Abstract of the PhD Thesis

Enikő Tóth:

Comparative chemical analysis of *Ballota* species, with special respect to *Ballota nigra*, our new official plant in Ph. Hg. VIII.

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1. Introduction

Ballota genus belongs to the Lamiaceae family. The first comprehensive publication about the division of the family derived from Bentham with the title *Labiatarum genera et species* (1832-36). The following article was Briquet's work, *Die natürlichen Pflanzen familien* (1895-97). Briquet reconstructed Bentham's division, raising some of his tribes and subtribes to the subfamilial level and merging Bentham's two largest tribes and two smaller ones into a single huge subfamily, "Stachyoideae". This name was corrected by Cantino and Sanders to Lamioideae in 1984. The Lamiaceae family can be divided into 8 subfamilies by Briquet. These are the followings: Ajugoideae, Prostantheroideae, Prasioideae, Scutellarioideae, Lavanduloideae, Stachyoideae, Ocimoideae, Catopherioideae. Erdtman suggested that the Labiatae is composed of two subfamilies, which differed from each other in the pollen morphology. Wunderlich's (1967) extensive pollen survey lent strong support to Erdtman's groupings through the addition of many new genera to the palynological data base. Briquet's widely used classification is highly incongruent with Erdtman's, and it is recommended that the former should be abandoned. *Ballota* takes place in the Stachyoideae/Lamioideae subfamily (Erdtman, 1945).

The subdivision of *Ballota* genus was made by Patzak in 1958 (Patzak, 1958, 1959, 1961). This resulted in ten sections: *Ballota* Patzak, *Acanthoprasium* Benth., *Rubiformis* Patzak, *Acetabulosa* Patzak, *Pseudodictamnus* Patzak, *Microphylla* Patzak, *Beringeria* (Neck.) Benth. *stricto sensu* Patzak, *Microselidae* (Briq.) Patzak, *Stachydiformis* Patzak, *Royleoides* Patzak. There are 33 species in the genus *Ballota* that are mainly distributed around the Mediterranean and Eurasia.

The most common species of the genus in Hungary is *B. nigra* L., a perennial herb widely distributed in Europe. *B. nigra* is ruderal plant, reaching almost one meter in height. *B. nigra,* since the adoption of the European Pharmacopoeia (IVth Edition), has become official in the Hungarian Pharmacopeia as well. In spite of this fact we knew little about this plant. The main therapeutic use of *B. nigra* is the neurosedative activity. Antibacterial, antifungal and antioxidant activities were also investigated with positive results.

The thorough research of the genus has started from the 1970s. The first diterpenes from *Ballota* genus was isolated by an Italian research group. Since 1976 several publications have appeared by Savona et al. They described the isolation and identification of ballonigrin, ballonigrinone, ballotinone, ballotenol, 7a-acetoxymarrubiin from *B. nigra* and *B. rupestris* (Biv.) Vis. (Section *Microselidae* (Briq.) Patzak). In connection with phenylpropanoid

glycosides from *B. nigra* publications have appeared since 1996. Veronique Seidel and her research group isolated verbascoside, forsythoside B, arenarioside from flowered aerial parts of *B. nigra*. A new publication reported the isolation of ballotetroside in 1997. They also isolated alyssonoside, lavandulifolioside and angoroside A and a non-glycosidic derivative (+)-(E)-caffeoyl-L-malic acid.

2. Determinations

In Hungary *Ballota nigra* cannot be found in domestic trade as a medicinal plant. Products which contain *B. nigra* extracts are not available in the pharmacies nowadays. Neither pharmacists nor patients know about the biological activities of this plant, that is why they do not use or look for it. A lot of publications deal with the isolation and structure elucidation of the main characteristic phenylpropanoids (PPs) in *B. nigra* and other *Ballota* species, but the scientific literature does not contain quantitative information about these compounds. PPs reported for *Ballota* species may be valuable taxonomic markers. So we intended to:

- isolate active ingredients from *B. nigra* (diterpenes, flavonoids, phenylpropanoids) and to enlarge our knowledge on the chemistry of the plant;
- elaborate a quick, efficient method (TLC-densitometric) for the quantitative determination of the characteristic PPs (responsible for the neurosedative effect);
- reveal the differences and /or similarities in the PPs accumulation in plant organs, to what extent the phenylpropanoid content of the official plant in the Pharmacopoeia Hungarica VIII. Edition is advantageous;
- follow the variation of these compounds during the vegetative period (from May till October) so that the optimal time of collection should be determined;
- compare the PPs content of *B. nigra* samples collected from different locations of Hungary so that the differences (if there were any) due to chemotaxonomic or geographical origin in the PPs accumulation should be disclosed;
- make comparative chemical analysis of *Ballota* species *(B. nigra, B. rupestris, B. hirsuta*) for PPs content;
- prove the antioxidant activity of the phenylpropanoids.

3. Materials and methods

3.1. Plant material

Plant material was collected from several places and in different times depended on the aims of the work. For the isolation procedure *B. nigra* was collected in May 2004 from the experimental field of the Institute of Ecology and Botany of the Hungarian Academy of Sciences, Vácrátót (30 km north of Budapest, Hungary). The plants were mainly grown from seeds obtained via a botanical garden seed exchange programme. For study of the variation of the PPs during the vegetative period, parts of *B. nigra* (except roots) were gathered every week from 23rd May to 29th October in 2007. The fresh plants were separated into different parts (shoots, leaves, flowers/reproductive parts, stems), then dried at 40°C. The plant material was collected in the summer of 2007 from 11 locations for the country wide investigation.

3.2. Materials

All used solvents were commercial products of analytical grade (Merck, Germany). The dried aerial parts of *B. nigra* (500 g) were extracted three times with methanol (3 x 6000 ml) at room temperature. The dried extract was dissolved in water. This aqueous fraction was extracted successively with diethyl ether, chloroform, ethyl-acetate, and *n*-buthanol. Then extracts were chromatographed on a silica gel (Kieselgel 60) open column (column size 33 x 3 cm) (granule size 0.063-0.200mm), TLC, preparative TLC, Sephadex coloumn. CM was purchased from PhytoLab, Germany. FB and VE were isolated by us from *B. nigra* (voucher specimen no.: BN0607). The purity of both compounds was controlled by TLC and NMR spectroscopic* studies.

For the densitometric investigations the samples were heated in 25 ml of methanol under a reflux condenser on a water bath for 30 min. Densitograms of the PPs were obtained by using an IBM PC-controlled Shimadzu CS-9301PC densitometer (Japan). Quantification was performed in fluorescence mode by exploiting UV light-induced emission of the PPs.

4. Results

4.1. Isolation from *B. nigra*

Martynoside (Fig.3.), ladanein (Fig.2.) and 7a-acetoxyroyleanone (Fig.1.), isolated from the diethyl ether fraction, have not previously been reported for *B. nigra*. Phenylethanoid glycosides, including FB, have been found in other *Ballota* species, but this is the first finding

of martynoside in the genus. Labdane and clerodane type diterpenoids have been isolated from *Ballota* species, but 7a-acetoxyroyleanone is the first quinonoid type diterpene to be reported for the genus. Ladanein has been identified in *Ballota* species, for example *B. saxatilis* C. Presl. and *B. hirsuta* Benth., as well as from a species of the closely related genus *Marrubium* [from *M. trachyticum* Bois.]. Our findings support not only the infrageneric relationship of *Ballota* species, but also the close connection between the genera *Ballota* and *Marrubium* in the Tribe Stachyodeae (according to Bentham in Hegi 1962). Phenylethanoid glycosides, including martynoside, are widely distributed in the Subfam. Lamioideae (according to the two subfamiliar Erdtman systems). Sporadic data justify their presence in the Subfam. Nepetoideae, but martynoside was only found in *Salvia officinalis*. Our observations support the chemotaxonomic similarities and differences suggested by Erdtman's (1945), and Cantino (1986), Harley and Wagstaff's (1992) classifications. In the isolation procedures iridoids, characteristic to the subfamily, have not been confirmed by us.

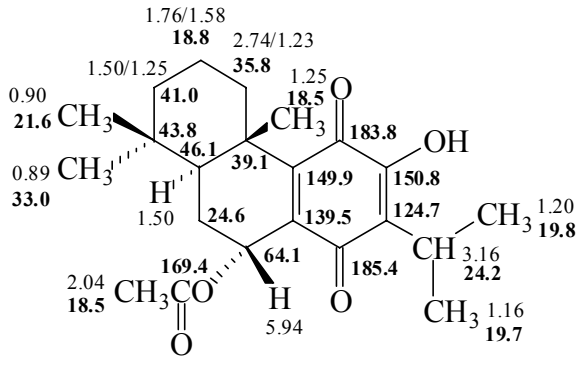

Fig.1. ^{1}H and ^{13}C chemical shifts of compound 7a-acetoxyroyleanone

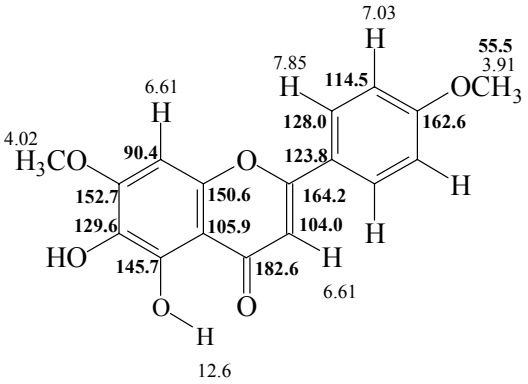

Fig.2. ^{1}H and ^{13}C chemical shifts of compound ladanein

4.2. TLC-densitometric investigations of PPs in *B. nigra* L.

Most of the beneficial biological effects (e.g. neurosedative, antioxidant, and antimicrobial) of *B. nigra* L. are due to its PPs content. We could not detect iridoids similar to the *Marrubium* genus and differently from the other Lamioideae species. There is numerous literature data on the isolation of PPs from *Ballota* species, but apart from one LC-MS investigation there have been no publications dealing with the distribution of these compounds. A rapid TLC-densitometric method with acceptable accuracy has been established for quantitative analysis of the most characteristic components of *B. nigra*. Quantitative data of these compounds were not available in the scientific literature before.

Experiments were performed to establish the optimum conditions for densitometric measurement and for storage of extracts.

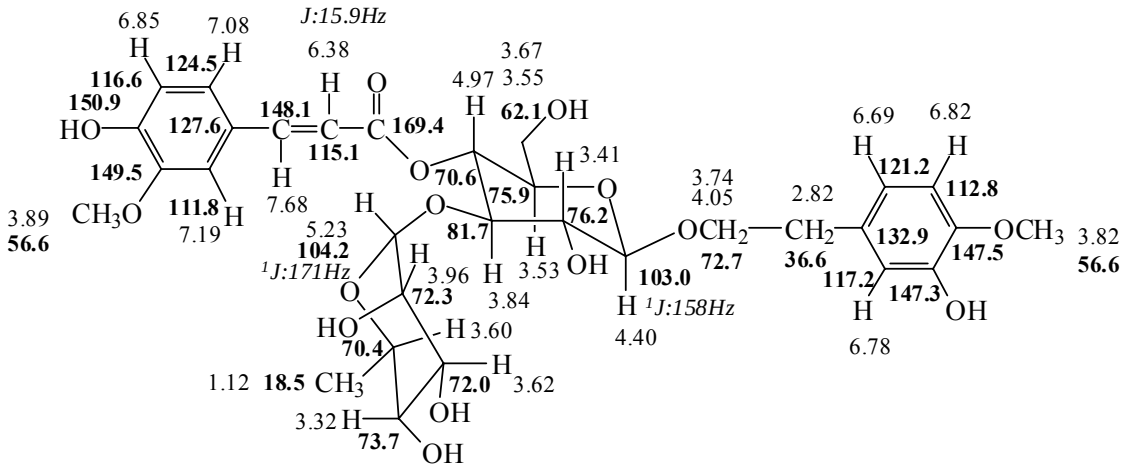

Fig.3. 1H and 13C chemical shifts of compound martynoside

To get the maximum of the investigated PPs we tried two methods of extraction. First the samples were heated in 25 ml of methanol under a reflux condenser on a water bath for 30 min. Second the samples were extracted three times with 8 ml of methanol applying ultrasonic bath for 10 minutes. The extracts were filtered and made up to 25 ml. The extraction with reflux condenser was more efficient. In our investigations we used this method. In the optimization of the densitometric method we determined the mode of detection, in which Naturstoff and PEG 400 reagent gave the best results (Fig.4.). The optimal wavelength of the densitometric measurements was at 395 nm. The period of colour stability was between 10 and 40 min after derivatization, so we used this time interval for the measurements.

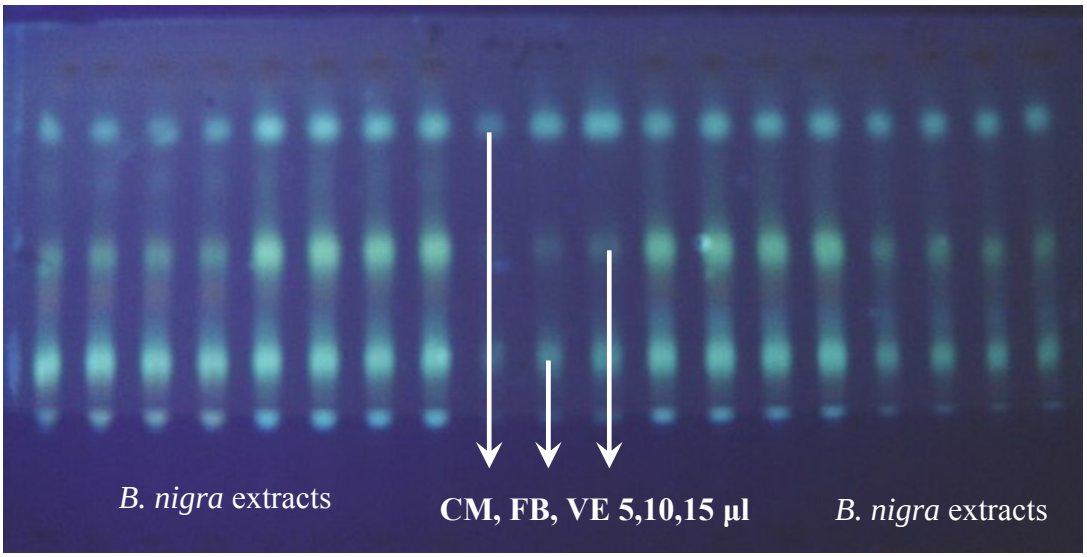

Fig.4. A developed plate detected in UV (395nm) after visualization

With the calibration lineal of each investigated PPs we determined the linear relationships (0-1.65 µg for CM, 0-13.00 µg for FB, and 0.60-10.50 µg for VE). To establish the sensitivity of the method, the smallest detectable amounts of the three PPs were determined (0.05 µg for CM, 0.3 µg for FB, and 0.6 µg for VE). The reproducibility of densitometric evaluation was examined by tenfold replicate scanning of a mixture of authentic compounds on a plate. The results were good (1.90% for CM, 0.88% for FB, and 1.57% for VE). The best conditions for storage of PPs proved that the PPs content of the methanolic extracts of *B. nigra* were more stable in the dark than in the light. The extracts proved stable when they were stored in the dark at either 5°C or at 25°C for 14 days.

4.3. Variation of PPs in *B. nigra* during a vegetative period

B. nigra, the common representative of the genus in Hungary, was chosen for thorough study for the variation of PPs during the vegetative period. In this investigation we wanted to obtain information on possible temporal fluctuations of PPs in all plant organs of *B. nigra* L. (leaves, stems, shoots, flowers/reproductive parts, roots). The developed TLC-densitometric method was used to test the PPs content in the *B. nigra* during a vegetative period. The studied compounds were VE, FB and CM.

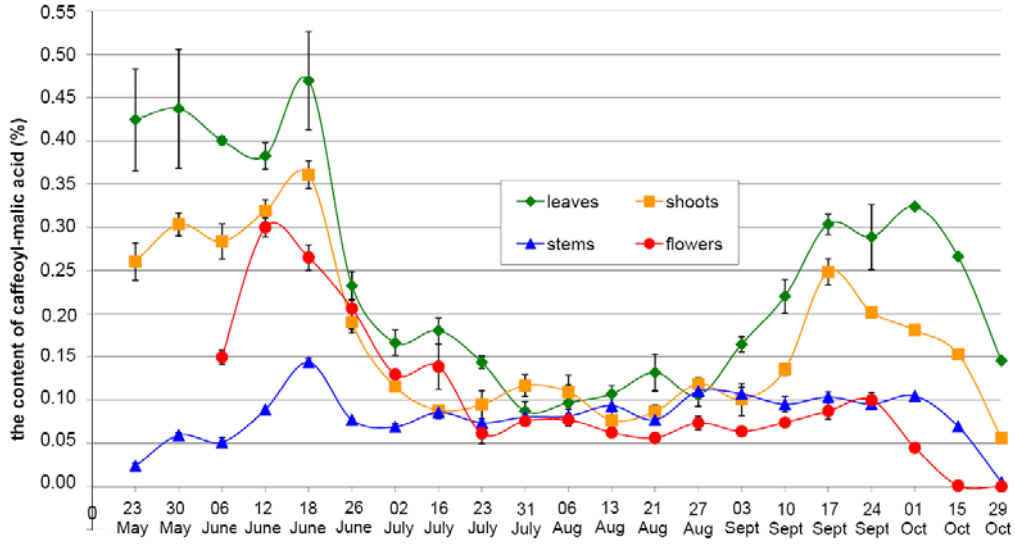

Fig.5. Variation of the content of CM in *B. nigra* in the vegetative period

For all of the three investigated compounds, we observed similar tendencies in every single plant organ. Increased amounts of PPs were found during the main and secondary flowering periods in June and September. The values for CM were lower by an order of

magnitude than those of FB and VE. The amount of CM was under 0.5 % (Fig.5.). The VE content reached the 12 % (Fig.6.). The highest quantity of FB was 7 % (Fig.7.). Our results give important evidences of the utilization of the plant. *B. nigra* contained the phenylpropanoids in the highest quantity in the flowering stage, so this period can be suggested for the plant collection.

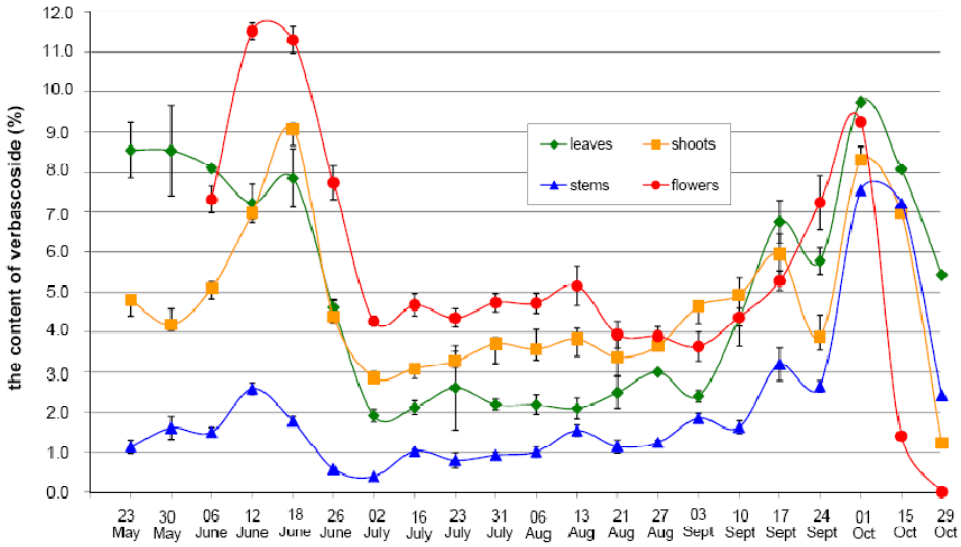

Fig.6. Variation of the content of VE in *B. nigra* in the vegetative period

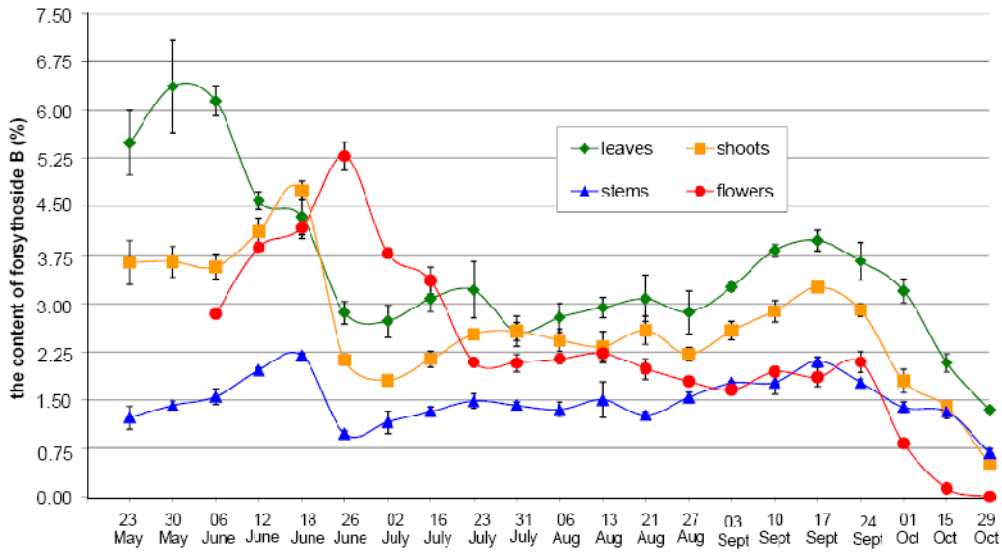

Fig.7. Variation of the content of FB in *B. nigra* in the vegetative period

4.4. Comparative analysis of *B. nigra* collected from different locations of Hungary

The plant material was collected in the summer of 2007 from 11 locations. The results showed the same tendencies as the analysis of the vegetative period of *B. nigra*. Of the compounds investigated, CM, when present was always in the smallest quantity adequate to

our former investigations, but the amount reached 1 % by the samples from Brasov. The samples gathered in June produced the PPs in advanced quantity. In July we could measure high VE values in Fót, Ópályi, Brasov, Nagyvázsony. The *B. nigra* samples gathered in August from Gyenesdiás, Rezi, Sződliget and Szeged produced the PPs in 1-2 % (Table 1, 2, 3).

date and place of collection		Forsythoside B (%)				
		leaves	flowers	stems	shoots	roots
9th June	Szomolya	1.19	2.25	0.33	0.89	-
17th June	Fót	1.04	1.55	0.18	0.56	-
25th June	Fót	1.74	1.94	0.97	1.65	-
30th June	Gyöngyössolymos	1.07	1.99	0.45	0.97	-
2nd July	Fót	0.76	2.2	0.59	1.17	-
8th July	Ópályi	2.02	2.63	0.96	1.56	1.78
11th July	Kerecsend	-	-	-	3.04	0.49
14th July	Brasov	1.07	2.82	0.9	1.34	1.9
26th July	Nagyvázsony	0.69	1.73	0.15	1.65	0.9
18th August	Gyenesdiás	-	-	-	0.24	0.3
19th August	Gyenesdiás	-	-	-	0.62	-
19th August	Rezi	-	-	-	1.14	-
21st August	Sződliget	0.34	-	-	1.04	1.27
23rd August	Szeged	-	-	-	2.1	-

Table 1. Forsythoside B content of *B. nigra* collected from different places and time

date and place of collection		Verbascoside (%)				
		leaves	flowers	stems	shoots	roots
9th June	Szomolya	5.88	10.07	1.41	4.38	-
17th June	Fót	1.77	7.64	0.12	2.45	-
25th June	Fót	1.64	8.23	1.07	5.39	-
30th June	Gyöngyössolymos	0.86	2.49	0.35	1.03	-
2nd July	Fót	0.22	7.34	0.31	2.92	-
8th July	Ópályi	2.63	10.48	2.06	4.05	6.64
11th July	Kerecsend	-	-	-	1.03	1.23
14th July	Brasov	0.71	9.35	1.19	10.38	11.26
26th July	Nagyvázsony	0.53	9.71	0.19	4.02	15.19
18th August	Gyenesdiás	-	-	-	0.47	2.28
19th August	Gyenesdiás	-	-	-	0.78	-
19th August	Rezi	-	-	-	0.88	-
21st August	Sződliget	0.42	-	-	0.98	1.63
23rd August	Szeged	-	-	-	0.47	-

Table 2. Verbascoside content of *B. nigra* collected from different places and time

date and place of collection		Caffeoyl-malic acid (%)				
		leaves	flowers	stems	shoots	roots
9th June	Szomolya	0.33	0.15	0.007	0.15	-
17th June	Fót	0.24	0.07	0.004	0.03	-
25th June	Fót	0.2	0.16	0.001	0.15	-
30th June	Gyöngyössolymos	0.07	0.04	0.006	0.06	-
2nd July	Fót	0.22	0.12	0.01	0.09	-
8th July	Ópályi	0.24	0.19	0.12	0.15	0.01
11th July	Kerecsend	-	-	-	0.14	0
14th July	Brasov	1.08	0.13	0.16	0.96	0.03
26th July	Nagyvázsony	0.14	0.22	0.004	0.11	0
18th August	Gyenesdiás	-	-	-	0.005	0
19th August	Gyenesdiás	-	-	-	0.03	-
19th August	Rezi	-	-	-	0.05	-
21st August	Sződliget	0.27	-	-	0.17	0.02
23rd August	Szeged	-	-	-	0.05	-

Table 3. Caffeoyl-malic acid content of *B. nigra* collected from different places and time

4.5. Comparison of PPs profiles of *B. nigra*, *B. rupestris* and *B. hirsuta*

Different parts of three *Ballota* species (*B. nigra*, *B. hirsuta* and *B. rupestris*) were analysed for PPs using a simple method of extraction and the newly developed TLC-densitometric method. The studied compounds were VE, FB and CM. The highest contents were detected in the leaves of *B. rupestris*, followed by *B. nigra*, while *B. hirsuta* had the least amounts. The lack of CM was a characteristic only of the roots. In the roots of *B. nigra* we could measure high amounts of VE (3.96 %). In conclusion the roots of *B. nigra* contained VE in higher amounts, but in all the other species FB was dominant. In comparison with other parts of *B. nigra* and *B. rupestris*, the stems showed significantly the lowest FB and VE values. From among the three investigated *Ballota* species the *B. nigra* has the most favourable characteristics in Hungary.

4.6. Antioxidant activity

The antioxidant activity was proved by us also with two methods (assay of lipid peroxidation on rat brain and DPPH radical scavenging assay). The investigated compounds were verbascoside, forsythoside B, caffeoyl-malic acid and martynoside. The 50 % methanolic extract of *B. nigra* was efficient by both method in a concentration dependent way. The percentage inhibition of lipid peroxidation was 91 % of CM, 84 % of FB and 58 % of VE by 60µg/ml concentration.

We could establish that the PPs production of *B. nigra* from different places was similar. Consequently, any region of the country seems to be suitable for cultivation of *B. nigra* and all the *Ballota* populations growing in Hungary can provide good quality of drugs as far as their PPs content concerned. Correspondently the surveys of the PPs content in *B. nigra* during a vegetative period we suggest the early summer plant collection. In May and June the PPs content in the plant reached the maximum. This evidence was also proved in the country-wide survey. Our newly developed TLC-densitometric method gives accurate, quick, straight information about the PPs content. We suggest its use in the Pharmacopoeia. From among the three investigated *Ballota* species the *B. nigra* has the most favourable characteristics in Hungary.

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Publications form the basis of the thesis

I. Enikő Tóth, Gábor Tóth, Imre Máthé, Gerald Blunden: **Martynoside, forsythoside B, ladanein and 7a-acetoxyroyleanone from *Ballota nigra* L**. Biochemical Systematics and Ecology 35 (2007) 894-897 i.f.:1.048

II. Gábor Janicsák**, Enikő Tóth** and Imre Máthé**: TLC-densitometric Investigations of Phenylpropanoid Glycosides in Black Horehound (*Ballota nigra* L.)** Journal of Planar Chromatography 20 (2007) 6. i.f.:0.683

III. Enikő Tóth, Gábor Janicsák, Imre Máthé, and Gerald Blunden: **Determination of phenylpropanoids in three *Ballota* species** Journal of Planar Chromatography (2009) 4. (under publication) i.f.:0.683

Other publications are related to the thesis

E. Háznagy-Radnai, P. Léber, **E. Tóth**, G. Janicsák, I. Máthé: **Determination of Stachys palustris iridoids by a Combination of Chromatographic Methods**, Journal of Planar Chromatography, 18 (2005) 314-318

Tóth E., Tóth G., Máthé I., Blunden G.: **Martynoside, forsythoside B, ladanein and 7a-acetoxyroyleanone from Ballota nigra L**. Planta Medica 73 (2007) 950 (P-403 55th International Congress and Annual Meeting of the Society for Medicinal Plant Research September 2-6, 2007, Graz, Austria)

Presentations in the theme of the thesis

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