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QUANTITATIVE DETERMINATION OF ELLAGIC ACID AND GALLIC ACID IN *GEUM RIVALE* L. AND *G. URBANUM* L.

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Hydrolyzable tannins and products of their hydrolysis, ellagic acid (EA) and gallic acid (GA), are important constituents of many medicinal plants and exhibit various biological activities. *Geum rivale* and *G. urbanum* are traditional herbal remedies rich in tannin compounds. The aim of the study was to quantitate free and total EA and GA in aerial and underground parts of *G. rivale* and *G. urbanum*. After optimization of extraction, both compounds were quantitated by reversed phase HPLC (RP-HPLC). EA was more abundant than GA in the investigated material, and underground parts of *G. rivale* were the richest source of total EA and GA.

Key words: *Geum rivale*, *Geum urbanum*, phenolic acid, Rosaceae.

INTRODUCTION

Ellagic acid (EA) and gallic acid (GA) are two important phenolic compounds present in many medicinal plants. They show a large variety of biological activities including antioxidant, antimicrobial and anticancer activity (Faried et al., 2007; Wansi et al., 2010; Hagiwara et al., 2010). EA and GA can exist in plant material in free form but the main sources of these compounds are hydrolyzable tannins (ellagitannins and gallotannins), which upon hydrolysis release a sugar and a respective acid moiety (Haslam, 2007; Serrano et al., 2009). Hydrolytic degradation of hydrolyzable tannins can occur in the gastrointestinal tract after ingestion of the medicine (Clifford and Scalbert, 2000; Cerdá et al., 2005). EA and GA, as the major products of hydrolyzable tannin degradation, might therefore have a significant effect on the activity of herbal remedies containing large amounts of hydrolyzable tannins.

Rosaceae is a plant family known to contain large amounts of tannin compounds (Hegnauer, 1973). Here we study the chemical composition of two species of the genus *Geum* (Rosaceae) – *Geum rivale* and *G. urbanum*. Both plants are perennial herbs widely distributed in temperate regions of the Northern Hemisphere. Rhizomes of the plants are used in traditional medicine as astringent, anti-inflammatory and antiseptic agents. Due to its characteristic clove-like aroma the rhizome of *G. urbanum* is sometimes used to flavor alcoholic beverages (Macků and Krejča, 1989; Strzelecka and Kowalski, 2000).

Analyses of the chemical composition of those species have revealed the presence of some triterpenes, sterols and flavonoids, as well as small amounts of essential oil (Vollmann and Schultze, 1995; Panizzi et al., 2000). It is tannins, however, that are considered to be the main group of constituents responsible for the activity and medicinal usefulness of the plants (Blinova, 1954; Strzelecka and Kowalski, 2000). Previously we determined total tannin content (by gravimetric method with the use of hide powder) in the plants, at 31.4–136.1 mg/g depending on the species and plant organ (Owczarek and Gudej, 2013). Most of them are believed to be hydrolyzable gallo- and ellagitannins (Blinova, 1954). Free EA and GA have also been isolated from the plants (Gstirner and Widenmann, 1964; Panizzi et al., 2000). *G. rivale* and *G. urbanum* could be rich sources of EA and GA and this might be reflected in their activity. To the best of our knowledge, EA and GA content has been studied in *G. rivale* and *G. urbanum* only fragmentarily, and the methods used were not optimized or validated (Oszmianski et al., 2007; Kuczerenko et al., 2011).

The aim of our study was to determine the content of free EA and GA, as well as their total content after hydrolytic degradation of tannins, in aerial and underground parts of *G. rivale* and *G. urbanum*.

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MATERIALS AND METHODS

PLANT MATERIAL

Aerial and underground parts of *G. rivale* and *G. urbanum* were collected in Lodz in May 2010. The material was identified by Prof. Jan Gudej, Department of Pharmacognosy, Medical University of Lodz, Poland. Voucher specimens are deposited in the Department of Pharmacognosy, Medical University of Lodz, Poland.

The plant material was dried under normal conditions and powdered using an electric grinder.

CHEMICALS AND REAGENTS

EA and GA were purchased from Sigma-Aldrich (Germany). Solvents used for HPLC analysis were obtained from POCH (Gliwice, Poland). Other solvents were of analytical grade and were obtained from Chempur (Piekary Slaskie, Poland).

PREPARATION OF STANDARD CURVE

Stock solutions of EA and GA were prepared by dissolving the reference substances in methanol to final concentrations of 103.28 μg/mL for EA and 221 μg/mL for GA. The stock solutions were then diluted to appropriate concentration ranges and used to establish calibration curves. Each concentration was injected three times and the calibration curves were plotted using linear regression of the mean peak area versus the concentration.

OPTIMIZATION OF SAMPLE PREPARATION

To optimize extraction of free EA and GA we compared different extraction solvents [methanol, 75%, 50%, 25% methanol/water (v/v), water] and two extraction methods (mechanical shaking, refluxing).

To optimize hydrolysis we compared different solvents [methanol, 75%, 50%, 25% methanol/water (v/v), water], acid concentrations [6, 8, 10 mL 25% (v/v) HCl in 20 mL solvent] and extraction times (60, 90, 120 min).

DETERMINATION OF FREE EA AND GA

Dried and ground plant material (500 mg) was mechanically shaken with 30 mL methanol for 20 min. The obtained extract was filtered and the residue was shaken twice with 20 mL methanol for 15 min. The combined extracts were diluted with methanol to 100 mL.

DETERMINATION OF TOTAL EA AND GA

Dried and ground plant material (50–100 mg) was refluxed with 20 mL water with 6 mL 25% (v/v) HCl for 60 min. The obtained extract was filtered and the residue was heated twice with 20 mL water for 15 min. The combined extracts were diluted to 100 mL.

HPLC ANALYSES

The analyses employed a Hewlett-Packard 1100 series chromatograph equipped with an HP1311A quaternary pump, HP1322A vacuum degasser, HP1314A variable wavelength UV/VIS detector and 20 μl manual injector. A Nucleodur C18 HPLC column (250 × 4.6 mm, 5 μm; Machery-Nagel) was used. The mobile phase consisted of solvent A [0.5% (v/v) water solution of H3PO4] and solvent B (methanol) with the following elution profile: 0–8.5 min: 5–14% B in A, 8.5–25 min: 14–70% B in A, 25–30 min: 70% B in A. Elution was carried out at room temperature at a flow rate of 1.0 mL/min. Injection volume was 20 μl and detection wavelength 254 nm. Before HPLC analysis all samples were filtered through a PFTE syringe filter (13 mm, 0.2 μm, Whatman). Identification of EA and GA was based on comparison of retention time with standards, and their content was calculated based on the corresponding peak areas and injected concentrations.

VALIDATION

Measurements of intra- and inter-day variability were used to determine the precision of the methods. The assessments were made for a sample of *G. rivale* rhizomes before and after hydrolysis. Each solution was injected five times. Intra-day repeatability was examined by analyzing each sample on the same day within 24 h. Inter-day repeatability was determined on five different days. The measure of repeatability was the relative standard deviation (RSD).

A recovery study was made to determine accuracy. Known quantities of the standard solutions at three different concentrations were added to known amounts of pulverized rhizomes of *G. rivale*, and the samples were extracted and analyzed by the established HPLC method. Percentage recovery was calculated as the ratio of detected versus added amounts.

The limit of detection (LOD) and the limit of quantification (LOQ) were found by successively diluting the standard stock solutions to the concentration giving the smallest detectable peak. These concentrations were used to calculate the 3-σ signal-to-noise (S/N) ratio for the LOD, and the 10-σ S/N ratio for the LOQ.

STATISTICAL ANALYSIS

All analyses were done in triplicate and are expressed as means ±SD. Statistical analyses employed StatisticaPL software (StatSoft Inc.,

TABLE 1. Linearity of standard curves and retention parameters of ellagic and gallic acid

Constituent	Regression equation	r	Linearity range (μg/mL)	Retention time ± SD (min)	k'
Ellagic acid	y=243456x	0.9999	3.23-51.64	8.549±0.223	2.403
Gallic acid	y=46968x	0.9999	3.45-110.50	23.192±0.182	8.232

TABLE 2. Precision data, LOD and LOQ for ellagic and gallic acid

Constituent	Intra-day variability RSD (%)		Inter-day variability RSD (%)		LOD (μg/mL)	LOQ (μg/mL)
	Free acids analysis	Total acids analysis	Free acids analysis	Total acids analysis		
Ellagic acid	1.89	2.58	3.34	2.18	0.045	0.150
Gallic acid	–	2.33	–	4.83	0.384	1.280

Poland). The significance of differences between means was analyzed by ANOVA.

RESULTS AND DISCUSSION

Preliminary optimization studies showed that adding water to the extraction solution caused a steady increase of the concentrations of both acids, especially during refluxing, when the extraction time was extended. This suggested that hydrolysis of the tannins might occur to some extent during extraction with water or hydromethanolic solution, and that water is not a proper solvent for free EA and GA determination. Therefore we used methanol for the analyses. There was no difference in extraction efficiency between refluxing and mechanical shaking but we chose the latter as the less invasive method.

The maximum wavelengths of EA and GA UV spectra are 254 nm and 271 nm respectively; we chose 254 nm as the wavelength for the analyses as EA is much the more abundant and more important compound in the samples.

For total EA and GA determination water proved to be the best solvent. Extending hydrolysis time and increasing the acid concentration did not enhance the efficacy of the process so we chose the lowest of the tested acid concentrations (6 ml 25% HCl in 20 ml solvent) and the shortest extraction time (60 min) for hydrolysis.

The calibration curves for EA and GA showed good linearity over the test ranges, with r = 0.9999. The precision of the methods was good, with intra- and inter-day variation of less than 5% for all test samples. Recovery was 85–90% for all test samples (Tabs. 1, 2, Fig. 1).

Free EA content ranged from 0.43 mg/g (underground parts of *G. rivale*) to 0.57 mg/g (aerial parts of *G. urbanum*) (Tab. 3). It was slightly higher in the aerial than in the underground parts. The free GA content of aerial parts of *G. urbanum* was too low for quantitation and in the other plant materials was virtually undetectable (Tab. 3). In their screening of various *G. urbanum* populations, Kuczerenko et al. (2011) reported average 0.4 mg/g free EA and 0.3 mg/g GA in aerial parts of the plant, and 0.5 mg/g EA and 0.6 mg/g GA in underground parts. The relatively small differences between studies are probably explained by variability between plants growing in different habitats.

Total EA content ranged from 32.19 mg/g (underground parts of *G. urbanum*) to 60.64 mg/g (underground parts of *G. rivale*) and was about 6 times higher than total GA content, which ranged from 5.25 mg/g (underground parts of *G. urbanum*) to 9.57 mg/g (underground parts of *G. rivale*) (Tab. 3). This suggests that ellagitannins might be much more abundant in the *Geum* species we investigated. The content of both compounds was highest in underground parts of *G. rivale*. The same plant material has also been shown to contain the largest amounts of tannins (Owczarek and Gudej, 2013). Oszmiański et al. (2007) reported 2.70 mg/g EA and 0.33 mg/g GA in underground parts of *G. rivale* after hydrolysis. The proportion of the compounds is similar to our findings but the difference in values is conspicuous; probably it is due to the difference in extraction procedures.

Ellagitannins and gallotannins are known constituents of plants of the genus *Geum*. Their exact content in *G. rivale* and *G. urbanum* has not been comprehensively studied before. Our results show that *G. rivale* and *G. urbanum* are rich sources of EA, but mostly in the bound form. As hydrolyzable tannins easily undergo hydrolysis in the gastrointestinal tract (Clifford and Scalbert, 2000; Cerdá et al., 2005), its actual bioavailability could be quite substantial. EA is an important natural compound whose potent antioxidant properties may be benefi-

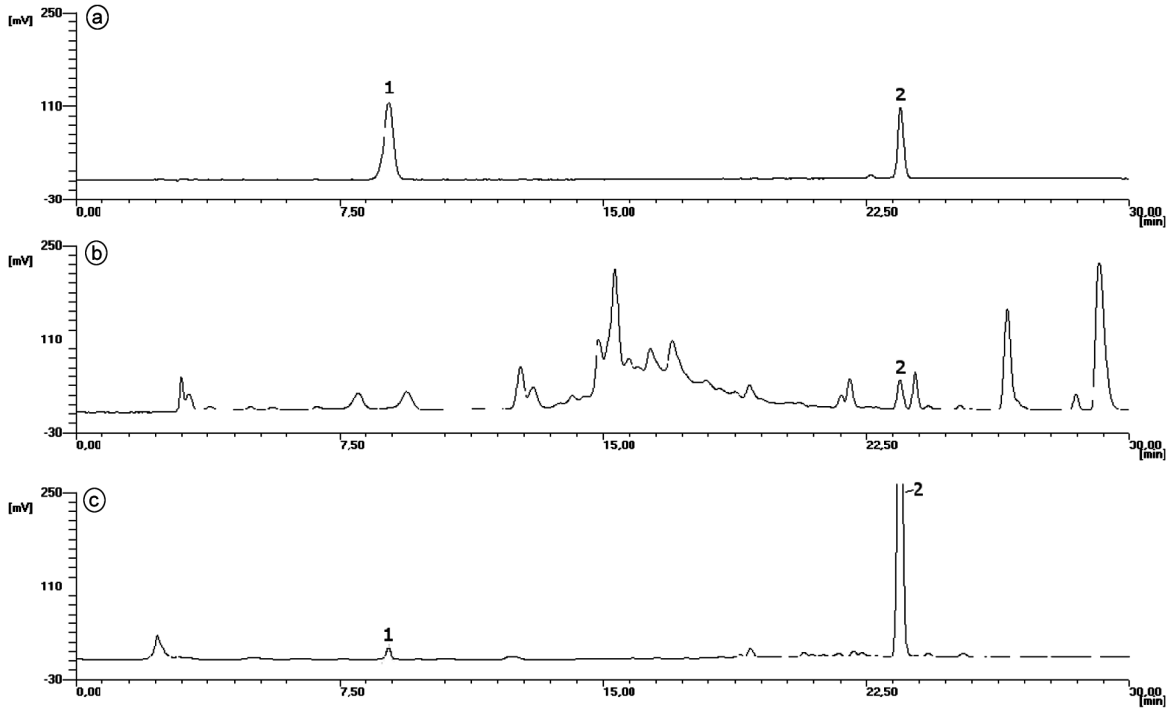

Fig. 1. Sample chromatograms: (**a**) Standards, (**b**) Extract from underground parts of *G. rivale* – determination of free EA and GA, (**c**) Extract from the underground parts of *G. rivale* – determination of total EA and GA. 1 – GA; 2 – EA.

TABLE 3. Free and total phenolic acids content

Constituent	Free phenolic acids content ± SD (mg/g)				Total phenolic acids content ± SD (mg/g)			
	Geum rivale		*Geum urbanum*		*Geum rivale*		*Geum urbanum*	
	aerial	underground	aerial	underground	aerial	underground	aerial	underground
Ellagic acid	0.52±0.010	0.43±0.002[a]	0.57±0.10	0.44±0.004[a]	40.31±1.08	60.64±0.87	46.71±0.51	32.19±0.50
Gallic acid	nd	nd	< LOQ	nd	7.45±0.08	9.57±0.27	8.35±0.29	5.25±0.11

[a] Means not significantly different ($p<0.05$); nd – not detected.

cial to human health, exerting cardioprotective, hepatoprotective and chemopreventive effects (Khanduja et al., 1999; Hwang et al., 2010; Kannan and Quine, 2012). The high content of this compound in the investigated plants may have a significant effect on their biological activity. GA is, like EA, a potent antioxidant, and also shows some antiviral activity (Choi et al., 2010; Giftson et al., 2010). Total GA is lower than total EA in the studied plant material but is still relatively high, making GA another potentially important factor in the activity of *G. rivale* and *G. urbanum*.

In traditional medicine the rhizome of *G. urbanum* is regarded as superior to other plant materials obtained from *G. urbanum* and *G. rivale* and is used more frequently (Gruenwald, 2000; Duke et al., 2002). Our results show that in terms of EA and GA content the rhizome of *G. rivale* is the most valuable material.

CONCLUSION

Plant material from *G. rivale* and *G. urbanum* is a rich source of EA and GA. Both compounds may have a strong influence on the biological activity of the plants.

AUTHORS' CONTRIBUTIONS

JG, AO study conception and design; AO acquisition of data; AO, MO analysis and interpretation of data;

AO drafting of manuscript; MO, JG critical revision of the manuscript.

All authors declare that there are no conflicts of interest.

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