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ANTIOXIDANT ACTIVITY OF *GEUM RIVALE L.* AND *GEUM URBANUM L.*

ALEKSANDRA OWCZAREK*, JAN GUDEJ and MONIKA ANNA OLSZEWSKA

Department of Pharmacognosy, Medical University of Lodz, Muszynskiego 1 St., 90-151 Łódź, Poland

Abstract: Two species of the Rosaceae genus *Geum*, comprising high amounts of tannins and phenolic acids, were investigated in terms of their antioxidant activity. Antioxidant potential of different extracts from aerial and underground parts of *Geum rivale* and *G. urbanum* was studied using various *in vitro* methods (FC, DPPH, FRAP and linoleic acid peroxidation test). The hydromethanolic extract from the roots of *G. rivale* with high total phenolic content of 17.48% was proven to be about twice as effective as the other extracts tested. Ethyl acetate and *n*-butanol were demonstrated to be the best solvents for optimal concentration of antioxidant constituents.

Keywords: *Geum*, Rosaceae, DPPH, FRAP, linoleic acid peroxidation

Reactive oxygen species (ROS) are highly reactive chemical molecules containing an atom of oxygen. They are constantly produced in every aerobic cell during the normal metabolic processes and most of them are quickly scavenged by cellular antioxidant systems consisting of enzymatic and non-enzymatic antioxidants. This natural mechanism, however effective, can sometimes fail, especially when the formation of ROS is enhanced by harmful factors like some chemicals, UV radiation or inflammatory processes. The resulting imbalance leads to the state known as "oxidative stress", during which the excessive ROS can damage cell structures, inhibit enzymes or oxidize nucleic acids. The destructive power of this phenomenon is widely acknowledged, and it is believed to be the cause of many diseases such as atherosclerosis, neurodegenerative diseases or cancer, as well as accelerated ageing (1).

In order to help maintain the fragile balance between the production and the destruction of the ROS, exogenous antioxidants can be applied, and plants area is considered to be a good source of natural, safe compounds with a large antioxidant potential (2-4).

G. rivale and *G. urbanum* are perennial herbs belonging to the Rosaceae family, widely distributed across the Europe, and commonly found either in forests and parks (*G. urbanum*) or on wet meadows and river banks (*G. rivale*) (5, 6). They are valued in folk medicine for their astringent and antiseptic properties, and scientific inquiries, motivated by the traditional use, have revealed the presence of tannins, phenolic acids, triterpenes, flavonoids and essential oil, in aerial and underground parts of the plants alike (7-9). Our investigations concerning the chemical composition of the two species showed, that particularly tannins and phenolic acids are present in significant quantities, both groups of compounds belonging to the wide class of polyphenols, that are known to play an important role in the antioxidant activity of plant materials.

There are only a few studies on the antioxidant activity of the plants from the genus *Geum*. The activity of methanolic extract from the roots of *G. rivale* was studied by Oszmianski et al. (10), using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] assays. The results proved, that the extract was three times more effective in scavenging free radicals than extracts from underground organs of two other Rosaceae species – *Filipendula ulmaria* and *Aruncus silvestris*. On the other hand, its activity was weaker than the activity of extracts from the roots of *Potentilla alba* and *Waldstenia geoides* but only by a factor of 1.5. The antioxidant potential of *G. urbanum* was, in turn, investigated by Mantle et al. (11). The hydromethanolic extract form the leaves of the plant, tested with ABTS assay, was demonstrated to have higher activity than 37 out of 38 other extracts evaluated.

Above results, although promising, give only a partial insight into the problem. Because of different

* Corresponding author: e-mail: aleksandra.owczarek@umed.lodz.pl; phone: +48 42 677 91 72

methodology used, their results are difficult to compare, and, as both plants are used in traditional medicine to some extent interchangeably, their direct comparison would definitely be of some interest. Moreover, there is no information about the best solvent to concentrate the antioxidant constituents of the plants. The aim of our study was, therefore, better characterization of the antioxidant potential of aerial and underground parts of *G. rivale* and *G. urbanum*, by assessing the activity of extracts of different polarities using various *in vitro* tests.

EXPERIMENTAL

Plant material

Aerial and underground parts of wild growing *G. rivale* and *G. urbanum* were collected from locations in Lódź during the flowering of the plants. The material was identified by Prof. Jan Gudej, Department of Pharmacognosy, Medical University of Lodz, Poland. Voucher specimens were deposited in Department of Pharmacognosy, Medical University of Lodz, Poland.

Plant material was dried under normal conditions, powdered with electric grinder and sieved through a 0.315 mm sieve.

Chemicals and instrumentation

Chromatographic grade purity reagents and standards: 2,2-diphenyl-1-picryl hydrazyl (DPPH), 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), 2,4,6,-tris-(2-pyridyl)-s-triazine (TPTZ), linolenic acid and (±)-6-hydroxy-2,2,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) were obtained from Sigma-Aldrich (Germany/USA). All other chemicals and solvents of analytical grade were purchased from POCh (Poland).

Samples were incubated in a constant temperature using a BD 23 incubator (Binder, Germany). Absorbance was measured using a Lambda 25 spectrophotometer (Perkin-Elmer, USA), in 10 mm quartz cuvettes.

Preparation of hydromethanolic extracts

Samples of plant material (100 mg or 1 g depending on the assay) were refluxed with 30 mL of 70% methanol for 30 min, and then twice with 20 mL of 70% methanol for 20 min. Combined extracts were filled up to 100 mL and left for further analysis (hydromethanolic extract – HME).

Preparation of dried extracts and fractions

Samples of plant material (30 g) were first exhaustively extracted with petroleum ether (250 mL) and then with chloroform (250 mL) in a Soxhlet apparatus. Subsequently, they were refluxed trice with 100 mL of methanol. The methanol extract was evaporated to dryness *in vacuo* (dry methanolic extract – DME, 4.5-5.3 g depending on the plant material). A part of DME (1 g) was left to further analysis and the rest was suspended in water and extracted successively with diethyl ether, ethyl acetate and *n*-butanol. The fractions were evaporated and left for further analysis (diethyl ether fraction – DEF, 0.2-0.5 g; ethyl acetate fraction – EAF, 0.7-1.1 g; *n*-butanol fraction – BF, 1.8-2.3 g).

Determination of total phenolic content (TPC)

The amount of total phenolics was determined according to the Folin-Ciocalteu (FC) method (12) with the use of HME (prepared from 100 mg of dry plant material) or methanolic solutions of DME, DEF, EAF and BF (60-200 μg/mL). Results were calculated with the use of eight-point calibration curve of gallic acid and expressed as gallic acid equivalents (GAE) per dry weight of the plant material or extract/fraction.

DPPH free radical-scavenging test

The scavenging activity was determined based on the method of Brand-Williams, Cuvelier, and Berset (13) with slight modifications as described previously (14). The range of concentrations used was 30-120 μg/mL for HME and 5-40 μg/mL for DME and fractions thereof. The activity of samples was expressed as EC_{50} value which is the concentration of the sample that reduces the amount of DPPH radicals by 50%. To improve accuracy of the measurements we used, similarly as in our previous paper, normalized EC_{50} values, that are not influenced by slight differences in the initial condition of the reaction.

Ferric reducing antioxidant power (FRAP) assay

The FRAP was determined according to the method of Pulido et al. (15), with some variations described previously (12). The concentration used was 30 μg/mL for HME and 1.9-4.0 μg/mL for DME and fractions thereof. The antioxidant activity was expressed in micromoles of ferrous ions produced by 1 g of the dry extract, fraction or standard, which was calculated from the eight-point calibration curve of ferrous sulfate.

Linoleic acid (LA) peroxidation test

The ability of the analytes to inhibit AAPH-induced LA peroxidation was assayed according to the method of Azuma et al. (16) with some modifi-

cations described previously (14). The range of concentrations used was 1.25-10.0 mg/mL for HME and 0.3-6 mg/mL for DME and fractions thereof. The activity of the analytes was expressed as IC_{50}, which is the concentration of the sample that decreases the degree of LA oxidation by 50%.

Statistical analysis

The samples of each analyte (extract, fraction or standard) were analyzed for LA-peroxidation test in triplicate and data are reported as the mean (n = 3 × 1) ± SD (standard deviation). For other photometric methods, three samples of each analyte were assayed, each sample was analyzed in quintuplicate and data are reported as the mean (n = 3 × 5 × 1) ± SD. The statistics (calculation of SD, one-way analysis of variance, Duncan's tests, and linearity studies) were performed using the software Statistica PL for Windows (StatSoft Inc., Poland).

RESULTS AND DISCUSSION

A variety of methods have been developed to evaluate the antioxidant potential of plant materials. The underlying reactions can proceed according to two main mechanisms. Single electron transfer (SET) reaction mechanism is connected with the transfer of a single electron from the molecule of the antioxidant to other molecule or ion, resulting in its reduction. In hydrogen atom transfer (HAT) dependent reaction the same effect is caused by the transfer of a hydrogen atom (17, 18). In our studies we used SET as well as HAT-based methods, in order to characterize different aspects of the antioxidant activity of the samples.

The DPPH assay is a discoloration test, in which the ability of the extract to scavenge the DPPH radical is measured. It is based largely on SET reaction mechanism, with hydrogen atom transfer playing only a marginal role. It is a simple and fast assessment, extensively used in many research laboratories, often as a preliminary method for estimating the antioxidant activity of natural products. DPPH is however, a synthetic radical that bears no similarity to free radicals occurring in living cells (13, 17).

The FRAP assay is a method, that depends entirely on SET reaction mechanism. It measures the ability of the sample to reduce Fe^{3+} to Fe^{2+}, and as such, also bears no relation to physiological conditions. It still permits, however, to assess the reductive power of the compounds tested and reflect their capacity to modulate redox processes in general (15, 17).

The principle of FC assay is a redox reaction proceeding mostly *via* SET mechanism, and for that reason the method could be considered as an another measure of the antioxidant activity. However, it is also a useful procedure for estimating the total phenolic content in the sample (17). It is also the only assay among the selected ones, the results of which due to the standardized conditions are easily comparable with the literature data.

LA peroxidation test, as opposed to other methods described, depends fully on the HAT reaction. It models more accurately physiological conditions, as it evaluates the scavenging potential of the tested compounds towards peroxy radicals, that are one of the naturally occurring ROS. It also provides insight into the ability of the sample to prevent harmful effects caused by the free radicals (in this case peroxidation of LA) (17-19).

The selected methods described above were used to test the antioxidant activity of hydromethanolic extracts (HMEs) of aerial and underground parts of *G. rivale* and *G. urbanum*, as well as the antioxidant activity of dry methanolic extracts (DMEs) and fractions obtained from DMEs by extraction with solvents of different polarities.

Hydromethanolic extracts

The results of the determination of the antioxidant activity and the total phenolic content for

Table 1. Antioxidant activity and total phenolic content of HMEs.

Plant material		DPPH	FRAP	LA oxidation inhibition	TCP
		EC_{50} [μg/mL]	FRAP [mM/g]	IC_{50} [μg/mL]	[%] GAE
G. rivale	Aerial parts	23.97 ± 0.85^{b}	2.29 ± 0.10^{b}	413.47 ± 20.54^{c}	7.83 ± 0.11^{a}
	Underground parts	11.22 ± 0.36^{a}	3.84 ± 0.14^{c}	252.84 ± 11.32^{a}	17.48 ± 0.46^{b}
G. urbanum	Aerial parts	26.57 ± 1.24^{c}	1.89 ± 0.07^{a}	366.60 ± 14.47^{b}	7.61 ± 0.36^{a}
	Underground parts	26.92 ± 1.31^{c}	1.94 ± 0.06^{a}	544.74 ± 26.43^{d}	7.89 ± 0.27^{a}

Different superscripts in each column indicate significant differences in the mean values at $p < 0.05$.

HMEs are shown in Table 1. Among the HMEs tested, the extract from the underground parts of *G. rivale* exhibited the highest activity regardless of the method used. Its EC_{50} value in DPPH assay was 11.22 μg/mL, while the FRAP value was 3.84 mM/g. Other HMEs with EC_{50} in range 23.97-26.92 μg/mL and FRAP in range 1.89-2.29 mM/g had about two times weaker activity.

The IC_{50} values for the inhibition of LA peroxidation varied from 544.74 to 252.84 μg/mL. The HME from the rhizomes of *G. rivale* was still the most active extract, while the HME from the rhizomes of *G. urbanum* was the least active one.

The results of FC total phenolic assay were in agreement with above findings showing almost twice higher content of phenolic compounds in the underground parts of *G. rivale* (17.48% GAE) comparing with other plant material tested (7.61-7.89% GAE). They also correspond with our previous studies that showed much higher content of tannins and phenolic acids in the rhizomes of *G. rivale* (20, 21). These two groups of compounds have, therefore, probably the greatest significance in the antioxidant activity of investigated *Geum* tissues.

High TPC values are not uncommon for plant materials acquired from species of Rosaceae family. In the studies by Cai et al. (22) evaluating 112 Chinese herbs, the roots of *Sanguisorba officinalis* and flowers of *Rosa chinensis* were among the ones containing the highest amounts of phenolic compounds, with TPC values of 15.87% GAE and 18.75% GAE, respectively. These results were comparable to those obtained for the leaves of *Camellia sinensis* (TPC = 17.40% GAE) and fruits of *Punica granatum* (TPC = 22.56% GAE), that are recognized as a valuable source of phenolics. The high values of TPC were also reported for the inflorescences and leaves of selected *Sorbus* species (TPC = 6.06-11.83% GAE) tested in one of our previous studies (12). The research conducted by Buricova et al. (23) revealed as well, significant amounts of polyphenols in the leaves of *Fragaria vesca* (TPC = 6.24% GAE), *Rubus idaeus* (TPC = 6.89% GAE) and *Rubus fruticosus* (TCP = 7.54% GAE). The investigated *Geum* species are, therefore, another Rosaceae plants, which can be considered as a good source of polyphenol rich plant materials.

Table 2. Antioxidant activity and total phenolic content of DMEs and fractions thereof.

Plant material		Extract/ Fraction	DPPH	FRAP	LA oxidation inhibition	TPC
			EC_{50} [μg/mL]	FRAP [mM/g]	IC_{50} [μg/mL]	[%] GAE
G. rivale	Aerial parts	DME	12.47 ± 0.54^{h}	3.46 ± 0.16^{a}	176.54 ± 8.34^{j}	19.46 ± 1.16^{a}
		DEF	6.01 ± 0.17^{f}	9.40 ± 0.39^{c}	67.49 ± 3.27^{d}	49.83 ± 1.10^{e}
		EAF	2.92 ± 0.13^{a}	17.62 ± 0.68^{jk}	49.57 ± 2.21^{b}	63.05 ± 0.39^{h}
		BF	4.11 ± 0.18^{b}	12.41 ± 0.54^{ef}	70.80 ± 3.06^{d}	46.49 ± 2.35^{cd}
	Underground parts	DME	4.22 ± 0.15^{bcd}	13.00 ± 0.44^{f}	129.30 ± 5.97^{i}	75.39 ± 2.57^{j}
		DEF	4.62 ± 0.21^{d}	12.26 ± 0.57^{ef}	89.88 ± 4.15^{f}	63.43 ± 2.17^{h}
		EAF	2.99 ± 0.09^{a}	18.56 ± 0.62^{l}	59.16 ± 2.37^{c}	65.59 ± 0.76^{hi}
		BF	3.35 ± 0.15^{a}	15.79 ± 0.63^{h}	59.04 ± 2.29^{c}	82.41 ± 2.73^{k}
G. urbanum	Aerial parts	DME	13.34 ± 0.47^{i}	3.07 ± 0.11^{a}	189.22 ± 8.74^{k}	19.06 ± 0.91^{a}
		DEF	6.44 ± 0.23^{g}	6.29 ± 0.28^{b}	119.27 ± 5.67^{h}	38.55 ± 1.02^{b}
		EAF	4.18 ± 0.11^{bc}	14.07 ± 0.67^{g}	72.28 ± 3.48^{d}	53.17 ± 2.61^{f}
		BF	6.09 ± 0.26^{fg}	12.05 ± 0.38^{e}	73.91 ± 3.12^{de}	44.37 ± 1.83^{c}
	Underground parts	DME	4.57 ± 0.20^{cd}	10.42 ± 0.43^{d}	92.03 ± 4.51^{f}	48.53 ± 1.25^{de}
		DEF	5.56 ± 0.22^{e}	9.29 ± 0.37^{c}	74.53 ± 3.49^{de}	48.28 ± 1.20^{de}
		EAF	3.16 ± 0.07^{a}	16.87 ± 0.65^{ij}	68.05 ± 3.11^{d}	57.11 ± 2.50^{g}
		BF	3.32 ± 0.13^{a}	16.28 ± 0.72^{hi}	80.96 ± 3.79^{e}	67.13 ± 1.33^{i}
Trolox			3.27 ± 0.10^{a}	9.42 ± 0.31^{c}	22.45 ± 1.10^{a}	-

Different superscripts in each column indicate significant differences in the mean values at $p < 0.05$.

Dry methanolic extracts and fractions thereof

The results of the determination of the antioxidant activity and the total phenolic content for DMEs and the fractions are shown in Table 2.

The normalized EC_{50} values for DPPH assay were in range 2.92-13.34 μg/mL. In case of DMEs, the extracts from the underground parts of the plants had significantly lower EC_{50} values (4.22-4.57 μg/mL) than the extracts from the aerial parts (12.47-13.34 μg/mL). Taking into consideration fractions of different polarities, EAFs were the most active ones (EC_{50} = 2.92-4.18 μg/mL), followed by BFs (EC_{50} = 3.32-6.09 μg/mL). The least active fractions, regardless of the plant material tested, were DEFs (EC_{50} = 4.62-6.44 μg/mL). The activity of the most active fractions with EC_{50} ranging from 2.92 to 3.35 μg/mL was similar to the activity of the Trolox standard (EC_{50} = 3.27 μg/mL) with no statistically significant differences ($p < 0.05$).

The FRAP values varied from 3.07 to 18.56 mM/g and corresponded largely to the EC_{50} values from DPPH assay, which was confirmed by significant correlation ($r = 0.8443$, $p < 0.05$) between the two variables. DMEs from the rhizomes of the plants (FRAP = 10.42-13.00 mM/g) were again over three times more active than DMEs from the aerial parts and the order of antioxidant potential of fractions was parallel to that of DPPH assay with AEFs being the most active ones, followed by BFs and DEFs. The most active fractions – EAFs and BFs (FRAP = 12.05-18.56 mM/g) had substantially higher reduction potential than Trolox standard (FRAP = 9.42 mM/g).

The IC_{50} values in LA peroxidation test ranged from 49.57 μg/mL to 189.22 μg/mL. The results of the test were significantly correlated with the results of the DPPH assay ($r = 0.8691$, $p < 0.05$) and also to some extent to the FRAP assay ($r = 0.6815$, $p < 0.5$). The DMEs from the underground parts (IC_{50} = 92.03-129.30 μg/mL) of the plants were still more active than the DMEs from the aerial parts (IC_{50} = 176.54-189.22 μg/mL), but the differences were not as substantial as in the previous tests. In the case of the aerial parts of *G. rivale* and the rhizomes of *G. urbanum*, EAFs and BFs were more active than DEFs, whereas in the case of the underground parts of *G. urbanum*, all fractions exhibited similar activity. As far as the aerial parts of *G. rivale* are concerned, the AEF was the strongest inhibitor of LA peroxidation. With EC_{50} = 49.57 μg/mL it was also the most active fraction of all fractions tested, but still over two times weaker than Trolox standard (IC_{50} = 22.45 μg/mL).

The TPC determined by FC assay varied from about 19% GAE in the DMEs from the aerial parts of the plants to about 80% in BF from the underground parts of *G. rivale*. EAFs were the fractions containing the highest amounts of polyphenols in the case of the aerial parts, and BFs in the case of the underground parts. The TPC results were significantly correlated with the results of DPPH ($r = -0.8523$, $p < 0.05$) and FRAP ($r = 0.8442$, $p < 0.05$) assay. The correlation was weaker, although still significant, in the case of LA peroxidation test ($r = -0.6649$, $p < 0.05$).

In general, ethyl acetate and *n*-butanol seem to be the best solvents to concentrate antioxidants from methanolic extracts of the investigated *Geum* species. Similar results were obtained in our previous studies concerning selected plants of the genus *Sorbus*, where EAFs and BF were the most active fractions in FC, DPPH, ABTS and FRAP assays (14). Moreover, in both cases strong correlations were identified between TPC values and SET-based antioxidant capacity. The findings varied, however, when it comes to HAT-type activity. The significant correlation between TPC values and the ability of the extracts to inhibit LA peroxidation was found for *Geum* species, while there was no clear correspondence between IC_{50} values of LA peroxidation test and total phenolic content in evaluated *Sorbus* extracts (14). The fact that the both genera vary in terms of their chemical composition could be probably accounted for these results.

CONCLUSION

The determination of the antioxidant activity of HMEs from aerial and underground parts of *G. rivale* and *G. urbanum* led to the identification the rhizomes of *G. rivale* as the plant material with the highest antioxidant potential. Significant correlations between TPC values and the antioxidant capacity evaluated by DPPH, FRAP and LA peroxidation assays suggest that, in the case of *Geum* extracts, polyphenols are mainly responsible for both SET and HAT-type antioxidant activity. Furthermore, ethyl acetate and *n*-butanol were proven to be the most efficient solvents to concentrate antioxidant compounds from methanolic extracts of evaluated plant materials. More detailed studies are required in order to identify the specific compounds responsible for this activity.

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