SZENT ISTVÁN UNIVERSITY

FACULTY OF HORTICULTURAL SCIENCE

MORPHOLOGICAL AND CHEMICAL DIVERSITY OF GROUND-IVY (*Glechoma hederacea* L.) COLLECTED IN HUNGARY.

PhD THESIS

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1. BACKGROUND AND OBJECTIVES

Nowadays the use of natural plant-based raw materials are increasing by the pharmaceutical and other industries. Worldwide the public interest in natural therapies has increased greatly. The use of traditional medicine are not limited to developing countries these have grown greatly in industrialized countries as well (WHO, 2011 in BERNÁTH, 2013). Due to this, medicinal plants that where used in folk medicine long ago but has now disappeared from the use, occur more frequently in products. The ground-ivy belongs to this group (*Glechoma hederacea* L.), which has been frequently examined over the past decade from different aspects.

In Europe the raw material of this species is still collected from the wild populations. Information on the plant material, concerning habitat, location, optimal harvesting time is practically incomplete. 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. In national aspect there aren't any information in terms of morphological characteristics and content of the species. In addition, the fact that the ground-ivy is ecologically adaptable, definitely is a positive starting point for the cultivation attempts to base. A detailed examination can help raise interest of farmers and processors about this currently neglected domestic flora with great potentials of usage.

Aims of our studies:

- Understanding the biological diversity of spontaneous species flocks of various origins by complex analysis with respect of morphological, production and nutritional characteristics.
- Establishment of cultivation involving the factors affecting the production and the quality with drug testing, particularly focusing on the year (weather conditions) and the effect of taxa (in the same habitat are sown).
- Development of cultivation and processing technology (seed storage, methods of stimulating germination, active agent extraction methods).

In order to achieve this, we conducted field and laboratory experiments for three years. With our work we want to enrich the literature with new data and publish internationally comparable results to contribute a growing potential, practical implementation and the quality of raw material production of the ground ivy.

2. MATERIALS AND METHODS

Wild populations: The aerial parts of *Glechoma hederacea* were collected from six remote Hungarian habitats - Soroksár (SBK), Vácrátót (VBK), Tatabánya (TAT), Várvölgy (VÁR), Kunadacs (KUN), Budapest (BUD), Nagykovácsi (NK), - in three different times in 2012, 2013 and 2014. Flowering shoots were cut in April, while collection of two further samples (only the leaves) was carried out in July and October.

Cultivated populations: From the wild growing populations cultivated ones where created in the experimental field of the Department of Medicinal and Aromatic Plants. Samples where collected three times in 2012 (April, July and October). In 2013 and 2014 only in April.

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 at 105 ºC for 3 hours.

2.1. Morphological features of the populations

- length of the hole flowering stems (cm)
- length of the flowering part (cm)
- length of the internode (cm)
- number of the nodes
- number of the flowering nodes

2.2. Germination tests

The seeds were purchased from Jelitto company in 2011. In 2013 their viability was 30% determined by TTC Test. The seeds were stored at +23°C and +4°C for 27 months. In every 3 months samples were taken and treated with germination stimulants - seeds were soaked in 200 or 1000 ppm gibberellic acid (GA_3) solution for 24 hours, or washed with 0.2% potassium nitrate (KNO_3)-. After this they were placed into Petri dishes (50-50 pieces seeds) with filter paper and transferred to a Sanyo MLR-351 Environmental Test Chamber providing visible radiation at flux of 5000 lux (170 µmol/m^2/s) over 8 hours/day with 30°C and night temperature was 20°C.

2.3. Laboratory analysis

Preparation of aqueous and alcoholic extracts:

The dried and powdered drug (1g) was infused with 100 °C distilled water (100 ml) and was left to stand for 24 hours. Then the extracts were filtered, and until the analysis they were stored in a freezer. In the case of alcoholic extracts the drug (1g) was extracted by ethanol (20%) and was left to stand for 72 hours. Then the extracts were filtered and stored in freezer until the chemical experiments. All measurements were done in three replications.

Determination of essential oil content:

It was analyzed by Clevenger type hydrodistillation according to the PhHg VII, its amount was given in ml/100 g dry material.

Determination of essential oil composition

The identification of the samples essential oil composition were carried out by GC-MS-method based on mass spectra, with the help of mass spectra of librarian references (NIST, Wiley and own essential library), and the calculation of their linear retention indexes.

Determination of total polyphenol content

The total polyphenol content were determined by the modificated method of SINGLETON and ROSSI (1965). The reaction indicator blue colour's intensity was determined photometrically at λ=760 nm. The results were determined to GAE mg/g d.w.. 3 replicates were used in each measurement in both years.

Determination of FRAP antioxidant activity

For the determination of the total antioxidant activity from the dried plant material the modified methodology of BENZIE and STRAIN (1966) was used. The purple colourisation was detected by spectrophotometer at λ=593 nm. Measurements were carried out in 3 replicates in both years. The results were provided in ascorbic acid equivalence (mg AAE/100g dw).

Analysis of rosmarinic-, chlorogenic acid and rutin content

The mass spectrometric identification of the compounds 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.

2.4. Statistical analysis

Data were analyzed by the program IBM SPSS Statistics 21. If the criteria of parametric probes were met, one way or multiple factor analysis of variance were carried out depending on the number of factors. For the pairwise comparisons of the variances – if the homogeneity of deviation was accepted - the Tukey HSD post hoc test was used. If the homogeneity of deviation did not meet for the pairwise comparison Games-Howel test was used.

3. RESULTS

3.1. Morphological characteristic

By all the observed morphological characteristics we found significant differences in habitat and taxa ($p = 0.0001$), the year ($p = 0.0001$) and their interaction ($p = 0.0001$). The flowering shoots average length between 10 and 30 cm was achieved, including the inflorescence length varied between 5 and 15 cm. The stems are divided up to 4 to 8 parts by the nodes, among which from the shoot tip develops flowers and it is calculated peak drives from 2 to 6 nodes.

3.2. Germination characteristic

Detectable difference was found in the storage time ($p = 0.0001$), the temperature ($p = 0.0001$) and the combination of the two effects ($p = 0.0001$) as well. At the start of storage, germination of the seeds was 78%, which decreased at the end of the experiment. After 3 and 6 months after the entry into store to store samples of both treatments had germinated poorly but still above 60% germination was measured.

We calculated significant differences in the case of the germination stimulants ($p = 0.0001$). During the test, the KNO3 solution treated seeds retained the germination rate. By the control we observed a minor decreasing (-4%) but in the case of gibberellic acid the values where significantly lower (-18%).

3.3. Essential oil composition

The essential oil content in the flowering shoots of the examined ground ivy population varied between 0.053 and 0.054 ml / 100g (d.w.). We identified most abundant sesquiterpene type compounds (min. 65%) in both wild and cultivated plants. The main component was germacrene-D (39.78 to 61.84%). The rate of plant parts has changed due to the production sites and the growing involvement, but always remained as the main component.

3.4. Total polyphenol content

Statistically significant difference was found in aqueous and alcoholic extraction methods ($p = 0.0001$) as in the aspect of the year ($p = 0.0001$). In each cases the higher values (49,78 - 77,58 GSE mg / g d.w.) was measured in the aqueous extracts.

During the examination of the various parts of the flowering shoots of both wild and cultivated plants, we have found that in the case of ground-ivy higher levels of phenolic compounds accumulate in the flower (150.00-185.00 mg GAE/g) and the leaf (110.00-145.00 mg GAE/g), and a lower in the stem (40.00-80.00 GAE mg/g). The total phenolic content of the wild populations inflorescence ranged between 41.44 and 108.30 GSE mg/g for the years under review. With the exception of data from the population from Budapest, we calculated significantly higher outcome in 2013 than in the previous year. These higher values originate from the fact that more flower and leaf pair developed which are characterized by high total phenol content. In the different collection time we got the opposite results with respect to the two years.

3.5. Chlorogenic acid, rutin, and rosmarinic acid content

Chlorogenic acid (CGA) was present in the majority of the extracts. Highest level of chlorogenic acid was found in VBK 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. 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 phenolic compound, rosmarinic acid (RA) was also detected. These populations were VBK (148.4 mg/100 g), GLE 2 (66.6 mg/100 g) (Fig. 4), and TAT (92.5 mg/100 g). However, RA was missing in all other samples.

3.6 Total antioxidant capacity:

Statistically significant difference was found in aqueous and alcoholic extraction methods ($p = 0.0001$) and in the aspect of the year ($p = 0.0001$). In each cases the higher values (20,14–40,49 mg ASE/g d.w.) was measured in the aqueous extracts.

During the examination of the various parts of the flowering shoots of both wild and cultivated plants, we have found that in the case of ground-ivy the stem has the lowest antioxidant capacity (8,53-61,09 mg ASE/g). Between the wild populations, significant differences were measured both in terms of habitat ($p = 0.0001$), years ($p = 0.0001$). For most habitats, the highest values were measured (ASE 50.41 to 64.62 mg/g) in the year 2013. We found similar results in the cultivated plants.

New scientific results

During four years of our research work we collected data from 7 Hungarian natural ground-ivy population, and 8, in the Soroksár Experimental Station raised plants. Based on the results achieved by the following new scientific results:

1. With the analyze of the Hungarian wild populations of ground-ivy we found that:
   - average length of the flowering shoots vary between 10 and 30 cm, the inflorescence length varied between 5 and 15 cm. The stems are divided up to 4 to 8 parts by the nodes,
   - the populations show morphological differences. This manifests itself in the length of the flowering shoots and inflorescent as in the number of the flowering buds. The average coefficient of variation value was 25%,
   - the chlorogenic acid and total phenolic content of the populations differ significantly with about 1.6-fold as well as the antioxidant capacity.

2. We defined first time the factors that significantly affect the properties and phenolic components accumulation in ground-ivy. Thus, we found that:
   - weather conditions are able to significantly influence the morphological properties and the total phenolic components of the ground-ivy,
   - the chlorogenic acid, total phenolic content and the antioxidant capacity differ statistically in the growing period, but the dynamics may change annually,
   - the distribution of the active agents differ significantly in various parts of the flowering shoots. Smallest amount is in the stem (40- 80 mg GSE/g) highest in the flowers (150-180 mg GSE/g).

   The effect of the geological location was determined to be of paramount importance. We found significant differences in every attribution by the wild and cultivated plants:

   - length of flowering shoot reduce with the average of 49.82 percent,

   - length of inflorescence reduce with the average of 44.81 percent,

   - length of the internodes reduce with the average of 47,32 percent,

   - the total phenol content increased (130-150%) as well as the antioxidant capacity(110-160 %).

3. We characterized first time in Hungary the composition of essential oil of the ground-ivy wild populations, and we found that:

- in the essential oil of flowering shoots, the sesquiterpene type compounds accumulate mainly (more than 70%),
- the main component of germacrene-D, which at least 25% is present in the essential oil,
- the environmental factors and habitat can influence the germacrene-D content.

4. By improving of cultivation technique in the aspect of seed germination we conclude:

- the storage temperature has a significant effect to the seed germination rate. For long term storage (more than 18 months) seed should be keep under +4 °C,
- the ground-ivy seed germination can be enhanced with 0,2 % KNO_3 solution,

5. By improving processing technique according to different extraction methods we conclude:

- aqueous extracts have significantly higher total phenolic content and antioxidant capacity as the alcoholic.

4. CONCLUSIONS AND PROPOSALS

It was the first time when the Hungarian wild populations of ground-ivy were analyzed. The morphological characteristics with regard to both the habitat and the years in respect of the values showed a varied picture. By the parameters of flowering shoots lengths the population of Tatabanya (TAT) rose from the other populations. With respect of the two years data we did not calculate a significant difference. For this reason, it is assumed that this test area is the optimal habitat for this species.

The plants installed from their natural habitat to the same environmental conditions (temperature, number of hours of sunshine) in respect of the inflorescence shoots (-49.82%), the inflorescence (-44.81%) and internodes (-47.32%) length shortened. These results are in accordance with SLADE and HUTCHINGS (1987) states, that the plant which had more light had a more dense texture and developed branched clones.

The storability test seed germination rates compared to the initial (78%) the values significantly decreased both at room temperature as well as refrigerated conditions in the case of stored cores.

However, for the last conditions the germination normalized around 50%, however at 24 °C temperature this has dropped to 10 to 12%. Based on these results, it appears that room temperature can be a good solution for 1.5 years to stored the seeds of the species, refrigerated conditions (+ 4 °C) should be provided is storing should take more time.

We were studying the effect of germination on germination stimulant procedures with seeds stored under refrigerated conditions in case of the KNO3 solution showed a significant increase in the germination (9-30%), 500 ppm solution concentration gibberellic acid was more inhibitory. However these stimulant procedures didn't have any effect on the germination, their values were very similar. There are no data available on international and domestic level about the determination of ground-ivy seeds germination, not even in the current Hungarian Standard (MSZ 6354-3) either. Therefore, the following test parameters are recommended: germination temperature of 20-30 ° C as regards the case of the former 16, for the later is 8 hours of illumination. The 14th day is the first day of seedling evaluation (germination vigor), the last one is done at day 35 (germination). On the dormant seeds KNO3 solution pre-treatment can be used.

We found that the essential oil components of Hungarian ground-ivy populations are mostly sesquiterpenes (with rates above 65 %), the main compound is the germacren-D which can fill out 25% - 60% of the oil. The accumulation of component can greatly influence by the environmental factors, habitat.

The ground-ivy grown in stock (strong light, high temperature) had a significant increase in final drug levels which varied between 60 and 230 mg GAE/g due to changed environmental conditions. We investigated the effect of the different collection time on the cultivated populations and a trend similar to the wild populations was observed. Here, we could not make a general statement.

Analyzing of chlorogenic acid content in terms of both wild and cultivated plants of the measured values it was concluded that the ground-ivy leafs collected in the summer had a greater amount of chlorogenic acid content than which were picked in the spring and autumn.

In the case of total antioxidant capacity the observed tendency was similar as in change of the total phenol content.

Based on our results, we suggest that to a higher total phenol content in wild populations of flowering plants worth collecting more as material, and preferably those shoots which have short internode, with lots of flowers and leaves.

Other objectives included the development of cultivation and each step of processing technology.

With regard of the efficiency of maceration in every case showed that the aqueous extracts had higher phenol content and stronger antioxidant capacity compared to alcohol (20%) extracts. Theoretical and practical results of our research will hopefully be a good foundation for the future and can provide the same field work to be carried out.

LIST OF USED LITERATURE

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5. PUBLICATIONS CONNECTED WITH THE TOPIC OF THE THESIS

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