SUPPLEMENTARY MATERIAL

Multidirectional characterization of chemical composition and health-promoting potential of *Rosa rugosa* hips

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Abstract

Rugosa rose provides one of the largest hips frequently used in the preparation of pharmaceutical and food products. The aim of work was to conduct multidirectional study of biological activity and chemical composition of *Rosa rugosa* hips. Antiradical, cytotoxic (against cervical and breast cancer cell lines), antibacterial (against eight bacterial strains) and antifungal potential of the species in question was evaluated. Total contents of phenolics, phenolic acids, flavonoids, tannins, carotenoids and ascorbic acid were determined. LC–ESI–MS/MS analysis was performed in order to investigate closely phenolic acids and flavonoid glycosides.

As a result, interesting selective cytotoxic effects on cervical (HeLa) and breast cancer (T47D) cell lines, significant antiradical activity (EC_{50} 2.45 mg mg⁻¹ DPPH[•]) and moderate antimicrobial potential (MIC 0.625 – 1.25 mg mL⁻¹) were observed. Nine phenolic acids and eleven flavonoid glycosides were qualitatively and quantitatively determined, including seven compounds previously not reported in *R. rugosa* hips.

Keywords

rose hips, phenolics, LC–MS, antiproliferative, antiradical, antibacterial

Experimental

Plant material

Hips (pseudofruits) of *R. rugosa* were collected in Serock (Lublin Voivodeship, Poland) in August 2013. The botanical material was authenticated by Prof. Tadeusz Krzaczek and a voucher specimen was deposited at the Chair and Department of Pharmaceutical Botany, Medical University of Lublin, Poland (voucher No. R-039/13). The nuts were separated and removed from the red hypanthium. The plant material was dried at ambient temperature and powdered according to the European Pharmacopoeia 6th edition (2007).

Chemicals

Standards of gallic, gentisic, caffeic, protocatechuic, synapic, *p*-coumaric, salicylic acid and pyrogallol, quercetin, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]), Trolox, hide powder were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). Flavonoids: quercetin-3-*L*-arabinofuranoside (avicularin), quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-rutinoside (rutin), quercetin-3-*O*-galactoside (hyperoside), kaempferol-3-*O*-glucoside (astragalin), kaempferol-3-*O*-(6''-*O*-(*E*)-*p*-coumaroyl)-glucoside (tiliroside), quercetin-3-*O*-rhamnoside (quercitrin), kaempferol-3-*O*-rutinoside (nicotiflorin), apigenin-7-*O*-glucoside were from ChromaDex (Irvine, USA). Ethanol, methanol, hexane, chloroform, diethyl ether, ethyl acetate, sodium molybdate, sodium nitrite, dimethyl sulfoxide (DMSO), isoamyl alcohol and Folin–Ciocalteu reagent were from Avantor Performance Materials (Gliwice, Poland); β -carotene was from Fluka (Buchs, Switzerland).

All the chemicals were of analytical grade. LC grade methanol (MeOH), acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). LC grade water was prepared using a Millipore Milli-Q purification system (Millipore, Bedford, MA, USA).

Analysis of the major groups of active ingredients in plant material

Total phenolic, phenolic acid, flavonoid and tannin content

Analysis of total phenolic, phenolic acid, flavonoid and tannin content was conducted using spectrophotometric methods. The total phenolic content in plant material was determined according to the Singleton and Rossi method (1965), with some modifications (Olech et al. 2012). The results were expressed in mg of gallic acid g⁻¹ of dry plant material.

In order to evaluate the content of phenolic acids, the method described by Nichiforesco and Coucou (1965) was used. The phenolic acid content was calculated using a reference curve plotted for caffeic acid and expressed in mg of caffeic acid g⁻¹ of dry plant material.

Total flavonoid content in plant material was determined colorimetrically according to the method described by Lamaison and Carret (1990). Results were expressed in mg of quercetin g⁻¹ of dry plant material.

Determination of tannin content in *R. rugosa* pseudofruits was performed according to the method described in the Polish Pharmacopoeia 6th edition (2005). Tannins were estimated indirectly after adsorption on and precipitation with insoluble hide powder. Result was expressed in mg of pyrogallol g⁻¹ of dry plant material.

All the spectrophotometric assays were conducted in the Thermo Scientific Evolution 300 spectrophotometer (Lafayette, CO, USA).

Total carotenoid content

The carotenoid content in *R. rugosa* pseudofruits was determined spectrophotometrically by means of the modified method described in Current Protocols in Food Analytical Chemistry (Scott 2000) and expressed in mg of β-carotene (reference standard) per g of dry plant material.

Content of ascorbic acid

The content of ascorbic acid in the plant material was assayed according to the method from European Pharmacopoeia 6th edition (2007). Results were expressed as % of dry plant material.

LC–ESI–MS/MS analysis of phenolic composition of rose pseudofruits

Preparation of samples

Twenty-five grams of dried and pulverized rose pseudofruits were extracted four times (maceration with stirring for 8 h each), at ambient temperature with 150 mL of 80 % (v/v) aqueous methanol. During the final extraction, a sonication step at 50°C (30 min) was applied in order to achieve exhaustive extraction. Extracts were combined, filtered and evaporated to dryness under vacuum. The residue was weighted and redissolved in volume flask (100 mL) to 80% (v/v) methanol at a concentration of 100 mg mL⁻¹ (M). Part of this solution (20 mL) was lyophilized in a Free Zone 1 apparatus (Labconco, Kansas City, KS, USA) and stored in the refrigerator prior to further analysis.

The remaining amount of M was fractionated into diethyl ether, ethyl acetate and water fractions. Briefly, 80 mL of the M solution was evaporated to dryness under vacuum. Syrupy residue was

dissolved in hot water, left to cool (12 h, 4°C), filtered and filled up with distilled water to the volume of 100 mL. Subsequently elution with solvents with increasing polarity was carried out. Initially, the aqueous fraction was extracted with six portions of diethyl ether (50 mL). Collected ether extracts were combined, the solvent was evaporated and the residue was lyophilized to give the ether fraction (FE).

The aqueous solution after extraction with diethyl ether was further washed six times with portions (50 ml) of ethyl acetate. Eluates were combined, concentrated in a vacuum evaporator and the residue was lyophilized to give the acetate fraction (OE).

The aqueous solution remaining after the extractions was also evaporated, freeze-dried and used for further analysis as the water fraction (W).

Prior to LC-MS analysis, the methanolic extract and its fractions were cleansed of ballast substances using solid phase extraction on octadecyl SPE columns (500mg, JT. Baker Inc., Philipsburg, USA). All extracts were prepared in duplicate.

LC-ESI-MS/MS analysis of phenolic acids and flavonoid glycosides

Phenolic acids and flavonoid glycosides contents in methanolic extract (M), diethyl ether (FE), ethyl acetate (OE) and water (W) fraction were determined by reversed-phase ultra-high-pressure liquid chromatography-mass spectrometry, performed on an ACQUITY UPLC System (Waters Corporation, Milford, MA, USA) according to the previously described methods (Nowak et al. 2014). For the specific detection of analytes, a Waters DAD and TQ detector in negative electrospray ionization mode with Metastable Reaction Monitoring (MRM) were used. Argon was used as a collision gas (collision energies are given in Table S1) and nitrogen as a desolvation gas (500 L h⁻¹ and 1000 L h⁻¹ for phenolic acids and flavonoids, respectively). The data was acquired and processed using MassLynx software (Waters Corp., Milford, MA, USA). Data acquisition was carried out using different retention time windows. Triplicate injections were made for each standard solution and sample. Calibration curves were obtained by plotting the peak area of phenolic compound at each level prepared against the concentration of standard solutions and showed linear relationships. Compounds were quantified using the external standard method.

For phenolic acids, chromatographic analyses were carried out on a Waters ACQUITY UPLC[®] HSS T3 column (1.0 x 100 mm; 1.8 µm; Waters Corporation, Milford, CT, USA) utilizing the gradient elution programme with a mobile phase A (water containing 0.1% HCOOH) and a mobile phase B (ACN). The solvent A concentration was changed as follows: 0 min (95%); 0.8

min (90%); 2.0 min (85%); 3.6 min (79%); 5.3 min (73%); 7.6 min (50%); 8.6 min (5%); 10.0 – 11.0 min (95%). The flow rate was 0.06 mL min⁻¹ and the column temperature was 30°C.

The injection volume was 3 µL. In the MRM, the mass range from 50 to 400 *m/z* was recorded and the following parameters were applied: capillary voltage 2.4 kV, source temperature 110°C, desolvation temperature 350°C.

In the case of flavonoid glycosides, separations were carried out on a Waters ACQUITY UPLC® BEH C18 (2.1 x 100 mm; 1.7 µm; Waters Corporation, Milford, CT, USA). Gradient elution programme with a mobile phase A (water containing 0.1% HCOOH) and a mobile phase B (ACN containing 0.1% HCOOH) was used. The solvent A concentration was changed as follows: 0 min (87%); 0.5 min (87%); 6.0 min (85.5%); 8.5 min (60%); 9.5 min (60%); 9.6 – 11.6 min (87%). The flow rate was 0.04 mL min⁻¹, and the column temperature was 50°C. The injection volume was 5 µL. The mass range from 200 to 800 *m/z* was recorded and the following parameters were applied: capillary voltage 2.8 kV, source temperature 120°C, desolvation temperature 350°C.

The limits of detection (LOD) and quantification (LOQ) for phenolic compounds were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations.

DPPH[•] assay

The radical scavenging activity of methanolic extract was determined according to slightly modified method of Olech et al. (2012). Aliquots of 180 µl of a freshly-prepared 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) coloured solution in methanol (0.07 mg mL⁻¹) were mixed with 20 µl of the extract diluted to various concentrations in 96-well microplates. The solutions were shaken and incubated at 28°C for 30 minutes in the dark. A decrease in DPPH[•] absorbance induced by the sample was measured at 517 nm using an Elisa reader (Infinite Pro 200F, Tecan Group Ltd., Männedorf, Switzerland). To determine EC₅₀, a dose response curve for five prepared dilutions of M was plotted. Test was carried out in triplicate and averaged. Results were expressed as standard equivalents using Trolox (TE) and ascorbic acid (VCE) based on their EC₅₀ values. Moreover, the antiradical efficiency (AE = 1/ EC₅₀) was calculated.

***In vitro* cytotoxic assay**

The methanolic extract prepared from *R. rugosa* pseudofruits was evaluated for its cytotoxic activity against cervical (HeLa ECACC 93021013 human cervical epithelial cell line derived from cervical carcinoma of a 31-year-old Black) and breast cancer (T47D ECACC 85102201

human breast carcinoma cells) cell lines. Normal human skin fibroblast cells in vitro (HSF primary cell culture isolated from the skin a 25-year-old female 5th passage) were included in the cytotoxicity test as a control group.

Each cell line was inoculated at 10^4 cells per mL density on a microtiter plate (Nunc, Roskilde, Denmark) in the RPMI 1640 medium (Sigma, St. Louis, MO, USA) with 10% FBS (Sigma) and the extracts added at concentration of $100\text{ }\mu\text{g mL}^{-1}$, $75\text{ }\mu\text{g mL}^{-1}$ or $50\text{ }\mu\text{g mL}^{-1}$. The cultures were incubated for 48 h under standard conditions (37°C, 5% CO₂, 90% humidity). End-point determinations were performed with 5-bromo-2-deoxy-uridine (BrdU) labelling and the detection kit III (Roche Diagnosis, Mannheim, Germany) using an Elisa reader (Genesys 20, Thermo Spectronic, Madison, WI, USA) (Olech et al. 2012).

The growth percentage was evaluated spectrophotometrically versus the untreated controls using the cell viability assay. The results of each spectrophotometric measurement were expressed as the percentage of cells with growth inhibition compared to untreated samples. All experiments were carried out in triplicate.

Antibacterial and antifungal assay *in vitro*

The antibacterial potential of *R. rugosa* extracts was evaluated using the micro-broth dilution method, which enables determination of minimal inhibitory concentration (MIC) according to the early described procedure (Nowak et al. 2014). Eight reference strains, including Gram-positive bacteria (*Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 10240) and Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 9027, *Proteus mirabilis* ATCC 12453) were used. The plant material was dissolved in dimethyl sulphide (DMSO) and the series of the two-fold dilutions, ranging from 0.02 to 2.5 mg mL^{-1} , was prepared in Mueller-Hinton broth (Biocorp, Poland) in 96-well microtitre plates. The wells were inoculated with the bacterial suspension (the final inoculum size of 10^6 colony forming units – CFU mL^{-1}). Following the 24-hour incubation at 35°C, MIC was defined as the lowest concentration of extract at which no visible growth was observed. DMSO was used as a negative control, while as a positive control only plant material in broth and broth with inocula was included. Gentamicin was applied as a reference compound. Minimal bactericidal concentrations (MBCs) were determined by collecting 20 μL from each well with growth inhibition and placing onto duplicate Mueller-Hinton agar plates and incubating at 35°C for 24 h. MBC was defined as the lowest extract concentration at which there was no bacterial growth.

Determination of antifungal activity of M (quantitatively dissolved in DMSO) was carried out for two reference yeast strains derived from the ATCC collection i.e. *Candida albicans* ATCC 10231 and *Candida parapsilosis* ATCC 22019. MICs values were designated using the serial twofold dilution method in 96–well microtiter plates according to the method described above with modifications of the substrate, inoculum density and incubation conditions. Serial dilutions of M were performed in Mueller–Hinton agar (Biocorp, Poland) supplemented with 2% glucose. The wells were inoculated with the yeast suspension (the final inoculum size of 1.5×10^5 colony forming units – CFU mL⁻¹). After 48 h of incubation at 30°C, the MICs were assessed visually as the lowest extract concentration showing complete growth inhibition. Fluconazole was used as a reference compound. In order to determine the minimal fungicidal concentration (MFC) of M, 20 µL from each tube that showed growth inhibition was streaked onto Sabouraud dextrose agar plates. After 48 h of incubation at 30°C, the MFC was assessed visually as the lowest drug concentration at which there was no growth. Experiments were done in triplicates.

Table S1. Analytical parameters of LC–ESI–MS/MS qualitative and quantitative method. Limits of detection (LOD), limits of quantification (LOQ) and linearity ranges for identified compounds. Compounds confirmed by comparison with authentic standards.

Peak	t _R (min)	UVmax (nm)	[M–H] [–] (m/z)	Fragment ions (m/z)	Collision energy (eV)	Compound	LOD (ng µl ^{–1})	LOQ (ng µl ^{–1})	Linearity range (ng µl ^{–1})
Phenolic acids									
1	3.42	271.0	169	124.8	15	Gallic acid	0.028	0.093	0.10 – 10.00
2	4.48	260.0 294.0	153	108.0	25	Protocatechuic acid	0.018	0.059	0.10 – 10.00
				109.0	15				
3	5.56	255.7	137	93	15	4–hydroxybenzoic acid	0.017	0.056	0.10 – 10.10
4	5.65	237.0 328.0	153	109.0	15	Gentisic acid	0.017	0.057	0.10 – 10.00
5	6.10	322.7	179	134.9	20	Caffeic acid	0.017	0.057	0.10 – 10.30
6	6.35	236.7 296.7	137	93	15	3–hydroxybenzoic acid	0.017	0.058	0.10 – 10.00
7	7.28	225.7 308.7	163	118.9	15	<i>p</i>–Coumaric acid	0.018	0.061	0.10 – 10.20
8	7.64	236.7 323.7	223	163.8	15	Synapic acid	0.032	0.107	0.10 – 10.00
				207.8	15				
9	8.72	235.7 300.7	137	93.0	15	Salicylic acid	0.017	0.057	0.10 – 10.20
Flavonoid glycosides									
1	3.00	255.7 355.7	609	300	35	Rutin	0.86	2.60	0.98 – 9.65
2	3.09	255.7 353.7	463	300	25	Hyperoside	1.12	3.40	1.00 – 10.00
3	3.36	256.7 356.7	463	300	25	Isoquercitrin	0.47	1.42	1.00 – 10.00
4	4.41	264.7 349.7	447	284	25	Astragalin	1.13	3.43	1.00 – 10.00
5	4.62	257.7 349.7	433	300	25	Avicularin	0.89	2.69	0.89 – 8.72
6	4.81	264.7 349.7	593	285	30	Kaempferol–3–O– rutinoside	0.44	1.34	1.00 – 10.00
7	5.41	256.7 352.7	447	300	25	Quercitrin	1.07	3.23	1.00 – 10.00
8	5.93	252.7 366.7	463	301	25	Spireoside	0.99	3.00	1.10 – 10.76
9	6.15	255.7 351.7	505	300	25	Quercetin–3–O–β–D– glucopyranosyl–6''–	0.06	0.18	1.00 – 10.00
10	8.28	266.7 314.7	593	285	30	Tiliroside	0.86	2.61	1.00 – 10.00

Table S2. Content of phenolic acids and flavonoid glycosides in different extracts from rugosa rose pseudofruits. Mean values of three replicate assays with standard deviation. Abbreviations: M – methanolic extract, FE – diethyl ether fraction, OE – ethyl acetate fraction, W – water fraction, tr – trace amount, nd – not detected.

Phenolic acids [$\mu\text{g mg}^{-1}$ of dry extract]	M	FE	OE	W
Caffeic acid	tr	0.70 ± 0.04	0.01 ± 0.00	nd
Gentisic acid	–	0.17 ± 0.01	0.07 ± 0.01	nd
Protocatechuic acid	0.01 ± 0.00	1.10 ± 0.05	0.06 ± 0.00	nd
Gallic acid	0.13 ± 0.01	8.30 ± 0.42	4.01 ± 0.18	0.01 ± 0.00
Salicylic acid	tr	0.20 ± 0.01	nd	nd
Synapic acid	nd	0.21 ± 0.01	nd	nd
<i>p</i> -Coumaric acid	0.02 ± 0.00	2.04 ± 0.11	0.07 ± 0.00	nd
3-hydroxybenzoic acid	nd	0.07 ± 0.00	nd	nd
4-hydroxybenzoic acid	nd	1.37 ± 0.08	nd	nd
Flavonoids [$\mu\text{g mg}^{-1}$ of dry extract]	M	FE	OE	W
Quercetin-3-L-arabinofuranoside (avicularin)	0.18 ± 0.01	3.08 ± 0.15	6.34 ± 0.32	nd
Quercetin-3-O-rutinoside (rutin)	0.07 ± 0.00	nd	0.91 ± 0.03	0.08 ± 0.00
Quercetin-3-O-galactoside (hyperoside)	0.12 ± 0.00	1.30 ± 0.05	3.27 ± 0.12	0.01 ± 0.00
Quercetin-3-O-glucoside (isoquercitrin)	0.17 ± 0.02	1.95 ± 0.08	6.22 ± 0.23	0.01 ± 0.00
Quercetin-4'-O- glucoside (spireoside)	0.03 ± 0.00	0.14 ± 0.01	nd	0.04 ± 0.00
Kaempferol-3-O-(6''-O-(<i>E</i>)- <i>p</i> -coumaroyl)-glucoside (tiliroside)	0.10 ± 0.01	4.36 ± 0.17	nd	nd
Kaempferol-3-O-glucoside (astragalin)	tr	0.38 ± 0.02	0.23 ± 0.01	nd
Quercetin-3-O-rhamnoside (quercitrin)	0.03 ± 0.00	0.30 ± 0.01	1.04 ± 0.03	nd
Kaempferol-3-O-rutinoside	tr	nd	0.11 ± 0.01	nd
Quercetin-3-O- β -D-glucopyranosyl-6"-acetat	0.06 ± 0.00	nd	0.44 ± 0.02	0.09 ± 0.01
Apigenin-7-O-glucoside	nd	nd	nd	nd

Table S3. Antiradical activity of methanolic extract (M) from *R. rugosa* hips. EC₅₀ expressed as mg of dry extract per mg DPPH[•], mean values of three replications; AE – antiradical efficiency (1/EC₅₀); TE – Trolox equivalent; VCE – ascorbic acid equivalent. Equivalents were calculated by dividing extract EC₅₀ by standard EC₅₀. For Trolox EC₅₀ = 0.244 mg mg⁻¹ DPPH[•]; for ascorbic acid EC₅₀ = 0.198 mg mg⁻¹ DPPH[•].

Extract	R ²	EC ₅₀	AE	TE	VCE
M	0.9986	2.45	0.41	10.04	12.37

Table S4. The cytotoxic activity of rosehip methanolic extract (M) added at a concentration of 100 $\mu\text{g mL}^{-1}$, 75 $\mu\text{g mL}^{-1}$ and 50 $\mu\text{g mL}^{-1}$ after 48 h incubation. Data are expressed as the percentage of dead cells. Abbreviations: HeLa – cervical cancer cell line; T47D – breast cancer cell line.

Concentration ($\mu\text{g mL}^{-1}$) \ Cell line	T47D (%)	HeLa (%)	Fibroblasts (%)
100	40	20	0
75	20	0	0
50	0	0	0

Table S5. Antibacterial and antifungal activity of different extracts obtained from *R. rugosa* pseudofruits. Abbreviations: MIC – minimal inhibitory concentration [mg mL⁻¹]; MBC – minimal bactericidal concentration [mg mL⁻¹]; MFC – minimal fungicidal concentration [mg mL⁻¹]; ATCC – American Type Culture Collection. MICs of gentamicin ranged from 0.03–0.12 x 10⁻³ mg mL⁻¹ and 0.25–1.0 x 10⁻³ mg mL⁻¹ for Gram–positive and Gram–negative bacterial strains, respectively. MIC for fluconazole ≤ 0.008 mg mL⁻¹. DMSO at the final concentration used had no influence on the growth of the tested strains; ‘–’ – not determined

Reference microbial strains	M			OE			W		
	MIC	MBC	MBC/MIC ratio	MIC	MBC	MBC/MIC ratio	MIC	MBC	MBC/MIC ratio
Gram (+)bacteria									
<i>S. epidermidis</i> ATCC 12228	1.25	> 1.25	1	0.625	> 2.5	4	1.25	5	4
<i>S. aureus</i> ATCC 25923	1.25	2.5	2	1.25	> 2.5	2	1.25	5	4
<i>B. subtilis</i> ATCC 6633	1.25	2.5	2	1.25	2.5	2	1.25	2.5	2
<i>M. luteus</i> ATCC 10240	0.625	1.25	2	0.625	1.25	2	0.625	1.25	2
Gram (–) bacteria									
<i>E. coli</i> ATCC 25922	1.25	1.25	1	1.25	1.25	1	0.625	0.625	1
<i>K. pneumoniae</i> ATCC 13883	1.25	2.5	2	1.25	> 2.5	2	1.25	1.25	1
<i>P. aeruginosa</i> ATCC 9027	1.25	1.25	1	1.25	2.5	2	1.25	1.25	1
<i>P. mirabilis</i> ATCC 12453	1.25	2.5	2	1.25	> 2.5	2	1.25	1.25	1
	MIC	MFC	MFC/MIC ratio	MIC	MFC	MFC/MIC ratio	MIC	MFC	MFC/MIC ratio
Yeasts									
<i>C. albicans</i> ATCC 10231	1.25	1.25	1	–	–	–	–	–	–
<i>C. parapsilosis</i> ATCC 22019	1.25	1.25	1	–	–	–	–	–	–

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