of antioxidant activity and phenolic content of *Sorbus* leaves. The study demonstrated that during the full course of the growing season, the leaves of *S. aucuparia* possessed significant and dose-dependent *in vitro* radical-scavenging activity towards DPPH radicals and the ability to inhibit AAPH-induced oxidation of linoleic acid, both of which correlate with their polyphenolic composition. Given that the highest antioxidant capacities and the highest phenolic contents were found for the leaf samples harvested in the three summer months (June, July and August), this period could be considered optimal for the cost-effective production of natural antioxidants in their potential use in food, medicine, cosmetics and other fields that require antioxidants. However, further research is needed to clarify possible toxicity and other biological properties of the extracts presented here. Moreover, the effects of the use of these natural extracts on food, cosmetic or drug sensory properties (such as odor and taste) should be addressed in future research.

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