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SEASONAL VARIATION IN PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF *GLECHOMA HEDERACEA* L. HARVESTED FROM SIX HUNGARIAN POPULATIONS

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Ground ivy (*Glechoma hederacea* L.) is one of the prosperous plants for the food-industry as natural antioxidant. This fact led us to examine the chemical diversity of six ground ivy populations situated in different natural habitats and to analyse the effect of the harvesting time. Total phenolic content, chlorogenic acid, and rutin content, as well as the antioxidant capacity showed significant differences due to the harvest time. The highest total phenol content (115 mg g^{-1} GAE) and the strongest antioxidant activity (53.3 mg g^{-1} AAE) were measured in the population originated from Budapest (GLE 6), harvested in July. The highest chlorogenic acid (357 mg/100 g) and rutin (950 mg/100 g) contents were detected in the July harvested samples from the Soroksár Botanical Garden population (GLE 1). According to our results, the collection time has significant effect on the total phenolic content – first of all on the chlorogenic acid and rutin accumulation levels of ground ivy, while the influence of the habitat seems to be less important.

Keywords: ground ivy, chlorogenic acid, rutin, vegetation time, habitat

Glechoma hederacea L. is a broad-leafed, creeping perennial plant, which is distributed in the temperate climate of the Northern Hemisphere. In the European folk medicine the flowering shoots and leaves were used as tonic and diuretic agent against gall or kidney stones (Grieve, 1976). Many studies highlight the significant antioxidant effect of its herbal extract (Matkowski, 2008; Barros et al., 2010). Milovanovic and co-workers (2010) proved a concentration dependent antioxidant activity in pork lard treated with ground-ivy alcoholic extract.

Among the bioactive compounds, chlorogenic acid (Belščak-Cvitanović et al., 2011), rosmarinic acid (Matkowski, 2008; Döring & Petersen, 2014; Xie et al., 2014), flavonoids as apigenin, luteolin, quercetagetin, rutin (Yamauchi et al., 2007; Xie et al., 2014), ascorbic acid and α-, β-, γ-, δ-tocopherols (Barros et al., 2010) have been isolated from these species.

In Europe the raw material of ground ivy is still collected from the wild populations. Information on the plant material, concerning habitat, location, optimal harvesting time, is practically incomplete. In the genus, the effect of these factors has only been studied in the closely related *Glechoma longituba* species: Liu and co-workers (2012) studied 29 different

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populations in China, and found significant differences among them concerning total flavonoid, oleanolic acid, and ursolic acid contents.

In addition to this, there are no universally accepted standards for the raw material and drug quality of ground ivy in Europe, although some national specifications exist. According to the Hungarian specification for drug quality (Hungarian Standard, 1967), flowering shoots of the plant should be collected in April–May; however, no scientific proof has ever been published in this respect.

The aim of our study was to investigate the variation in total phenol content, antioxidant capacity, and the main compounds of the phenoloid fraction related to the harvesting time in the water extracts of *Glechoma hederacea*. To detect the effect and eventual differences among the wild populations, six different locations have been included in the study.

1. Materials and methods

1.1. Plant material and water extraction

The aerial parts of *Glechoma hederacea* were collected from six remote Hungarian habitats in three different times in 2012. Flowering shoots were cut in April, while collection of two further samples (only the leaves) was carried out in July and October. The locations of the populations are indicated in Figure 1. The population GLE 1 was situated in an open site, on sandy soil, exposed to the sun, surrounded by pine trees in the Soroksár Botanical Garden, Budapest. The population GLE 2 was found in a semi-shaded place, on clay soil, in the Vácrátót Botanical Garden. Population GLE 3 was located in an open site area, on clay soil, near the city Tatabánya. Population GLE 4 was situated in a semi-shaded site near to a cemetery in Várvölgy on clay soil, while the natural habitat of population GLE 5 was located in a semi-shaded meadow, characterized by sandy soil, near to the village Kunadacs. The plant stand GLE 6 grew in an open-site park in Budapest, characterized by clay soil. For each location the average temperature, sum of precipitation, and hours of full illumination data for the period 4 weeks before are shown the Tables 1, 2, 3.

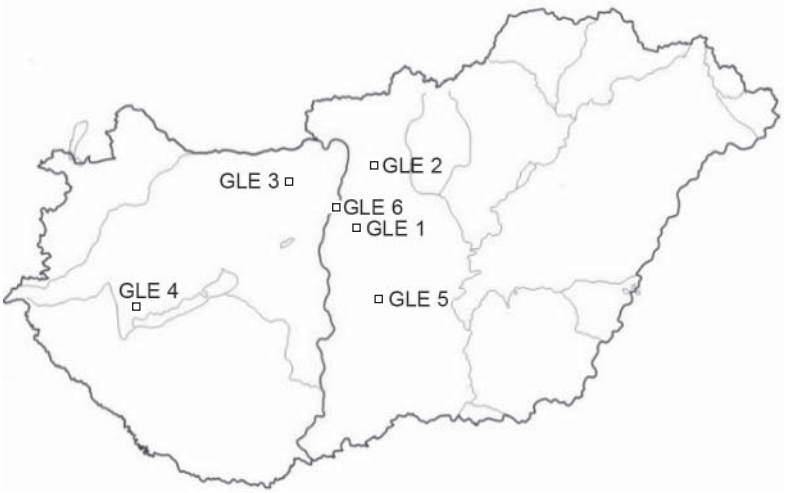

Fig. 1. Location of the studied *Glechoma hederacea* L. populations

Table 1. Average temperature of 4 weeks before collecting (°C)

	GLE 1	GLE 2	GLE 3	GLE 4	GLE 5	GLE 6
April	12.4	11.8	11.9	11.5	12.2	13.1
July	23.9	22.9	22.6	22.5	23.8	24.4
October	12.4	10.9	11.2	12.1	11.9	13.5

Table 2. Sum of precipitation of 4 weeks before collecting (mm)

	GLE 1	GLE 2	GLE 3	GLE 4	GLE 5	GLE 6
April	21	21	37	27	22	22
July	80	49	80	64	28	57
October	62	61	59	78	81	59

Table 3. Sum of fully illuminated hours of 4 weeks before collecting (h)

	GLE 1	GLE 2	GLE 3	GLE 4	GLE 5	GLE 6
April	211	203	196	201	194	200
July	282	269	230	275	297	272
October	160	155	121	115	160	157

The identification of the plant species was carried out according to the description of Simon (2000). After collection, the plant material was immediately dried in a plate chamber dryer at 45 ºC. The drug was powdered; 1 gram was infused with 100 °C distilled water. After 24 h, the extracts were filtered and stored in a freezer until analysis. For the determination of the dry matter content of the extracts, 20 ml was heated in a drying chamber at 105 ºC for 3 h.

1.2. Chemicals

Folin-Ciocalteu reagent, gallic acid, the tripyridyl-*s*-triazine, and for the HPLC analysis crystalline reference substances of chlorogenic acid (CGA) and rosmarinic acid (RA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Rutin was obtained from Carl Roth KG (Karlsruhe, Germany). HPLC-grade acetonitrile, formic acid, and methanol were purchased from Merck (Merck, Darmstadt, Germany). A Milli-Q ultrapure water system (Merck Millipore, Billerica, MA, USA) was used throughout the study to obtain high purity water (18 mΩcm) for the HPLC analysis. All other solvents and reagents were obtained from Reanal Ltd. (Budapest, Hungary).

1.3. Determination of total phenol content and investigation of the total antioxidant capacity

The total phenol content (TPC) was determined by the modified method of Singleton and Rossi (1965). Sample solution of 0.5 ml was introduced into a test tube and then 2.5 ml Folin-Ciocalteu's reagent (10%, v/v) was added. After incubation for 1 min, 2 ml of sodium carbonate (0.7 M) were added. The absorbance was measured at 760 nm after incubation for 5 min in hot water (50 °C). Gallic acid (0.3 M) was used as chemical standard for calibration.

The results were expressed as mg of gallic acid equivalents per g dry material (mg g^{-1} GAE). The measurements were carried out in three replications.

Determination of the total antioxidant capacity was done by using the FRAP method (Benzie & Strain, 1996). FRAP reagent was prepared by mixing 10 volumes of 300 mmol l^{-1} acetate buffer, pH 3.6, with 1 volume of 10 mmol l^{-1} TPTZ (2,4,5-tripyridyl-*s*-triazine) in 40 mmol l^{-1} hydrochloric acid and with 1 volume of 20 mmol l^{-1} ferric chloride. In a reaction tube, 5 µl sample solution was added to 2.5 ml FRAP reagent. Absorbance was measured after 5 min at 596 nm. Results were expressed in mg ascorbic acid equivalent per g of dry material (mg g^{-1} AAE). All measurements were carried out in three replications.

1.4. HPLC analysis

The extracts were filtered through a 0.22 µm PTFE membrane before injecting 10 µl into the HPLC. For standard solutions individual stock solutions (1 mg ml^{-1}) of rosmarinic acid (RA), chlorogenic acid (CGA), and rutin were prepared in methanol and stored at –4 ºC protected from light. A stock standard mixture was prepared in methanol with the final concentration of 250 µg ml^{-1} for each compound. Working standard solutions were prepared by dilution from the stock standard mixture.

The mass spectrometric identification of RA, CGA, and rutin was based on the method previously developed by Abrankó and co-workers (2012). The identification was carried out using HPLC system including a diode array detector (DAD) coupled to an Agilent (Santa Clara, CA, USA) 6530 quadruple – time-of-flight mass spectrometer (q-TOFMS), which was equipped with a dual spray ESI source.

Analysis of phenolic compounds was performed using a Waters Alliance high performance liquid chromatography (HPLC) system equipped with photodiode array detector (PDA) together with a quaternary pump, an auto-sample injector, an on-line degasser, and an automatic thermostatic column oven (Waters Corp., Milford, MA, USA). Chromatographic separation was carried out on a Phenomenex Kinetex Phenyl-hexyl, 4.6×150 mm, 2.6 µm column (Torrance, CA, USA). For the elution, 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B) were used as solvents at a flow rate of 500 µl min^{-1}. The gradient program started at 10% B, and after 5 min of isocratic run, solvent B was increased linearly and reached 45% at 35 min and then 100% at 40 min. Finally, 100% B was kept constant for 5 min. Detection wavelength was 330 nm. The sample injection volume was 10 µl. The chromatographic peaks of RA, CGA, and rutin were confirmed by comparing their retention times and UV spectra with those of their reference standards.

1.5. Statistical analysis

The results are presented as mean values and standard deviations (SD). Data were analysed by the program STATISTICA 10 using multivariate analysis of variance (MANOVA) by Tukey's HSD test (α=0.05) for checking the effects of habitat and harvest time on chemical properties. the homogeneity of variance was clarified with Brown–Forsythe test. A level of $P<0.05$ was used as the criterion for statistical significance.

2. Results and discussion

2.1. Total phenol content

The total phenol content (TPC) of the samples can be seen in Figure 2. TPC levels showed similar changes due to the different collecting times in each population. The highest value was observed in the summer (July) collected GLE 6 sample (109.8±5.8 mg g^{-1} GAE), while the lowest ones were detected in the extracts of the autumn (October) harvested samples of populations GLE 3–6 (with the average of 43.9±3.2 mg g^{-1} GAE). However, even these results exceeded the maximum levels (25 mg g^{-1} GAE) detected by Belščak-Cvitanović and co-workers (2011) in the water extracted ground ivy samples.

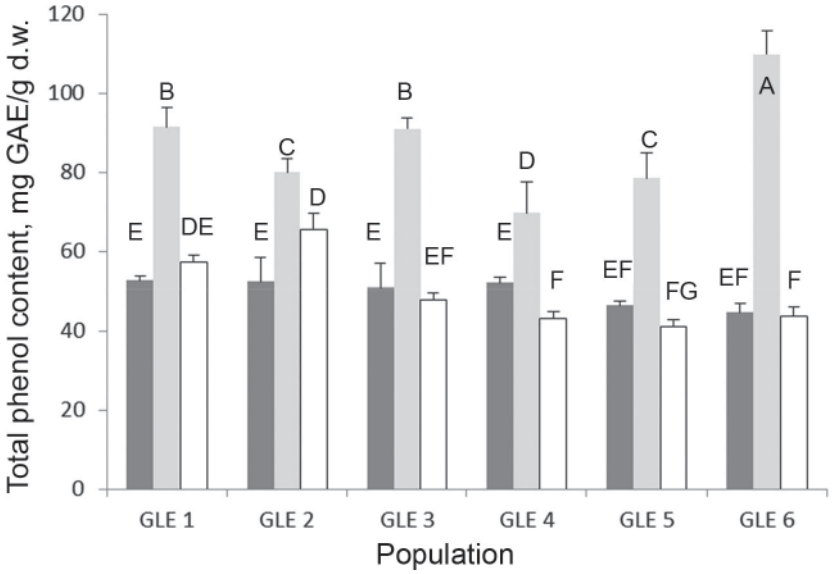

Fig. 2. Total phenol content (TPC) of the extracts of examined populations at different harvesting times. Different letters show significant differences ($P<0.05$) ■: April; ▨: July; □: October

Each value of the July collection time was significantly higher than the April and October ones. Significant differences among the habitats were found only in these samples. The mean value of the GLE 6 population was twice higher than in the GLE 4 population. However, no statistical difference could be found among the populations considering the samples collected in April and October. Comparing with the meteorological data in the way of temperature, we can observe that results are fluctuating more with the season as with the population. Through the illumination, the location and season together affect the TPC content. In the case of walnut (*Juglans regia*) (Solar et al., 2006; Cosmulescu & Trandafir, 2011) and tea (*Camellia sinensis* var. *sinensis*) (Erturk et al., 2010), the authors came to the conclusion that the light and the length of the illumination period may effectively stimulate the biosynthesis of phenolic compounds. According to this, the balance between the April values can be explained by the undeveloped surrounding plants that give later shade to the populations. In July they

are fully developed and that could cause the significant differences between the populations. The highest values can be detected in the populations located in the open sites (GLE 1; GLE 3; GLE 6). The differences by October values can be related with the defoliation level of the deciduous trees surrounding the populations. The precipitation was so diverse that no correlation could be found with the results.

2.2. Antioxidant capacity

The antioxidant capacities (AOC) of the water extracts are shown in Figure 3**.** Similarly to the TPC outcomes, the values of the summer (July) collection were higher than the spring (April) and autumn (October) ones. Significant interaction could be detected between the harvest time and the habitat. The strongest antioxidant capacity was observed in the GLE 2, GLE 4, GLE 5 and GLE 6 samples collected in summer (July) (varying between 48.69 and 53.06 mg g^{-1}AAE). The lowest value was detected in the autumn (October) collected sample of the GLE 1 population (7.88±1.56 mg g^{-1}AAE).

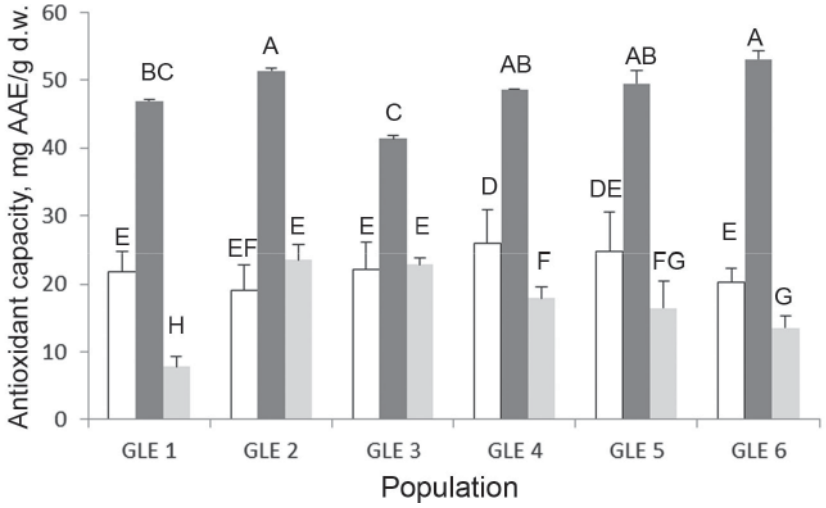

Fig. 3. Antioxidant capacity (AOC) of the extracts of examined populations at different harvesting times. Different letters show significant differences (P<0.05) □: April; ▒: July; ■: October

The connection between TPC and AOC values seems to be questionable, as a strong positive correlation was detected in three of the six investigated populations GLE 2 (r=0.800), GLE 5 (r=0.930), and GLE 6 (r=0.923), and only in case of the summer collections. Evaluation of all measurements did not show significant correlation (Table 4). This observation is in correspondence with former references. According to Kahkonen and co-workers (1999) and Kouřimská and co-workers (2014), the rate of antioxidant capacity does not necessarily correlate with total phenol content. Presumably other vitamin components like tocopherols can contribute to the strong antioxidant activity of ground ivy extracts.

Table 4. Results of correlation analysis of the TPC and AOC contents at different harvesting times based on all measurements

April			July			October		
	TPC I	AOC I		TPC II	AOC II		TPC III	AOC III
TPC I	1		TPC II	1		TPC III	1	
AOC I	–0.015	1	AOC II	0.144	1	AOC III	0.153	1

2.3. Chlorogenic acid, rutin, and rosmarinic acid content

Chlorogenic acid (CGA) was present in the majority of the extracts (Table 5). It could be detected in 34 out of the 36 investigated samples. In previous works in the case of flowering shoot, Dadáková and co-workers (2010) could not detect CGA in water extract, while Belščak-Cvitanović and co-workers (2011) reported a level of 1.30 μg g^{-1} (130.00 mg/100 g) in samples originating from commercial trade in Croatia.

Table 5. Chlorogenic acid (CGA) and rutin contents of the extracts of examined populations at different harvesting times

	April		July		October	
	CGA (mg/100 g)	Rutin (mg/100 g)	CGA (mg/100 g)	Rutin (mg/100 g)	CGA (mg/100 g)	Rutin (mg/100 g)
GLE 1	9.54±0.75g	n.d.	345.8±15.4a	929.6±29.5A	8.05±0.23g	n.d.
GLE 2	2.09±1.24h	n.d.	188.8±9.1c	197.9±10.25B	3.31±0.34h	n.d.
GLE 3	7.25±3.08gh	n.d.	293.5±12.2b	182.4±16.0B	4.55±0.61h	n.d.
GLE 4	5.39±0.58h	n.d.	36.0±7.6de	n.d.	2.78±0.65h	n.d.
GLE 5	10.30±0.71g	n.d.	23.4±5.93ef	n.d.	0.180±0.085i	n.d.
GLE 6	4.86±0.21h	n.d.	50.1±11.2d	37.30±4.12C	n.d.	n.d.

Different letters show significant differences ($P<0.05$); n.d.: not detected

Highest level of chlorogenic acid was found in GLE 1 sample collected in July (356.7 mg/100 g). The mean values of the samples collected in July exceeded the values of the ones collected in April or October. This could be related with the high solar radiation in summer, because other studies (Zucker, 1965; Percival & Baird, 2000) highlighted that the light may enhance the level of CGA in ground ivy plants and the increased accumulation level may correlate with the supposed function of CGA as UV-protectant (Clé et al., 2008; Döring & Petersen, 2014) as in other plants. The concentrations of both chlorogenic acid and rutin varied on a large scale (2.08–293.5 mg/100 g for CGA and 5.73–929.6 mg/100 g for rutin) depending on population and harvesting time. In three July collected samples the third main phenoloid compound, rosmarinic acid (RA) was also detected. These populations were GLE 1 (148.4 mg/100 g), GLE 2 (66.6 mg/100 g) (Fig. 4), and GLE 3 (92.5 mg/100 g). However, RA was missing in all other samples.

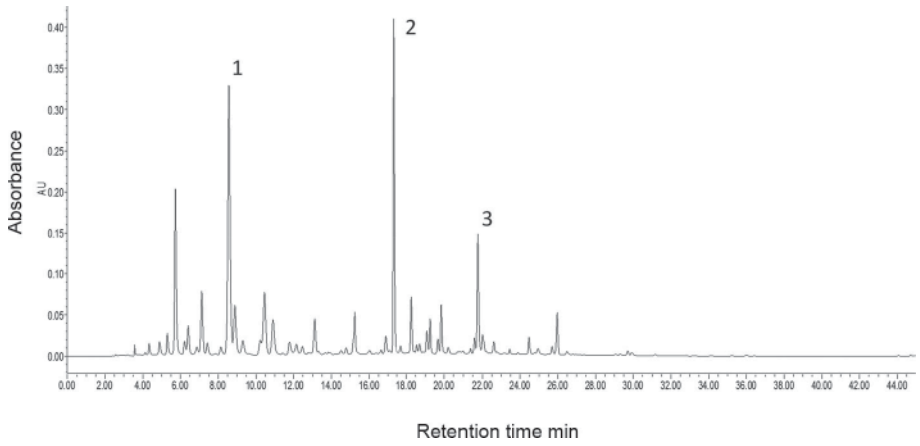

Fig. 4. HPLC chromatogram (at 330 nm) of the water extract of GLE3 sample collected in July

3. Conclusions

By the accumulation of TPC, chlorogenic acid, and rutin significant differences among populations appear only in July, which shows the effect of the environment. From the three meteorological factors, temperature and illumination may affect the level of phenolic substances in the ground ivy. Nevertheless, data indicate that the growing habitat might also have an influence on the content of phenolics in the drug. According to our results, harvesting time seems to be a more important factor in the accumulation than location.

Based on our results, the recommended harvest time for ground ivy shoots is the midsummer period.

Among the investigated locations, open sites exposed to sunlight, such as the meadow around the city Tatabánya, seem to be more advantageous for collecting good quality raw material. Although the public park of Budapest had good results too, a site like this cannot be recommended for collecting due to the danger of heavy metal contamination and other pollution.

We suggest further studies to clear up the role of the genotype and differences of the potential of phenoloid accumulation in ground ivy.

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