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SUPPLEMENTARY MATERIAL TO

Phenolic profiling and bioactivities of fresh fruits and jam of *Sorbus* species

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THE GENUS *Sorbus*

In recent years, there has been worldwide interest in functional food that can provide not only basic nutrition requirements but also physiological benefits. Therefore, it is worthwhile to estimate health benefits of plant-based foodstuffs as a rich source of bioactive compounds. The genus *Sorbus* belongs to the family *Rosaceae* and includes about 90 species spread over the Northern hemisphere, of which 8 species are found in Serbia (*Sorbus domestica* L., *S. aucuparia* L., *S. torminalis* (L.) Crantz., *S. chamaemespilus* (L.) Crantz., *S. aria* (L.) Crantz., *S. graeca* (Spach) Kotschy, *S. umbellata* (Desf) Fritsch and *S. austriaca* (Beck) Hedl.[1] *Sorbus* species form characteristic sour berry-like fruits with a unique flavour that are collected after the first autumn frost and consumed fresh and overripe. They are traditionally prepared and consumed as preserves, such as: jam, jelly, syrup, compote, liquor, wine or tea. Before consumption, the fruits are usually fermented, brewed or preserved by cooking with sugar.[2,3] Particularly popular is wild rowanberry jelly made from *S. aucuparia* fruits, which is widely consumed with game. Furthermore, the extract and syrup of *S. domestica* fruits are applied in the alcohol industry in liquor, cider or schnapps production.[4]

Apart from being consumed worldwide in human diet, the fruits of *Sorbus* species are alleged to express numerous medical properties, such as: antidiarrheal, diuretic, antidiabetic, astringent, vasoprotective, broncho- and vaso-relaxant, anti-inflammatory, antimicrobial, cytotoxic and antioxidant activities.[5–11]

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Furthermore, *Sorbus* fruits have been extensively used for the treatment of anaemia, oedema, dyspepsia and various digestive disorders.[12] They are also known as a rich source of vitamin C and are used in the treatment of a wide range of diseases, including respiratory tract infections, fever, cold, flu, rheumatism and gout,[6] while in Russia, the dry fruits of *S. aucuparia* are considered as a multivitamin supplement, taken twice a day as an infusion.[3]

EXPERIMENTAL DETAILS

Chemicals and reagents

All standards of phenolic compounds, as well as all other chemicals, were purchased from Sigma–Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland) or from ChromaDex (Santa Ana, CA, USA). All reagents used in this study were of analytical grade.

Plant material collection and extracts preparation

The fruits of *S. aucuparia* L. 1753 and *S. torminalis* (L.) Crantz. 1753 f. *semitorminalis* (Borb.) Jáv. 1925 were collected in October 2013, in Novi Sad, Republic of Serbia, while *S. torminalis* (L.) Crantz. f. *torminalis* 1753 was collected in October 2013, in the village Vrdnik, near Novi Sad, Republic of Serbia. Specimen vouchers (*S. aucuparia* No. 2-1568; *S. torminalis* f. *torminalis* No. 2-1575 and *S. torminalis* f. *semitorminalis* No. 2-1577) were prepared, identified and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Sciences, University of Novi Sad, Republic of Serbia.

The harvesting was performed in autumn when 1 kg of ripe, brownish fruits was collected (picked by hand) from three trees of each species. Three types of extracts of each species were prepared: methanol extracts of fresh fruits with seeds (M), water extract of fresh fruits with seeds (W) and jam extract (J); all in triplicate, making 27 extracts in total.

For preparation of the methanol extracts, 30 g of fresh fruits were grounded immediately after harvesting and extracted by maceration with 80 % aqueous methanol (1 mL of solvent/0.1 g of plant material), constantly shaken at 120 rpm/min during 72 h at room temperature. For the preparation of the water extracts, 30 g of fresh fruits were ground immediately after harvesting and extracted by maceration with boiling, distilled water (1 mL of solvent/0.1 g of plant material), constantly shaken at 120 rpm/min during 1 h at room temperature.[13] After filtration, solvents (80 % aqueous methanol and water) were evaporated *in vacuo* at 40 °C. The crude residues were dissolved in hot, distilled water (10 mL/1 g). In order to remove the non-polar compounds, the extracts were washed exhaustively with petroleum ether (fraction 40−60 °C) and concentrated to dryness under vacuum, yielding 17 and 17 % for M and W extracts of *S. aucuparia* fresh fruits, respectively, or 21 and 15 % for M and W extracts of *S. torminalis* f. *torminalis* fresh fruits, respectively, or 20 and 17 % for M and W extracts of *S. torminalis* f. *semitorminalis* fresh fruits, respectively.

Jam was prepared according to a traditional Serbian recipe by cooking the fresh fruits in boiling water and crushing them during stirring. Afterwards, the cooked fruits were sieved in order to remove the seeds.[13] According to Council Directive 2001/113/EC,[14] the obtained product corresponds to definition of fruit purée. Further, in order to prepare jam, the purée was boiled and 350 g of sugar per 1 L of purée was added.[14] The mixture was stirred until appropriate consistency of jam was achieved. In order to prepare jam extracts, 10 g of each jam sample were weighed and evaporated under vacuum at 40 °C. The crude residues of jam were dissolved in hot, distilled water (10 mL of water/1g of crude residue). The extracts were then

filtered and evaporated under vacuum at 40°C yielding 38, 54 and 85 % for *S. aucuparia*, *S. torminalis* f. *torminalis* and *S. torminalis* f. *semitorminalis* jam samples, respectively.

Dried mass of the final methanol, water and jam extracts were dissolved in distilled water to obtain 300 mg mL^{-1} stock solutions for evaluation of the total phenolic, flavonoid and vitamin C contents, as well as the antioxidant, anti-AChE, cytotoxic and antimicrobial activities. Additionally, the dried extracts were dissolved in distilled water to obtain 20 mg mL^{-1} stock solutions for LC–MS/MS analysis. All extracts were stored in a freezer until analysis.

LC–MS/MS analysis of single phenolic compounds

The samples and 45 standards (prepared in serial dilutions, ranging (1.53–25.0)×10^3 ng mL^{-1}, dissolved in a mixture of 0.5 % formic acid and methanol (in 1:1 ratio)) were analyzed using an Agilent Technologies 1200 Series HPLC instrument coupled with an Agilent Technologies 6410A QqQ mass spectrometer with an electrospray ion source and controlled by Agilent Technologies MassHunter workstation software (ver. B.03.01). The injection volume was 5 μL. Separation was performed using a Zorbax Eclipse XDB–C18 (Agilent Technologies) column, 50 mm×4.6 mm, 1.8 μm, held at 50 °C. The mobile phase, consisting of 0.05 % aqueous formic acid (A) and methanol (B) was delivered at a flow rate of 1 mL min^{-1} in the gradient mode (0 min 30 % B, 6 min 70 % B, 9 min 100 % B, 12 min 100 % B, post time 3 min). The ion source parameters were: nebulization gas pressure 40 psi*, drying gas flow 9 L min^{-1}, temperature 350 °C and capillary voltage 4000 V. All compounds were detected in the negative mode, using dynamic selected reaction monitoring with optimized compound-specific parameters (retention time, precursor ion, product ion, fragmentor voltage and collision voltage). The concentrations of the standard compounds in the extracts were determined from the peak areas by using the linear regression equations obtained from the calibration curves. Results are presented as the mean value of triplicate measurements.

Reduction of DPPH·

Ten μL of extract, in a series of different concentrations (0.08–5.0 mg mL), were added to 100 μL of 90 μmol L^{-1} DPPH solution in methanol, and the mixture was diluted with an additional 190 μL of methanol. Consequently, 100 μL of 90 μmol L^{-1} DPPH solution in methanol were added to each well. In the controls, the exact amount of sample was substituted with solvent. In blank probe, only methanol (290 μL) and extract (10 μL) were mixed, while in blank probe for control only 300 μL of methanol were added. Measurements of absorbance were taken at 515 nm after 1 h. The antioxidant activity of the extracts was expressed as concentration required to decrease the initial DPPH· concentration by 50 % (IC_{50}). All samples and the control were made in triplicate.

$O_2^{\bullet-}$ scavenging capacity

The capability of the extracts to neutralize $O_2^{\bullet-}$, formed by the reduction of nitroblue tetrazolium with NADH mediated by phenazine methosulphate under aerobic conditions, was measured. Briefly, a mixture of 0.2 mL nitroblue tetrazolium (144 μmol L^{-1}), 10 μL of extract (substituted with solvent in the control), 0.1 mL of NADH (0.68 mmol L^{-1}), and freshly prepared phenazine methosulphate (60 μmol L^{-1}) was diluted with 1.1 mL of phosphate buffer (0.017 mol L^{-1}, pH 8.3). The blank probe was prepared by mixing 1.5 mL of the buffer and 10 μL of extract. The absorbance was read at 560 nm after 5 min. All samples and the control were made in triplicate and IC_{50} values were determined.

* 40 psi = 275.8 kPa

•NO scavenging capacity

The •NO scavenging capacity was determined by an adapted Griess method. Briefly, the reaction mixture containing sodium nitroprusside (75 μL, 10 mmol L^{-1}), phosphate buffer (75 μL, 12.86 mmol L^{-1} KH_2PO_4, 54.13 mmol L^{-1} Na_2HPO_4, pH 7.4) and extract (10 μL, substituted with solvent in the control) was incubated at 25 °C for 90 min. Ten microlitres of extract and 150 μL of the phosphate buffer were added in the blank probe, while the extract was substituted with solvent in the blank probe for the control. After incubation, 150 μL of solution prepared by mixing equal amounts of sulphanilamide (2 % in 4 % phosphoric acid) and *N*-(1-naphthyl)ethylenediamine dihydrochloride (0.2 %) were added to the reaction mixture and allowed to stand for 3 min. The absorbance of these solutions was read at 546 nm against appropriate blanks. All samples and the control were made in triplicate. IC_{50} values were determined.

HO• scavenging capacity

A modified deoxyribose assay was applied to measure the HO• scavenging capacity of the extracts. Briefly, 0.1 mL of 2-deoxy-D-ribose (50 μmol L^{-1}) in phosphate buffer (12.86 mmol L^{-1} KH_2PO_4, 54.13 mmol L^{-1} Na_2HPO_4, pH 7.4) was mixed with 20 μL of the extract or solvent in the control, 0.1 mL H_2O_2 (0.015 %), 0.1 mL $FeSO_4$ (10 mmol L^{-1}) and diluted with 2.7 mL of the phosphate buffer (pH 7.4). Three mL of the phosphate buffer and 20 μL of the extract were added in the blank probe, or substituted with solvent in the blank probe for the control. After incubation at 37 °C for 1 h, 0.2 mL ethylenediaminetetraacetic acid (EDTA; 0.1 mol L^{-1}) was added to all wells. The thiobarbituric acid (TBA) reaction was performed by adding 2 mL of an aqueous mixture containing TBA (3.75 mg mL^{-1}), $HClO_4$ (1.3 %), and trichloroacetic acid (TCA; 0.15 g mL^{-1}), followed by heating at 100 °C for 10 min. The absorbances of the cooled mixtures were read at 532 nm. All samples and the control were made in triplicate and the IC_{50} values were determined.

FRAP assay

The FRAP reagent was prepared by mixing 10 mmol L^{-1} 2,4,6-tripyridil-*s*-triazine in 40 mmol L^{-1} HCl, 0.02 mol L^{-1} $FeCl_3$, and acetate buffer (22.78 mmol L^{-1} CH_3COONa, 0.28 mol L^{-1} CH_3COOH, pH 3.6) in a ratio of 1:1:10, respectively, whereas ascorbic acid ranging 1.25–160 μg mL^{-1} was used to create a standard curve. Following the addition of an extract or ascorbic acid (10 μL, substituted with solvent in the control) to 290 μL of FRAP reagent, absorbance was read at 593 nm after 6 min. In the blank probe, an extract or ascorbic acid (10 μL, substituted with solvent in the blank probe for the control) were mixed with 290 μL of distilled water. All samples and blank probes were made in triplicate and the mean values of reducing power are expressed as milligrams of ascorbic acid equivalents per gram dw, calculated according to the standard calibration curve.

Lipid peroxidation

The extent of Fe^{2+}/ascorbate induced LP was determined by the TBA assay using as a substrate polyunsaturated fatty acids, obtained from linseed by Soxhlet extraction (69.7 % linolenic acid, 13.5 % linoleic acid, as determined by GC–MS). The fatty acids were added to phosphate buffer (12.86 mmol L^{-1} KH_2PO_4, 54.13 mmol L^{-1} Na_2HPO_4, pH 7.4) in the presence of 0.25 % Tween-80, to obtain a 0.035 % suspension, and sonicated for 1 h. This suspension (3 mL) was mixed with 20 μL of $FeSO_4$ (4.58 mmol L^{-1}), 20 μL of ascorbic acid (87 μmol L^{-1}) and 100 μL of extract. In the control, instead of samples, 100 μL of 80 % aqueous methanol were added. The phosphate buffer (3.04 mL; pH 7.4) and 100 μL of extract were added in the blank probe. In the blank probe for the control, instead of samples, 100 μL of 80

% aqueous methanol were added. After incubation at 37 °C for 1 h, 200 μL of EDTA (37.2 mg mL^{-1}) were added to all samples followed by 2 mL of an aqueous mixture containing TBA (3.75 mg mL^{-1}), $HClO_4$ (1.3 %) and TCA (0.15 g mL^{-1}). Following heating at 100 °C for 15 min, the cooled mixtures were centrifuged at 1600*g* for 15 min, 250 μL of each mixture were transferred to 96-well microplates and the absorbance at 532 nm was read. All samples and the control were made in triplicate and IC_{50} values were determined.

Anti-AChE activity

In this work, a newly optimized procedure was developed. Namely, 110 μL of Tris–HCl buffer (20 mmol L^{-1}, pH 8.0) was mixed with 20 μL of AChE (0.5 U mL^{-1}, dissolved in Tris––HCl buffer (20 mmol L^{-1}, pH 7.5)) or 20 μL of Tris–HCl buffer (20 mmol L^{-1}, pH 7.5) in the blank probe. A 10 μL aliquot of extract, concentration ranging 25–250 mg mL^{-1}, or solvent in the control, were added. The 96-well plate was placed inside the plate reader, incubated at 37 °C, with agitation for 15 min. After incubation, 40 μL of a 5,5′-dithiobis(2-nitrobenzoic acid) solution (1.196 mg mL^{-1}) was prepared in Tris buffer (NaCl, $MgCl_2 \cdot 6H_2O$, pH 8.0) and was added in the plate. Consequently, 20 μL of acetylthiocholine iodide solution (15 mmol L^{-1}) was added in the plate and the absorbance was read at 412 nm. Galanthamine was used as the positive control. All samples and the control were made in triplicate and results are presented as IC_{50} values.

Statistical analysis

The percentage of inhibition achieved by different concentrations of extracts in the performed antioxidant assays was calculated using the following equation: I (%) = $100(A_0 - A)/A_0$, where A_0 is the absorbance of the control reaction and A is the absorbance of the examined samples, corrected for the value of the control. The potential of an extract to inhibit AChE was calculated by the following equation I (%) = $100(1 - A/A_0)$, where A_0 is the absorbance of the control reaction and A was the absorbance of the examined samples, corrected for the value of the control. The effect on cell growth was calculated as: I (%) = $100A_t/A_c$, where A_t is the absorbance of the test sample and A_c is the absorbance of the control, both obtained after subtracting absorbances at a reference wavelength. Corresponding inhibition—concentration curves, as well as calibration curves, were drawn using Origin software version 8.0 (OriginLab, MA, USA) and IC_{50} values (concentration of extract that inhibited $DPPH^{\bullet}$, $^{\bullet}NO$, $O_2^{\bullet-}$, $HO^{\bullet}$ and malondialdehyde production, AChE activity and cell growth by 50 %) were determined. For each assay and determinations of the extract composition, the final results are expressed as mean ± standard deviation (*SD*) of the measurements of three separate extracts, while measurements for each extract were performed in three or eight (cytotoxic activity) different trials. A comparison of the group means and the significance between the groups were verified by the Student's *t*-test. Statistical significance was set at $p \leq 0.05$.

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