SEASONAL VARIATION IN PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF GLECHOMA HEDERACEAE L. HARVAESTED FROM SIX HUNGARIAN POPULATIONS

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Abstract:
Ground-ivy (Glechoma hederacea L.) is one of the prosperous plants for the food-industry as an natural antioxidant. This fact led us to examine the chemical diversity of six ground-ivy populations situated in different natural habitats and to analyze 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 (114.95 mg/g GAE) and the strongest antioxidant activity (53.28 mg/g AAE) were measured in the population originated from *Budapest* (GLE 6), harvested in July. The highest chlorogenic acid (356.71 mg/100g) and rutin (950.38 mg/100g) 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 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

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Glechoma hederaceae (L.) is a broad-leafed, creeping perennial plant which is distributed in 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 AND 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 this 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 frequently 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 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 (MSZ 19885:1967) 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 detected in a semi-shaded site near to a cemetery in Várvölgy on clay soil, while the natural habitat of population GLE 5 was 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 by the **Table 1, 2,3**.

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 hours, 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 on 105 ºC for 3 hours.

1.2. Chemicals

Folin-Cicocalteau reagent, gallic acid, thetripyridyl-s-triazine, 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 (18mΩ.cm) 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-Cicocalteau's reagent (10% v/v) was added. After incubation of 1 min, 2 ml of sodium carbonate (0.7 M) was 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 GAE). The measurements were carried out in three replications.

Determination of the total antioxidant capacity was done by using the FRAP method (BENZIE AND STRAIN, 1996). FRAP reagent was prepared by mixing 10 volumes of 300 $mmol^{-1}$ aceatate buffer, pH 3.6, with 1 volume of 10 mmol/lTPTZ (2,4,5-tripyridyl-s-triazine) in 40 mmol/l hydochloricacid and with 1 volume of 20 mmol/l ferric chloride. In a reaction tube, 5 µl sample solution was added to 2.5 ml FRAP reagent. Absorbance was measured after 5 min. on 596 nm. Results were expressed in mg ascorbic acid equivalent per g of dry material (mg/g 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 to the HPLC. For standard solutions individual stock solutions (1 mg/ml) of rosmarinic acid (RA), chlorogenic acid (CGA) and rutin were prepared in methanol and stored protected from light,

at -4 ºC. 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. 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 analyzed 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 (109.818±5.826 mg/g 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 43.919±3.155 mg/g GAE). However, even these results exceeded the maximum levels (25 mg/g GAE) detected by BELŠČAK-CVITANOVIĆ AND CO-WORKERS (2011) in the water extracted ground ivy samples. 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 two times 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. By the illumination the location and season together affect the TPC content. In the case of walnut (*Juglans regia*) (SOLAR ET AL., 2006; COSMULESCU AND 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 explain 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 detect by the populations located in the open sites (GLE1;GLE3;GLE6). The differences by

October values can be related with the defoliation level of the deciduous trees surrounding the populations. The participation where so diverse that no correlation can be found with the results.

2.2. Antioxidant capacity

The antioxidant capacity (AOC) of the water extracts is shown on **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, GLE4, GLE 5 and GLE 6 samples collected in summer (July) (varied between 48.685 and 53.063 mg/g AAE). The lowest value was detected in the autumn (October) collected sample of the GLE 1 population (7.883±1.560 mg/g AAE).

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 KAHKONEN AND HIS CO-WORKERS (1999) and KOUŘIMSKÁ AND HIS 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.

2.3. Chlorogenic acid, rutin and rosmarinic acid content

Chlorogenic acid (CGA) was present in the majority of the extracts (**Table 5**.). We could detect it in 34 samples out of the 36 investigated ones. 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 (130.00 mg/100g) in samples originating from commercial trade in Croatia.

Highest level was found in July collected GLE 1 sample (356.70 mg/100g). 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 AND 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 AND PETERSEN, 2014) as in other plants.The concentrations of both chlorogenic acid and rutin varied on a large scale (2.08-293.45 mg/ 100g for CGA and 5.73-929.55 mg/100g 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 (148.41 mg/100) GLE 2 (66.63 mg/ 100g) (**Figure 4.**), and GLE 3 (92.52 mg/100g). However, RA was missing in all other samples.

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 factor 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, the harvesting time seems to be a more important factor in the accumulation as the location.

Based on our results, the recommended harvest time for ground ivy shoots is the mid summer period. During the process better to collect in the open air places.

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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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 full illumination hours of 4 weeks before collecting (hours)

	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

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

April		
	TPC I	AOC I
TPC I	1	
AOC I	-0,015	1

July		
	TPC II	AOC II
TPC II	1	
AOC II	0,144	1

October		
	TPC III	AOC III
TPC III	1	
AOC III	0,153	1

Table 5. Chlorogenic acid (CGA) and rutin contents of the extracts of examined populations at different harvesting times
Different letters show significant differences ($p < 0.05$)

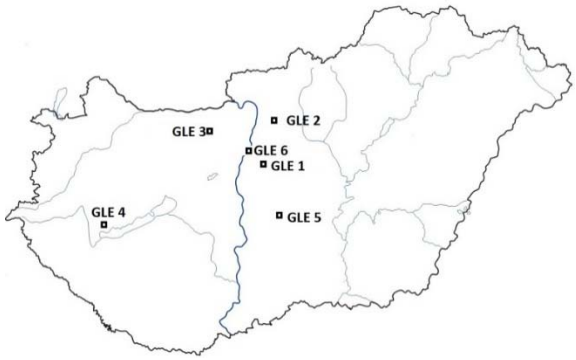
	April		July		October	
	CGA (mg/100g)	Rutin (mg/100g)	CGA (mg/100g)	Rutin (mg/100g)	CGA (mg/100g)	Rutin (mg/100g)
GLE 1	9.540±0.750g	n.d.	345.805±15.408a	929.550±29.458A	8.050±0.226g	n.d.
GLE 2	2.085±1.237h	n.d.	188.800±9.051c	197.925±10.204B	3.312±0.338h	n.d.
GLE 3	7.245±3.076gh	n.d.	293.450±12.233b	182.400±15.952B	4.550±0.608h	n.d.
GLE 4	5.390±0.580h	n.d.	36.020±7.608de	n.d.	2.780±0.651h	n.d.
GLE 5	10.300±0.707g	n.d.	23.390±5.926ef	n.d.	0.180±0.085i	n.d.
GLE 6	4.860±0.212h	n.d.	50.085±11.151d	37.295±4.122C	n.d.	n.d.

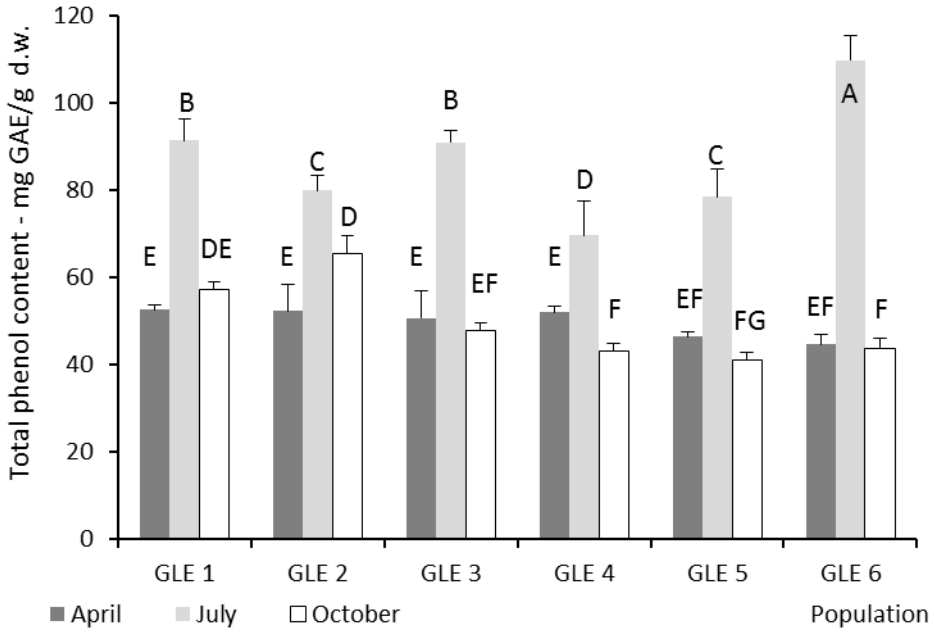
Figure 1: Location of the studied Glechoma hederacea L. populations

Figure 2: Total phenol content (TPC) of the extracts of examined populations at different harvesting times
Different letters show significant differences ($p < 0.05$)

Figure 3: Antioxidant capacity (AOC) of the extracts of examined populations at different harvesting times
Different letters show significant differences ($p < 0.05$)

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




Total phenol content - mg GAE/g d.w.
120
100
80
60
40
20
0
E
B
DE
E
C
D
E
B
EF
E
D
F
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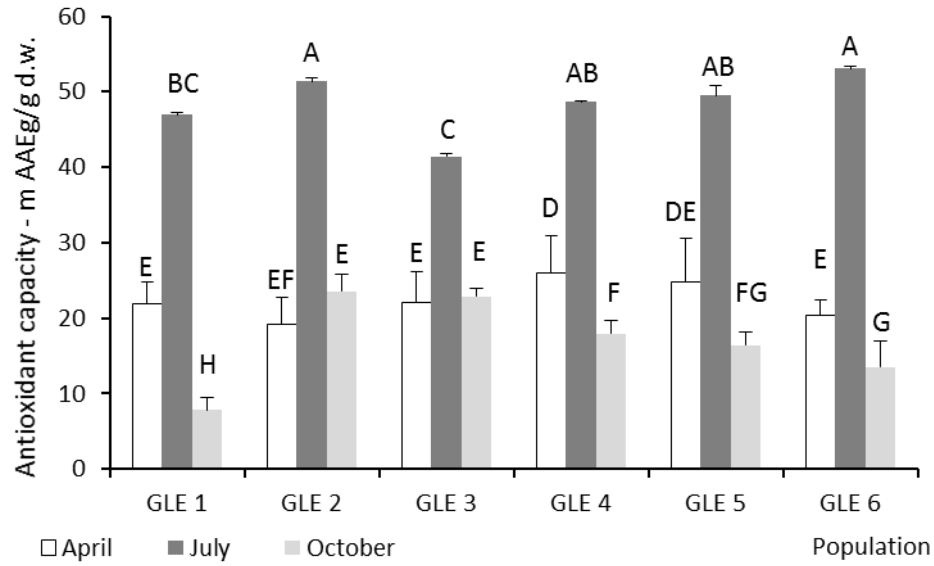
FG
EF
A
F
GLE 1
GLE 2
GLE 3
GLE 4
GLE 5
GLE 6
April
July
October
Population


Antioxidant capacity - m AAEg/g d.w.
60
50
40
30
20
10
0
E
BC
H
EF
A
E
E
C
E
D
AB
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FG
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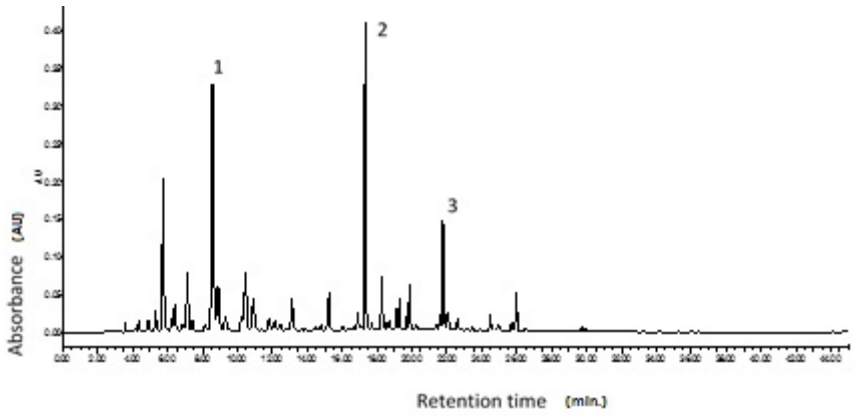
GLE 1
GLE 2
GLE 3
GLE 4
GLE 5
GLE 6
April
July
October
Population


1
2
3
Absorbance (AU)
Retention time (min.)