PhD Thesis

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

By

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**2009**

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**IF: 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, 443-446.

**IF: 0,683**

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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. In subfamily Lamioideae, pollen is usually tricolpate and shed in a two-celled stage; in subfamily Nepetoideae, it is usually hexacolpate and shed in a three-celled stage. 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) (Cantino and Sanders, 1986).

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 (Seidel et al., 1999). *B. nigra* is a ruderal plant, reaching almost one meter in height.

The most common species of the genus in Hungary is ***B. nigra* L.**, a perennial herb widely distributed in Europe (Tutin et al., 1972). *B. nigra,* since the adoption of the European Pharmacopoeia (IVth Edition), has become official in the Hungarian Pharmacopoeia as well. In spite of this fact we knew little about this plant. In Hungary *Ballota* cannot be found in

domestic trade as a medicinal plant. The scientific literature did not contain quantitative information about the main characteristic phenylpropanoids (PPs) in *B. nigra* and other *Ballota* species. PPs reported for *Ballota* species (Seidel et al., 1996; Seidel et al., 1997; Didry et al., 1999; Siciliano et al., 2005; Tóth et al., 2007) may be valuable taxonomic markers.

So we purposed to summarize the scientific knowledge about *Ballota*, to isolate new compounds, to elaborate a TLC-densitometric method for the quantitative determination of the characteristic PPs (responsible for the neurosedative effect) and to follow the variation of these compounds during a vegetative period. We collected *B. nigra* samples country-wide and surveyed the PPs content also.

## 2. LITERATURE

### 2.1. The main botanical features of the genus *Ballota*

Patzak determined the botanical features of the genus and the species also. *Ballota* genus comprises perennial herbs or small shrubs, verticillasters few to many-flowered. Bracteoles present, calyx 10-veined; limb undulate, or with 5-16 crenations or teeth, the lobes more or less mucronate or gradually narrowed into an awn, rarely entire. Corolla-tube is shorter than or equalling calyx, with a ring of hairs inside. Stamens parallel, the outer pair the longer; anther-cells diverging. Style-branches subequal. Nutlets oblong, rounded at apex (Tutin et al., 1972).

In Hungary *B. nigra* is the most expansively frequent. The main botanical features of *B. nigra* by the Pharmacopoeia Hungarica VIII and the Flora Europea are the following: *B. nigra* is a perennial herb up to 130 cm. Stems conspicuously four-angled, longitudinally striated, dark green or reddish-brown and more or less pubescent. Leaves greyish-green, petiolate, petioles usually short, rarely up to 80 mm, lamina ovate to orbicular, ovate-oblong; 2 cm to 4 cm wide, lower cauline leaves 3-8 x 2-6cm, margin irregularly crenate, cuneate to cordate at the base; both surfaces covered with abundant whitish hairs; venation pinnate, prominent on the lower surface, slightly depressed on the upper. Verticillasters are many-flowered. Flowers sessle or very shortly pedicellate, calyx infundibuliform, densely pubescent, with 10 prominent ribs and 5 subequal, broadly ovate teeth; 7-13 mm; limb

regularly dentate; lobes 5, awned or mucronate. Corolla 9-15 mm, pink, lilac, purple or white, tube slightly shorter than the calyx tube, bilabiate, the upper lip pubescent on the outer surface, the lower lip with 3 lobes, and the middle lobe notched. Bracteoles are 3-9 mm, subulate or filiform, membranous.

In our research field *B. hirsuta* and *B. rupestris* was well grown from seeds, so we purposed to investigate these species also. Both of these two species are perennial and woody at base. Stems are hirsute by *B. hirsuta* and pubescent by *B. rupestris* with glandular and eglandular simple and stellate, medifixed hairs. The lower and middle cauline leaves of *B. hirsuta* are 3-6 x 3-5 cm, cordate or truncate at base, ovate or suborbicular, crenate; petiole of lower leaves 5-40 mm. The lower and middle cauline leaves of *B. rupestris* are 4-6 x 4-5 cm, cordate at base, ovate or ovate-lanceolate, obtuse or subacute, and coarsely and irregularly crenate-dentate; petioles 10-25 mm. The bracteoles are similar by the two species, linear-subulate, membranous. Verticillasters are many-flowered. Calyx of *B. hirsuta* is 10-12 mm and campanulate; limb is 8-10 mm in diameter, irregularly dentate; lobes 10 or more, up to 2 mm, triangular-acuminate, sometimes dentate, mucronulate. Calyx of *B. rupestris* is 8-10 mm, subcylindrical; limb 6 mm in diameter, irregularly dentate, lobes 6-10, 2 mm, and patent, triangular-lanceolate, awned. Corolla is purple or white by both species.

## 2.2. Chemical ingredients of *Ballota* species

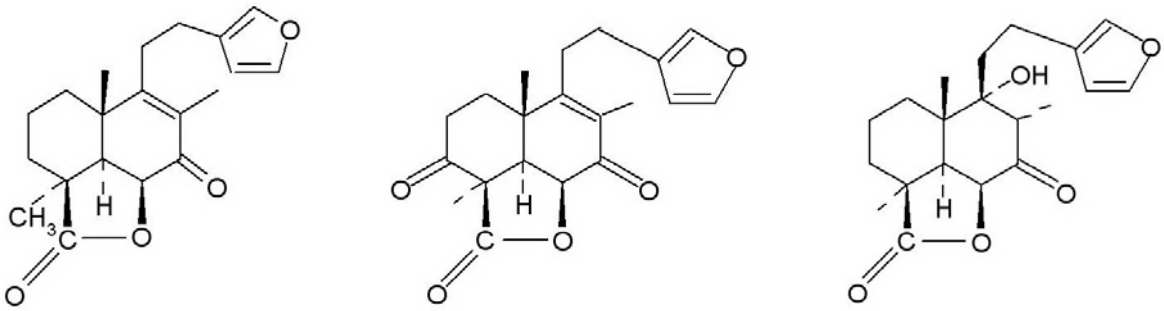
The *Ballota* genus was divided into 10 sections, which were described above. The first section is *Ballota. Ballota* section comprises *B. nigra* and its 7 subspecies [*uncinata* (Fiori et Beg.) Patzak; *velutina* (Posp.) Patzak; *kurdica* Davis; *sericea* (Vand.) Patzak; *anatolica* Davis; *nigra*; *foetida* Hayek].

The early publications of *B. foetida* appeared in 1921 and 1934. Leclerc studied the antispasmodic action of *B. foetida* with beneficial results and without evidences of secondary harmful effects (Leclerc, 1921). The title of Balansard's publication was Pharmacological study of *B. foetida*. The author defined the main groups of components, such as essential oil, bitter substance, neutral saponin, stachydrine, choline. The physiological effects were due to choline (Balansard, 1934).

The thorough research of the genus has started from the 1970s. A Polish publication appeared about anatomical investigations and preliminary phytochemical examinations of *Galeopsidis* and *Ballotae nigrae* shoots in 1975. The extracts and essential oils were tested by paper and thin-layer chromatography. The authors revealed the presence of basic, flavonoid and phenolic compounds in both plants. There was 0.01% essential oil in *B. nigra*. They found salicylic and caffeic acids in both *Galeopsis* and *Ballota* (Strzelecka et al., 1975).

## 2.2.1. Diterpenes

The first diterpenes from *Ballota* genus were isolated by an Italian research group. Since 1976 several publications have been published by Savona et al. They described the isolation and identification of ballonigrin, ballonigrinone (Savona et al., 1976b), ballotinone (Savona et al., 1976a), ballotenol (Savona et al., 1977a), 7a-acetoxymarrubiin from *B. nigra* and *B. rupestris* (Biv.) Vis. (Section *Microselidae* (Briq.) Patzak) (Savona et al., 1977b) (Fig. 1.).

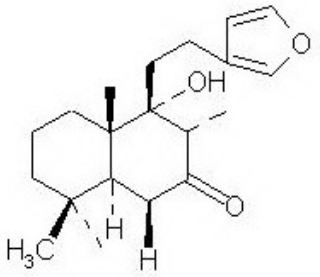


ballonigrin ballonigrinone ballotinone

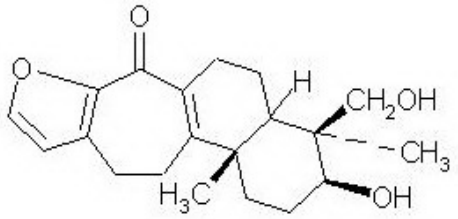
ballotenol 7a-acetoxymarrubiin

Fig. 1. The structures of the diterpenes isolated from *Ballota* species

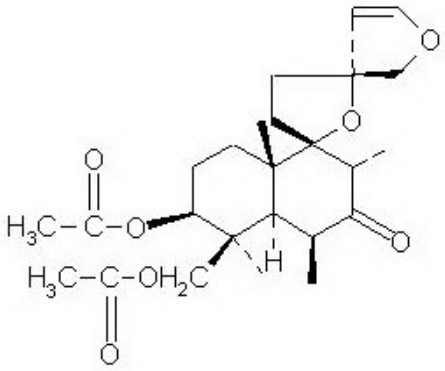
Savona et al. isolated hispanonic acid and hispaninic acid (Rodriguez et al., 1979) from *B. hispanica* (L.) Benth. (Section *Beringeria* (Neck.) Benth. *stricto sensu* Patzak), 18-Hydroxyballonigrin (Savona et al., 1978a) from *B. acetabulosa* (Section *Acetabulosa*), 13-Hydroxyballonigrinolide (Savona et al., 1978b) from *B. lanata*. They published hispanolone in *B. andreuzziana* Pamp. (Section *Rubiformis* Patzak) and ballonigrin, 18-hydroxyballonigrin, marrubenol, 7,4'-di-O-methylapigenin from *B. pseudodictamnus* (L.) Benth. (Section *Pseudodictamnus* Patzak) in 1982 (Savona et al., 1982). From the aerial parts of *B. nigra var. foetida* a new prefuranic labdane diterpene, preleosibirin was isolated and published in 1986 (Bruno et al., 1986).



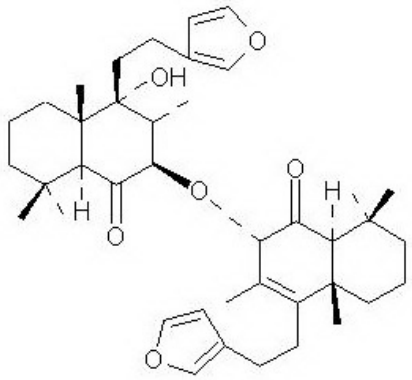

hispanolone




limbetazulone




preleosibirin




persianone

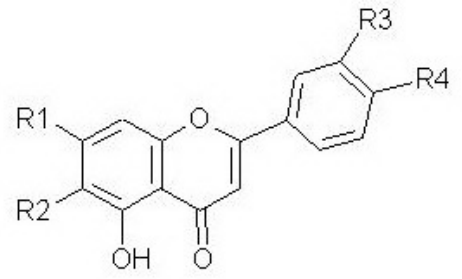
Fig. 2. The structures of the diterpenes isolated from *Ballota* species

Hispanolone was isolated from *B. africana* (L.) Benth. (Section *Beringeria* (Neck.) Benth. *stricto sensu* Patzak) in 1990 by Davies-Coleman et al.. The isolation of diterpenes continued in the next years, mainly furolabdanes was published. A dimeric diterpene, persianone was isolated from *B. aucheri* Boiss. (Section *Microselidae* (Briq.) Patzak) in 1995 (Rustaiyan et al., 1995). Ahmad et al. isolated clerodane type diterpenoids from *B. limbata* in

2004 (Ahmad et al., 2004). In the same year Riaz et al. reported the isolation of limbetazulone, with a very rare naphtho[2,1-f]azulen-7-one skeleton, from *B. limbeta* (Fig. 2.)

## 2.2.2. Flavonoids

The flavonoid determinations in the genus *Ballota* can be found in the scientific literature since 1986. Aerial parts of *B. hirsuta* Benth. (Section *Beringeria* (Neck.) Benth. *stricto sensu* Patzak) yielded 8 flavonoid aglycons (salvigenin, kumatokenin, genkwanin, ladanein, nuchensin, isokaempferide, apigenin and luteolin) (Fig. 3.) and 6 glycosides (apigenin-7-(p-coumaroyl)-glucoside, apigenin-7-glucoside, luteolin-7-glucoside, quercetin-3-glucoside, luteolin-7-rutinoside and vicenin 2) (Ferreres et al., 1986).




**Apigenin** R1 = R4 = OH; R2 = R3 = H
**Genkwanin** R1 = $OCH_3$; R2 = R3 = H; R4 = OH
**7-4'-dimethylapigenin** R1 = R4 = $OCH_3$; R2 = R3 = H
**Ladanein** R1 = R4 = $OCH_3$; R2 = OH; R3 = H
**Salvigenin** R1 = R2 = R4 = $OCH_3$; R3 = H
**Luteolin** R1 = R3 = R4 = OH; R2 = H
**Nuchensin** R1 = R4 = $OCH_3$; R2 = R3 = OH

Fig.3. The structures of some flavonoid aglycons from *B. hirsuta*

Six flavonoids were obtained from the aerial parts of *B. acetabulosa* by a Turkish research group (scutellarein 4',7-dimethyl ether, apigenin, apigenin-7-glucoside, acacetin-7-glucoside, chrysoeriol-7-glucoside and luteolin-7-glucoside) in 1988 (Mericli et al., 1988). The presence of these compounds provided explanation for the haemorrhoid-healing effect of *B. acetabulosa*. Tomas-Barberan et al. revealed in 1992 that flavonoid p-coumaroylglucosides were present in all the species studied of genera *Ballota*, *Phlomis* and *Marrubium*. The polymethoxyflavone tangeretin was isolated from *B. nigra* and published in 1995 by Kisiel et al.. Citoglu et al. published flavonoid aglycons form *B. saxatilis* subsp. *Saxatilis* (Section *Microselidae* (Briq.) Patzak) in 1999. They isolated 6-hydroxyapigenol 7,4'-dimethyl ether (ladanein), kaempferol 3,7,4'-trimethyl ether and quercetin3,7,3',4'-tetramethyl ether (retusin). Two lactoylated flavonoids, luteolin-7-lactate and luteolin-7-glucosyl-lactate were isolated from *B. nigra* by Bertrand et al. in 2000. Numerous polyphenols were published from

*B. acetabulosa* in 2002 by Saphaz et al. These were chrysoeriol-7-O-β-(3”-Z-p-coumaroyl)glucopyranoside, chrysoeriol-7-O-β-(3”-E-p-coumaroyl)-glucopyranoside, chrysoeriol-7-O-β-glucopyranoside, apigenin-7-O-β-(3”-E-p-coumaroyl)-glucopyranoside, apigenin-7-O-β-glucopyranoside and one phenylethanoid glycoside, eutigoside A. The flavonoids kumatakenin, pachypodol, 5-hydroxy-7, 3’, 4’-trimethoxyflavone, velutin, salvigenin, retusin and corymbosin were isolated from *B. glandulosissima* (Section *Microselidae* (Briq.) Patzak) by Citoglu et al. in 2003.

### 2.2.3. Phenylpropanoids

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 (Seidel et al. 1997). They also isolated alyssonoside, lavandulifolioside and angoroside A and a non-glycosidic derivative (+)-(E)-caffeoyl-L-malic acid (Didry et al. 1999).

## 2.3. Therapeutic use and biological activities

Leclerc investigated the antispasmodic action of *B. foetida* in 1921, the results were beneficial. *Ballota* species are used in folk medicine as antiulcer, diuretic and choleretic agents, and for the treatment of wounds and upper respiratory inflammation.

### 2.3.1. Neurosedative activity

The main therapeutic use of *B. nigra* is the neurosedative activity. Pieretti et al. demonstrated that a mixture of phenylpropanoid glycosides significantly prolonged sleep induced by pentobarbital, reduced locomotor activity in mice, and produced a slowing of the electroencephalographic trace (Pieretti et al., 1992).

The antidepressant and anxiolytic activities of *B. larendana* Boiss. (Section *Microselidae* (Briq.) Patzak) and *B. nigra var. anatolica* Davis (indigenous to Turkey) were

investigated by behavioural tests in rats by Vural et al. in 1996. Extracts of both of these species had antidepressant activity; *B. larendana* extracts also had anxiolytic activity.

The results of Daels-Rakotoarison et al.'s work in 2000 showed that 4 phenylpropanoid glycosides were able to bind to benzodiazepine, dopaminergic and morphinic receptors. This may be in relation with the *B. nigra* known neurosedative activities (Daels-Rakotoarison et al., 2000).

### 2.3.2. Antibacterial and antifungal activity

Some publications proved the antibacterial activity of *Ballota* extracts. Citoglu et al. studied the effects of hispanolone, dehydrohispanolone and ballonigrine against some Gram-positive and Gram-negative microorganisms and a fungus. The minimum inhibitory concentration of these compounds (25, 50 μg/ml) showed that all of them were active against bacteria and fungus (Citoglu et al., 1998). The phenylpropanoid glycosides were also investigated against bacteria by Didry et al. (Didry et al., 1999). They found moderate antimicrobial activity of verbascoside, forsythoside B and arenarioside. Arenarioside showed inhibition of the methicillin-resistant Staphylococcus strain. The antifungal activity of *B. inaequidens* (Section *Microselidae* (Briq.) Patzak) (Citoglu et al. 2004) and *B. glandulosissima* (Citoglu et al., 2003) was tested by Citoglu et al. The flavonoids showed activities against Candida species. The water extract of *B. glandulosissima* possessed promising protective activity against $CCl_4$-induced hepatic damage and anti-inflammatory activity in rats (Özbek et al., 2004).

### 2.3.3. Antioxidant activity

Phenylpropanoids from *B. nigra* L. inhibited in vitro LDL peroxidation by Seidel et al.'s article. The investigated phenylpropanoids (verbascoside, forsythoside B, arenarioside, ballotetroside, caffeoyl-L-malic acid) were strong inhibitors of $Cu^{2+}$-induced LDL peroxidation, independent of any capacity to act as $Cu^{2+}$ chelators (Seidel et al., 2000). The same components were tested against reactive oxygen species as superoxide anion, peroxide hydrogen, hypochlorous acid, hydroxyl radical. Results showed evidence ability to scavange

reactive oxygen species (Daels-Rakotoarison et al., 2000). Vrchovská et al. investigated the ability of *B. nigra* infusion to act as a scavenger of DPPH radical, reactive oxygen species and nitric oxide, which was published in 2007. *B. nigra* lyophilized infusion strongly scavenged DPPH in a concentration dependent way, the extract exhibited superoxide radical-scavenging activity also, but for hydroxyl radical no scavenging activity was observed. The study attributed this scavenging activity to the phenolic derivatives present in the infusion (Vrchovská et al., 2007).

## 3. AIMS

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.

# 4. INVESTIGATIONS, DISCUSSIONS

## 4.1. Preliminary studies

At the beginning of our investigations we decided to determine the main chemical compounds of *B. nigra.* Accordingly plant material was collected, dried and powdered. 3.0 g sample was extracted with methanol and made up to 100 ml. 25 µl of the extract was chromatographed on a silica gel plate (Kieselgel 60), using toluol-ethylacetate (95:5) as mobile phase. We used Salvia diterpene standards. The spraying reagent was sulphuric acid-anisaldehyde. The *B. nigra* extract contained spots related to diterpenes. With the same conditions, but with different mobile phases we tested the extracts to sesquiterpenes (hexan-acetone 98:2) and ecdysteroids (toluol-acetone-ethanol-ammonia 50:70:16:4.5). These compounds were not present in the extract. Alkaloid and pseudo alkaloid were not detectable with the reagent Dragendorff. The phenylpropanoid derivatives were tested with ethylacetate-methanol-water (100:16.5:13) developing system. We detected in UV the developed and dried plates and appeared 4 fluorescent spots. The presence of flavonoids was proved with the same mobile phase as described above. The locating reagent was 5 % methanolic solution of aluminium-trichloride. The characteristic yellow spots appeared and were detected under UV light. The standards were luteolin, apigenin 7-O-glycoside and kaempferol.

The mobile phase was chloroform-methanol-water (25:10:1) in the iridoid test. The locating reagent was 1%-hydrochloric solution of dimethylamino-benzaldehyde. The standards were aucubin, asperulozide, catalpol. They were not present in the extract. The iridoid content was also investigated with Trim-Hill reaction. For the reaction we made aqueous extract (50 ml) from *B. nigra* shoots. 1 ml of the aqueous extract was heated with 11 ml of Trim-Hill reagent for 5 min. We did not experience the blue colour. So we could establish that our *B. nigra* sample did not contain iridoids.

We have also investigated the rosmarinic acid, caffeic acid and ursolic/oleanolic acid (US/OS) content of *B. nigra*. Powdered leaf samples (0.4 g) were extracted with 60 % aqueous methanol (7 ml) for 10 min using an ultrasonic extractor (Tesla, Czechoslovakia). This process was repeated 4 times; the extracts were combined and made up to 25 ml. After measuring with densitometer the caffeic acid was 0.01 %, US/OS content was 0.38 %.

## 4.2. Isolation works from *Ballota nigra*

Plant material 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). Its climate is continental, with a mean annual temperature of 10.3 °C, and a mean annual precipitation of 525 mm. The plants were mainly grown from seeds obtained via a botanical garden seed exchange programme.

The dried aerial parts of *B. nigra* (500 g) were extracted three times with methanol (3 x 6000 ml) at room temperature by the following. Before and after the 1 night maceration we used for 10 minutes ultrasonic bath (Tesla, Czechoslovakia). After filtration the extracts were evaporated to dryness. The second maceration continued through 4 days. The third extraction was carried out till 1 night.

The dried extract was dissolved in water. This aqueous fraction was extracted successively with diethyl ether, chloroform, ethyl-acetate, and *n*-buthanol (Fig. 4.)

The diethyl ether and chloroform extracts were combined (2.5 g) and checked with TLC. This was performed on a silica gel (Kieselgel 60 $F_{254}$), the mobile phase was chloroform-methanol (19:1). We used sulphuric acid-anisaldehyde as locating reagent.

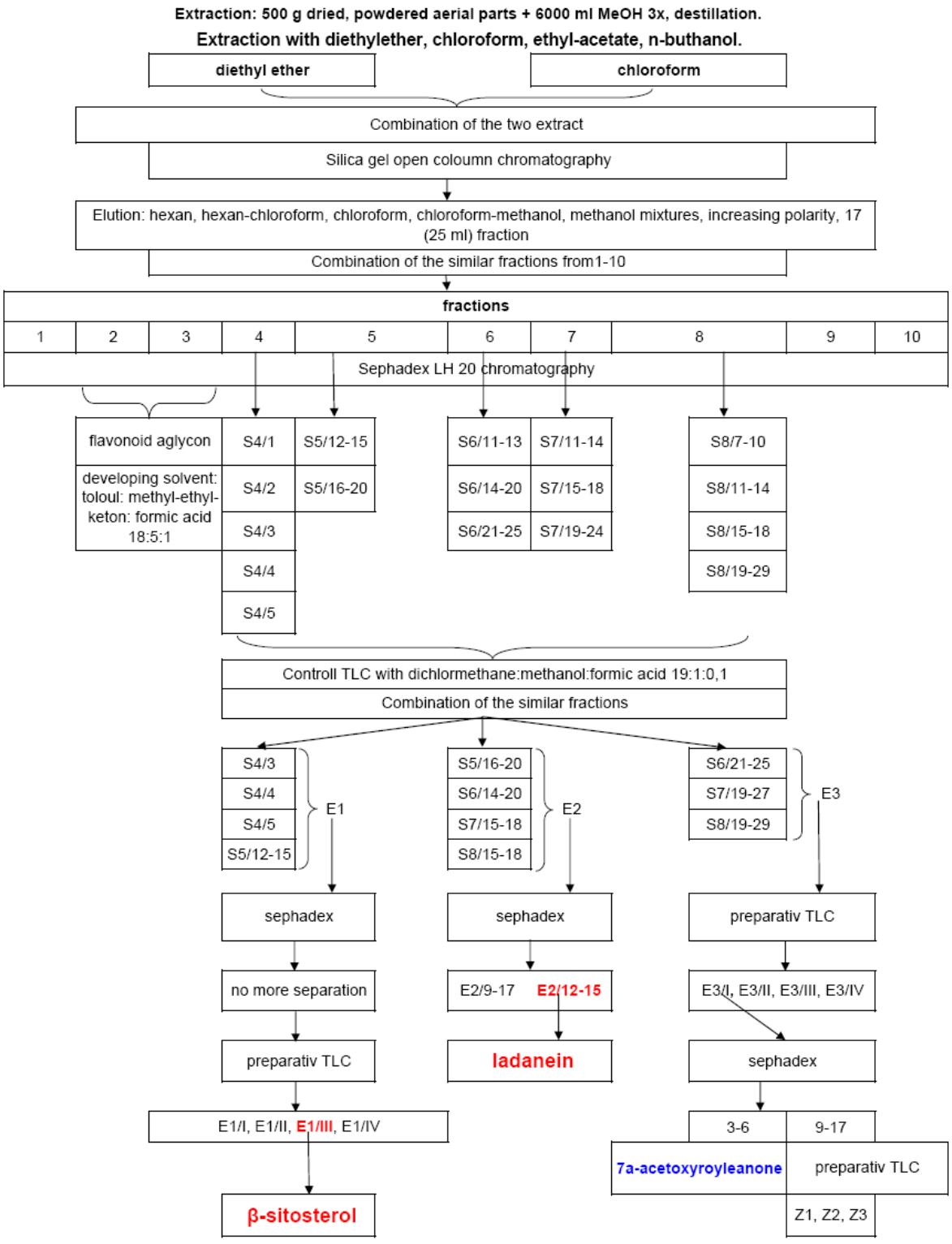



Fig.4. The isolation procedure of the diethyl-ether and chloroform fraction

The standards were β-sitosterol, ursolic acid, betulin, α-amirin. The combined extract contained β-sitosterol and α-amirin. The diethyl ether and chloroform extracts were chromatographed on a silica gel (Kieselgel 60) open column (column size 33 x 3 cm) (granule size 0.063-0.200 mm), eluting with mixtures of *n*-hexane, chloroform and methanol of increasing polarity, the eluent volume was 125 ml, the volume of each fractions were 25 ml. All the fractions were chromatographed on a silica gel plate (Kieselgel 60 $F_{254}$) (mobile phase: dichlormethane-methanol-formic acid 19:1:0.1, locating reagent: 1%-hydrochloric acid solution of dimethylamino-benzaldehyde). The similar fractions were combined which resulted in 10 fractions.

Each fraction was submitted to repeated gel filtration chromatography on Sephadex LH-20, eluting with chloroform-methanol (1:1). The combination of the similar fractions (from the gel filtration chromatography 4, 5, 6, 7, 8) resulted in three new fractions (code: E1, E2, E3). Repeated chromatography on Sephadex LH20 and preparative TLC led us the cristallized ladanein (Fig.6.) (6-hydroxy apigenin 7, 4'-dimethyl ether) (52 mg), 7a-acetoxyroyleanone (4.3 mg) (Fig.5.) and β-sitosterol (24.6 mg).

The purity of the components were tested by chromatographed on Merck Kieselgel 60 $F_{254}$ layers using chloroform-methanol (19:1) as the development solvent and 50% methanol-sulphuric acid as the locating reagent.

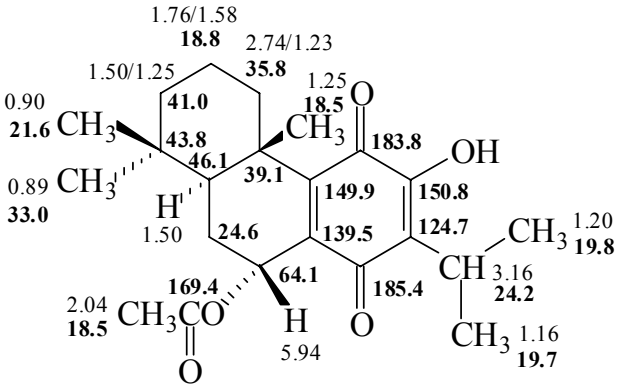



Fig.5. $^{1}H$ and $^{13}C$ chemical shifts of compound 7a-acetoxyroyleanone

The ethyl acetate extracts were chromatographed on a silica gel (Kieselgel 60) open column (column size 33 x 3 cm), eluting with mixtures of chloroform, ethyl acetate, methanol and water of increasing polarity. The phenylethanoid glycoside, martynoside (34 mg) (Fig.7.) was obtained from the ethyl acetate fraction (2.8 g) of the plant extract. During gel filtration,

the purity of martynoside was controlled by TLC using formic acid-acetic acid-water-ethyl acetate (7.5:7.5:18:67) as the developing system.

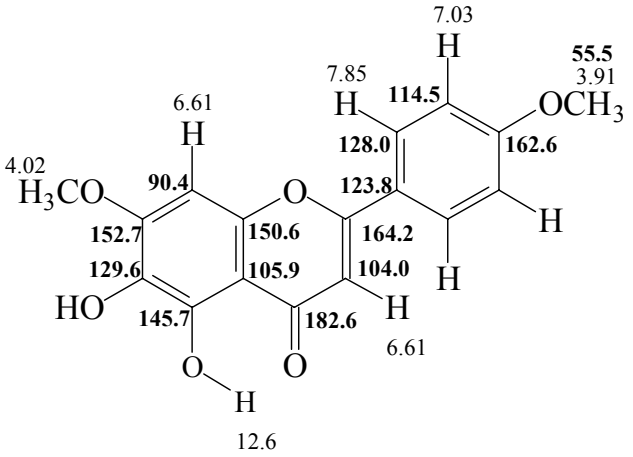



Fig.6. $^{1}$H and $^{13}$C chemical shifts of compound ladanein

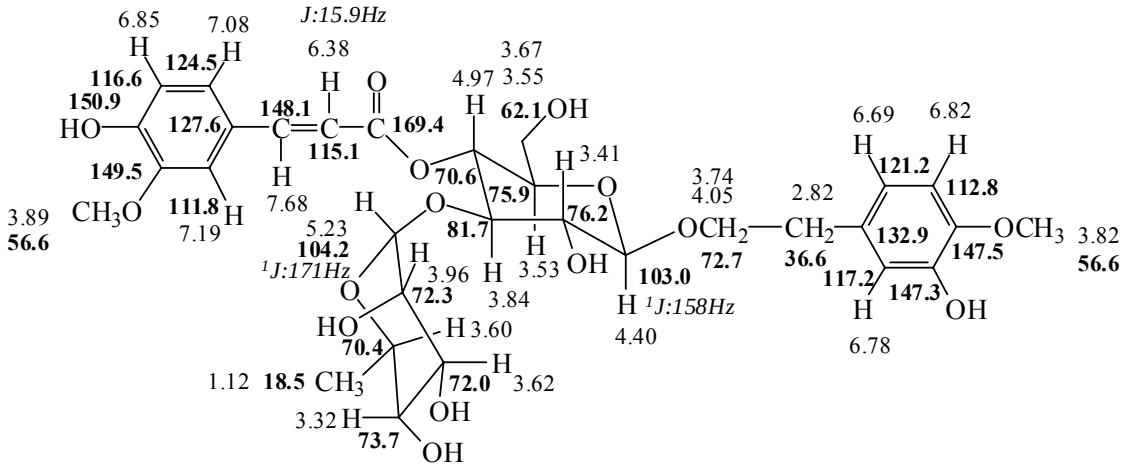



Fig.7. $^{1}$H and $^{13}$C chemical shifts of compound martynoside

The structures of the isolated compounds were elucidated by NMR spectroscopy using $^{1}$H, $^{13}$C, DEPT-135, 1-D selective TOCSY, 1-D selective NOESY, 2-D $^{1}$H-$^{13}$C HSQC, $^{1}$H-$^{13}$C HMBC, $^{1}$H-$^{1}$H COSY and $^{1}$H-$^{1}$H NOESY techniques (Pretsch et al., 2002; Duddeck et al., 1998). The investigations were made by Professor Gábor Tóth with a Bruker Avance 500 spectrometer in Budapest. The UV, $^{1}$H-, and $^{13}$C- NMR spectroscopic data obtained were identical with those previously described for these compounds in the literature (Citoglu et al., 1999; Skrzypek et al., 1999).

Further plant collection was carried out in July 2006 from the experimental field of the Institute of Ecology and Botany of the Hungarian Academy of Sciences, Vácrátót. The dried, powdered shoots of *B. nigra* (100g) were extracted with 1000 ml of 70%-aqueous methanol. The duration of maceration was four days. After filtration the procedure was repeated twice for one day. The dried extract was dissolved in water and extracted with diethyl-ether and subsequently with ethyl acetate. The main polar components of *B. nigrae* shoots remained in the residue. So we purified this fraction along. The residue was chromatographed on a silica gel (Kieselgel 60) open column, eluting with mixtures of *n*-hexane, ethyl acetate, methanol and water of increasing polarity. The similar fractions were combined. Repeated gel filtration chromatography and preparative TLC resulted in the isolation of forsythoside B (Fig.8.), verbascoside and caffeoyl-malic acid. The structures of the isolated compounds were elucidated by NMR spectroscopy, with authentic standards and by datas from the Hungarian Pharmacopoeia (VIIIth Ed.).

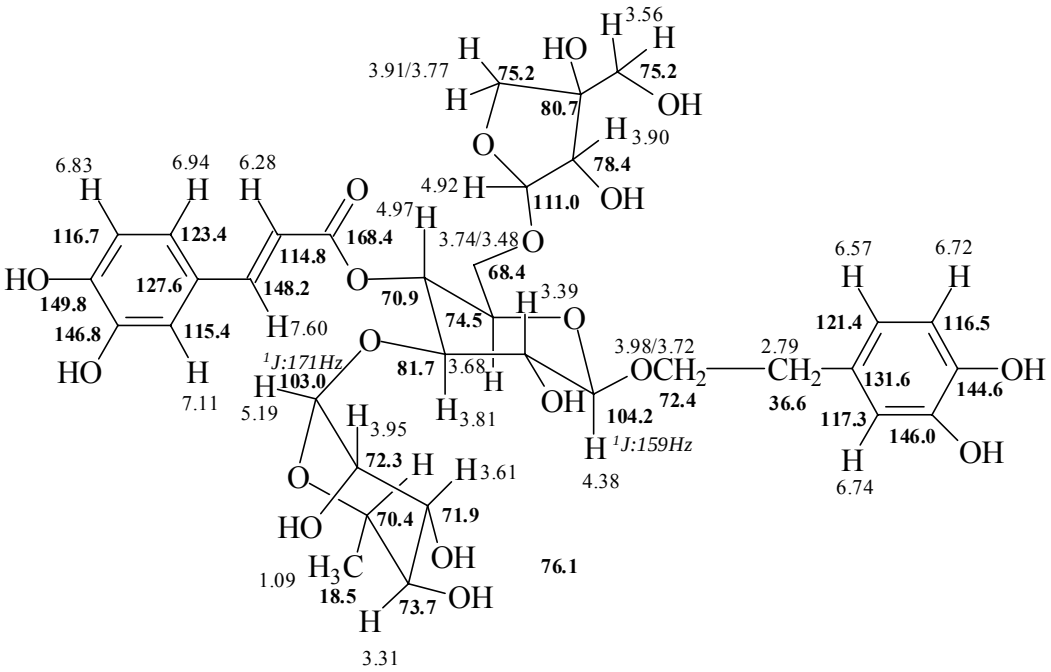



Fig.8. $^{1}H$ and $^{13}C$ chemical shifts of compound forsythoside B

## 4.3. Analitical investigations of PPs

### 4.3.1. Elaboration of TLC-densitometric method for the assay of PPs.

The quantitative analysis of the characteristic PPs in *Ballota* species was worked out. This kind of information about the genus was not available up to now. First we elaborated a rapid TLC-densitometric method with acceptable accuracy. Experiments were performed to establish the optimum conditions for densitometric measurement and for storage of extracts. During the optimization procedure, authentic samples of caffeoyl-malic acid (CM), forsythoside B (FB) and verbascoside (VE) were used. These PPs were present in the unpurified plant extract in well detectable quantity. CM was purchased from PhytoLab, Germany. FB and VE were isolated by us from *B. nigra*. The purities of both were monitored by TLC and NMR. The standard solution contained 0.305 mg CM, 1.785 mg FB and 2.015 mg VE in 5 ml methanol.

#### 4.3.1.1. Plant material and extraction methods

*B. nigra* shoots were collected in July 2007 from the experimental field (Vácrátót). The stand was produced and controlled by us with the same semi cultivated conditions for this purpose. The plant material was separated into organs (shoots, leaves, flowers, stems, and roots) and dried at 40°C.

Two kinds of extraction methods, first with reflux condenser and second with ultrasonic bath, were tested to obtain the maximum of the PPs from *B. nigra*. The tested solvents were methanol, ethyl acetate and acetone. The amount of the dried, powdered plant material was 0.5 g. The extracted plant material was leached three times with the solvent. The filtered extracts were made up to 25 ml in each case. Two parallel investigations were carried out.

Refluxation: The samples in 25 ml of methanol were heated under a reflux condenser on a water bath for 30 min. After cooling, the extracts were filtered and made up to 25 ml.

Ultrasonic extraction: 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 extracts were checked with TLC. It was performed on precoated aluminium plates (Kieselgel 60 $F_{254}$) in an unsaturated chamber (Desaga, Germany), by using the ascending technique at room temperature. The volumes applied to the plates were 25 µl from the extracts, 5, 10, 15 µl from the standards.

The mobile phase was formic acid-acetic acid-water-ethyl acetate (7.5:7.5:18:67) (Paál, 2004). The official mobile phase in the Ph.Hg. VIII. was the most suitable for the investigations; the separation of the components was acceptable.

The developed plates were dried under an intense air-stream in a fume cupboard for 10 min. The plates were then dipped into a 1% methanolic solution of aminoethyl diphenylborinate and heated at 40°C in an oven for 10 min. The dried plates were subsequently dipped in a 5% solution of PEG 400 in methanol and then heated in the same way as above. After measuring with densitometer we could establish that the extracts which were heated under a reflux condenser contained the components in higher amounts. Table 1 comprises contents of the PPs after extraction with the two methods. The most significant difference appeared in the case of VE, with ultrasonic bath the extracted amount was 4.84 mg/0.5g and with reflux it was 12.30 mg/0.5g. In the following we used this method for the extraction (Rf: CM: 0.83; FB: 0.35; VE: 0.54).

| | | **forsythoside B mg / 0.5g** | **verbascoside mg / 0.5g** | **caffeoyl-malic acid mg / 0.5g** |
|---|---|---|---|---|
| **extraction with ultrasonic bath** | U1 | 8,62 | 4,96 | 0,78 |
| | U1 | 8,19 | 5,50 | 0,76 |
| | U2 | 7,04 | 3,71 | 0,63 |
| | U2 | 7,72 | 5,19 | 0,69 |
| | average | **7,89** | **4,84** | **0,71** |
| **extraction with reflux condenser** | R1 | 12,51 | 11,53 | 1,05 |
| | R1 | 12,45 | 11,32 | 1,00 |
| | R2 | 13,33 | 13,50 | 1,11 |
| | R2 | 12,70 | 12,85 | 1,01 |
| | average | **12,75** | **12,30** | **1,04** |

Table 1. The amount of PPs after extraction with two different methods from *B.nigra*

U = ultrasonic; R = reflux with two parallel

Ethyl acetate and acetone were tested for extraction solvents by the same way. The results were negligible, because the investigated compounds were not detectable.

### 4.3.1.2. Mode of detection

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. The conditions used for densitometric determination were: scan mode, linear; beam size, 10 mm x 0.5 mm; zero set mode at start.

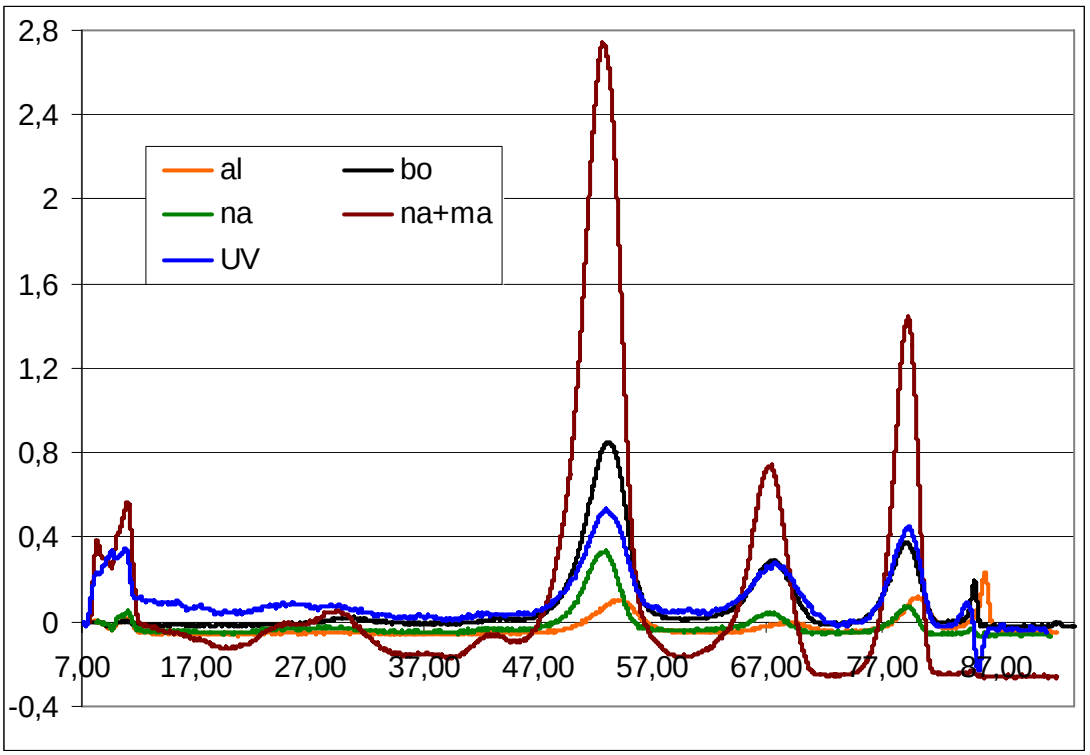



Fig.9. 5 different mode of detection

al = 5 % methanolic solution of aluminium-trichloride

na = 1% methanolic solution of aminoethyl diphenylborinate (Naturstoff reagent)

UV = detection in UV

bo = mixture of 10% aqueous solution of oxalic-acid and 3% aqueous solution of boric acid

na+ma = 1% methanolic solution of aminoethyl diphenylborinate and 5% solution of PEG 400 in methanol

4 locating reagents were tested and one layer was detected in UV. The reagents were the following: 5 % methanolic solution of aluminium-trichloride, mixture of 10% aqueous solution of oxalic-acid and 3% aqueous solution of boric acid, 1% methanolic solution of aminoethyl diphenylborinate (Naturstoff reagent), 1% methanolic solution of aminoethyl diphenylborinate and 5% solution of PEG 400 in methanol. The best results in the intensity of fluorescence were with Naturstoff and PEG 400 combination. Figure 9 shows the differences between the different reagents. PEG 400 fortified the intensity of fluorescence.

#### 4.3.1.3. Wavelength

To determine the optimum wavelength for excitation, i.e. that resulting in maximum emission, densitometric measurements on the spots was made between 330 and 430 nm in 5-nm steps. During this procedure, the appropriate emission filter was also selected, according to the wavelengths to be cut off. It was found that peak areas for the emission were maximum at 395 nm. Further investigations were performed at 395 nm. Selection of the emission filter was based on the requirement that it should cut off the exciting light but allow through as much of the fluorescent emission as possible. Accordingly, filter no. III was chosen which cuts off excitation wavelengths below 440 nm.

#### 4.3.1.4. Colour stability

The period of colour stability is the time during which the intensity of emission of the spots undergoes only negligible changes. For this purpose, we used plates developed, dried, and treated with the two visualization reagents. After visualization, densitometric evaluation of the three components was started immediately. The determination was repeated every 5 min for 30 min, then at 10-min intervals, from the moment of insertion of the plate into the instrument up to 2.5 h. During the 150-min observation period the change in emission intensity passed through three stages. In the first 10 min there was a steep decrease. This was followed by a period of negligible change for approximately 30 min and, finally, an increase for the remainder of the time (Fig.10.). The changes during the 30-min period were not large, as reflected by coefficients of variation of 3.43% for CM, 2.67% for FB, and 3.23% for VE.

Accordingly, we suggest densitometric measurement between 10 and 40 min after visualization.

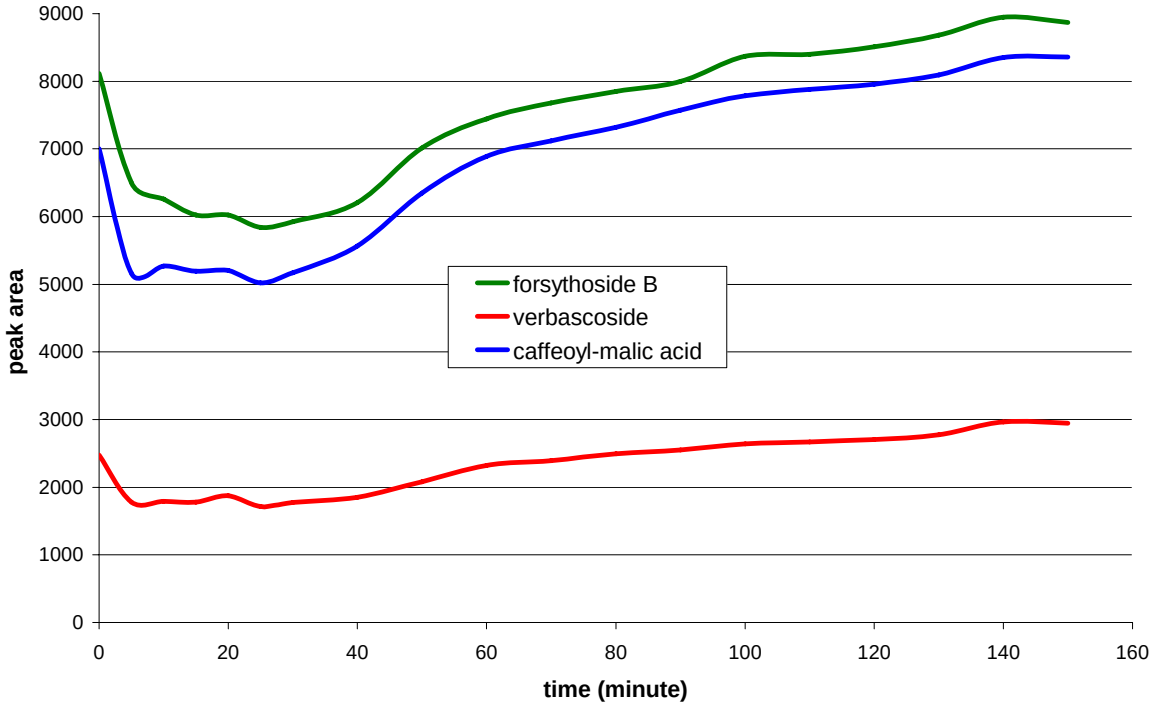



Fig. 10. Changes in the emission intensities of caffeoyl-malic acid, forsythoside B and verbascoside

## 4.3.1.5. Calibration

To determine the ranges of the quantities of the compounds for which there was a linear correlation with fluorescence intensity, calibration plots were drawn. The ranges were 0-2.75 µg for CM and 0-12.70 µg for FB and VE.

Every spotting was repeated twice. The calibration plots demonstrated the relationship between the amounts spotted and measured peak area.

Linear relationships were found in the ranges 0-1.65 µg for CM (Fig.11.), 0-13.00 µg for FB (Fig.12.), and 0.60-10.50 µg for VE (Fig.13.). The correlation coefficients ranged between 0.992 and 0.999.

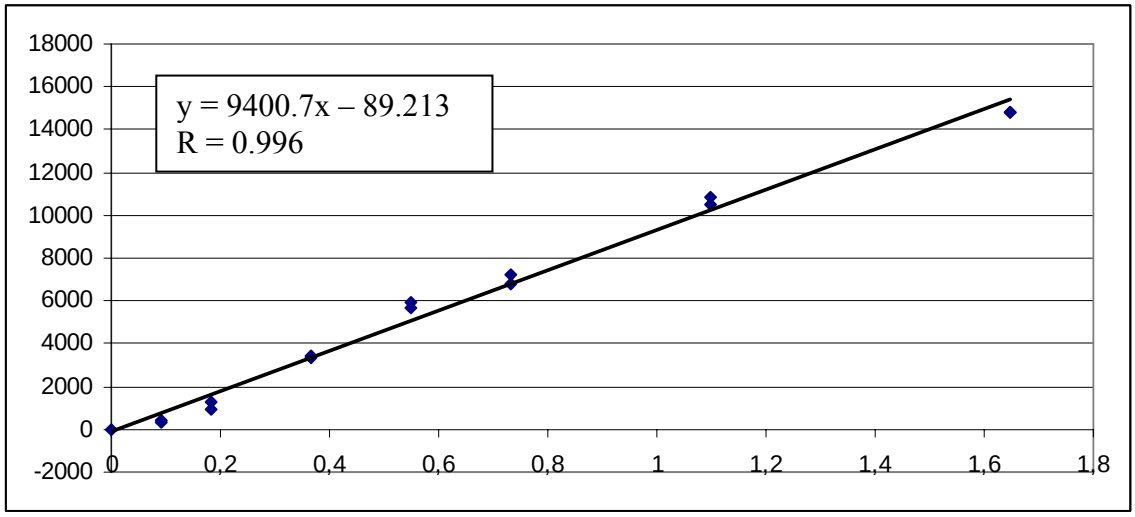



Fig.11. The calibration lineal of CM

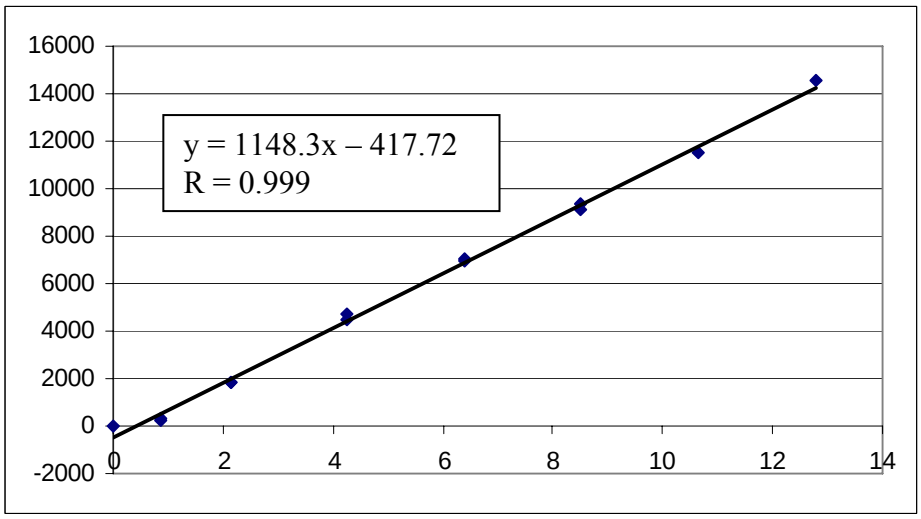



Fig.12. The calibration lineal of FB

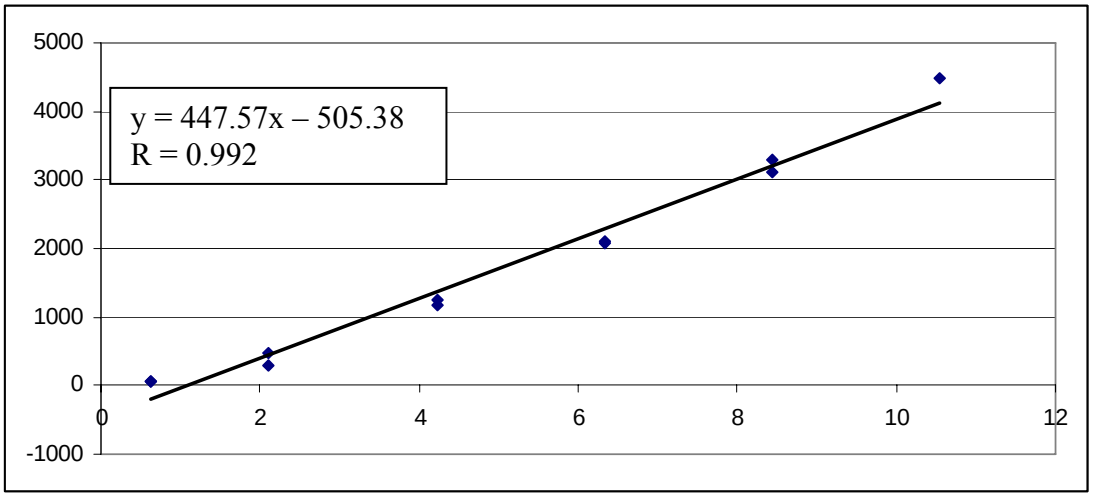



Fig.13. The calibration lineal of VE

### 4.3.1.6. Sensitivity

To establish the sensitivity of the method, the smallest detectable amounts of the three PPs were determined. The investigation was carried out with the 5 different mode of detection (Table 2.). The fluorescence of the components enabled exact quantification of even small amounts. Comparing the results we could establish that the lower limits of detection were 0.05 µg for CM, 0.3 µg for FB, and 0.6 µg for VE with the locating reagent Naturstoff and PEG 400. The sensitivity was the highest with the aluminium-trichloride reagent as table shows. This result confirmed our former evidence about the detecting reagent.

| Mode of detection | forsythoside B | verbascoside | caffeoyl-malic acid |
|---|---|---|---|
| aluminium-trichloride | 0.9 µg | 3.0 µg | 0.2 µg |
| boric acid and oxalic acid | 0.4 µg | 0.8 µg | 0.1 µg |
| naturstoff | 0.8 µg | 2.5 µg | 0.2 µg |
| **naturstoff + macrogol** | **0.3 µg** | **0.6 µg** | **0.05 µg** |
| UV | 0.8 µg | 1.5 µg | 0.2 µg |

Table 2. The smallest detectable amounts of the three PPs with 5 mode of detection.

### 4.3.1.7. Reproducibility

The reproducibility of densitometric evaluation was examined by tenfold replicate scanning of a mixture of authentic compounds on a plate. The result was good. Tenfold replicate densitometric estimation of the same spot yielded coefficients of variation of 1.90% for CM, 0.88% for FB, and 1.57% for VE (Table 3.). Thus, differences observed on replicate instrumental measurement were negligible.

| | peak area | | |
|---|---|---|---|
| | **forsythoside B** | **verbascoside** | **caffeoyl-malic acid** |
| **10 replicate** | 6135.3 | 1914.0 | 5442.5 |
| | 6145.6 | 1914.0 | 5448.3 |
| | 6139.2 | 1922.2 | 5484.5 |
| | 6107.4 | 1993.9 | 5507.2 |
| | 6149.6 | 1933.6 | 5535.1 |
| | 6187.6 | 1931.1 | 5548.5 |
| | 6093.0 | 1997.1 | 5640.6 |
| | 6226.5 | 1953.0 | 5667.9 |
| | 6231.2 | 1959.7 | 5707.9 |
| | 6248.8 | 1965.4 | 5721.5 |
| **average** | **6166.4** | **1948.4** | **5570.4** |
| **standard deviation** | **54.10** | **30.64** | **105.69** |
| **variation coefficient** | **0.877** | **1.572** | **1.897** |

Table 3. The reproducibility of the densitometric determinations with Naturstoff + PEG 400 reagent after 10 replicate

### 4.3.1.8. The optimal storage conditions

The best conditions for storage of PPs containing methanolic extracts of *B. nigra* were established. 1.0 g dried and powdered herb (collected in May 2007 in Vácrátót) was extracted with 50 ml methanol under reflux on a water bath for 30 min. After filtration and dilution to 50 ml, six 5 ml samples were separately exposed to three different storage conditions: in the dark under refrigeration at 5°C, in the dark at room temperature (25 ± 3°C), and in the light at room temperature. The amounts of CM, FB, and VE in the solutions were measured after 0, 1, 3, 6, 7, 10, and 14 days. The storage experiments 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 stored in the dark at either 5°C or at 25°C for 14 days; there were no losses whereas losses in the light were substantial-approximately 15% for both CM and FB and 37% for VE in 14 days (Fig.14., 15., 16.)

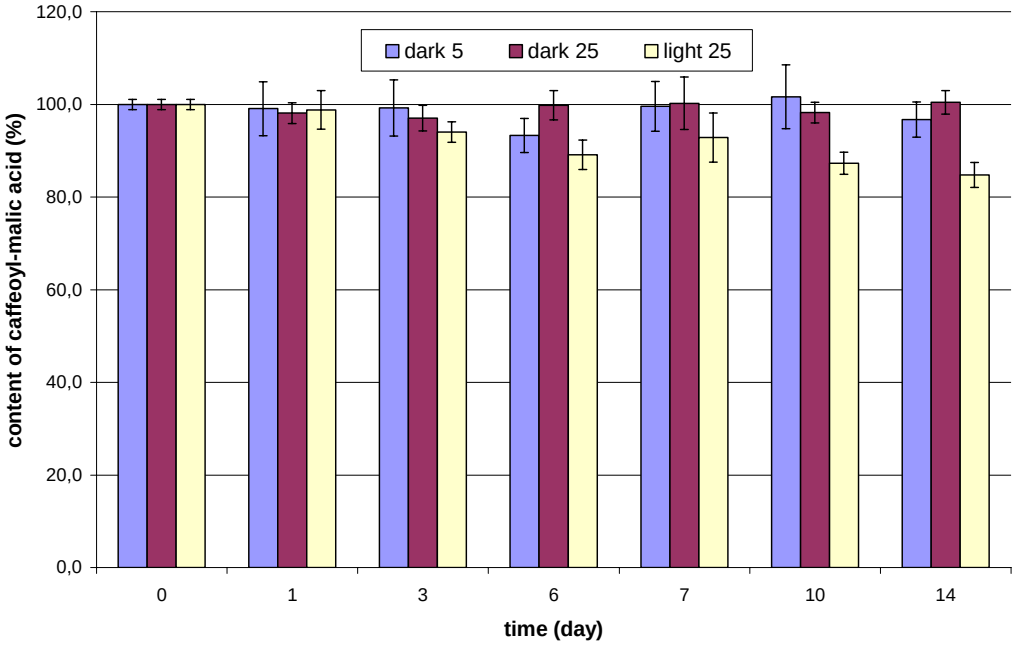



Fig.14. Variation of CM content of methanolic extracts of *B. nigra* stored in the dark at 5 °C (dark5), in the dark at room temperature (dark25), and in the light at room temperature (light25).

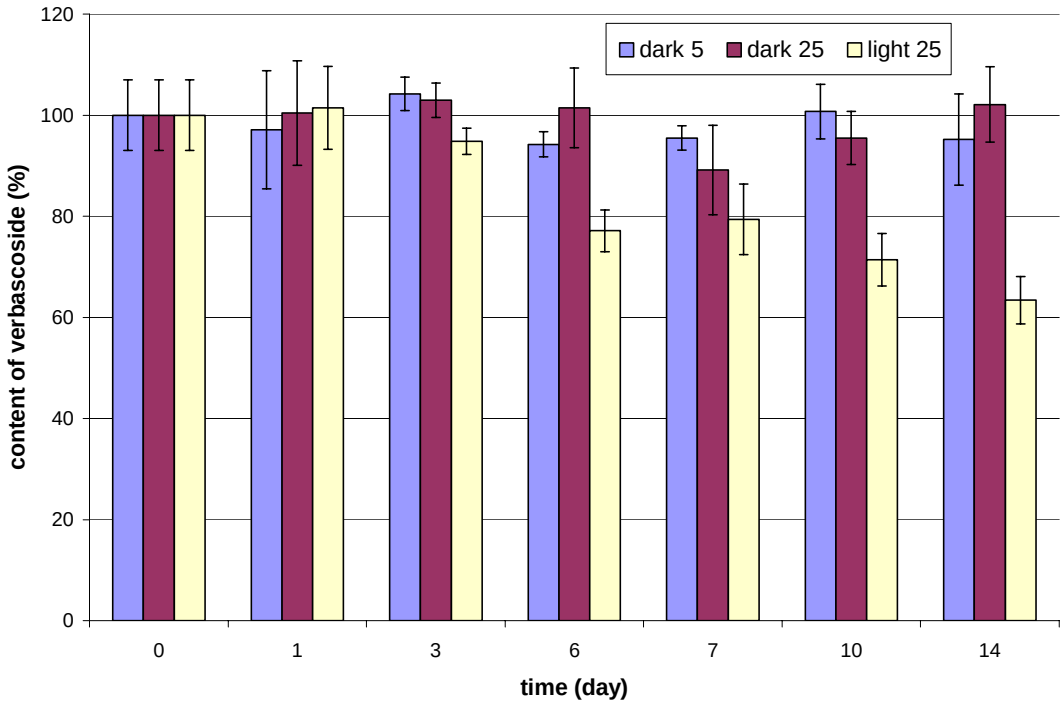



Fig.15. Variation of VE content of methanolic extracts of *B. nigra* stored in the dark at 5 °C (dark5), in the dark at room temperature (dark25), and in the light at room temperature (light25).

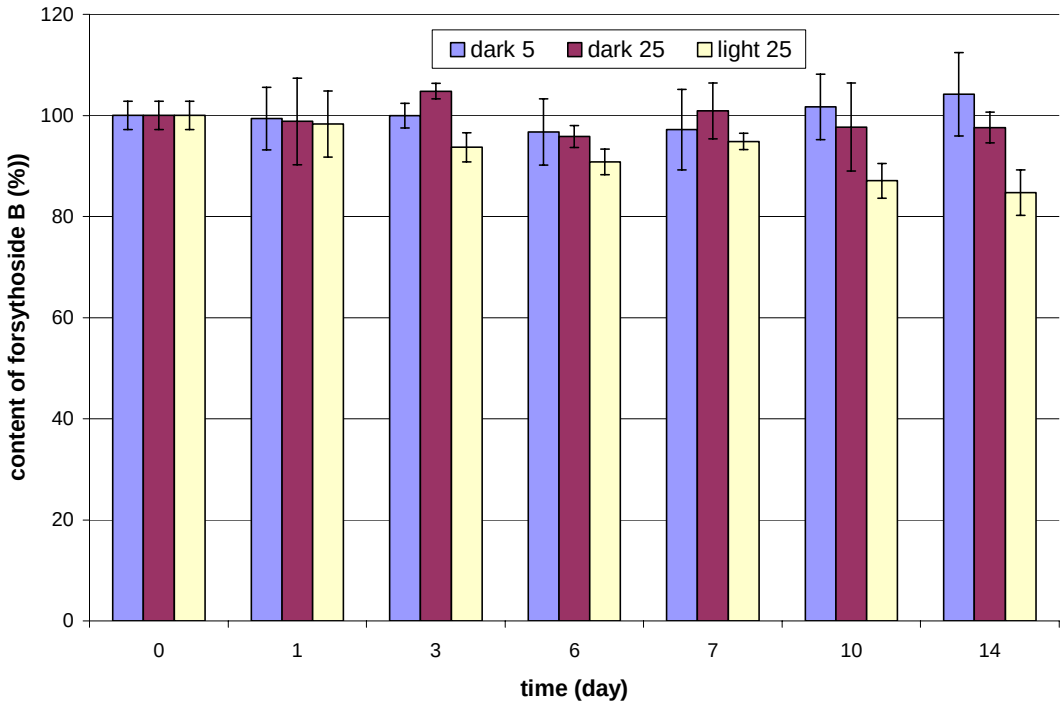



Fig.16. Variation of FB content of methanolic extracts of *B. nigra* stored in the dark at 5 °C (dark5), in the dark at room temperature (dark25), and in the light at room temperature (light25).

In further investigations the newly elaborated TLC-densitometric method was used. So the amount of the dried, powdered plant material was 0.5 g. The extracts were made with methanol (30 min heating under a reflux condenser). The filtered extracts were made up to 25 ml in each case. Two parallel investigations were carried out. The extracts were checked with TLC. It was performed on precoated aluminium plates (Kieselgel 60 $F_{254}$) in an unsaturated chamber, by using the ascending technique at room temperature. The volumes applied to the plates were 25 µl from the extracts, 5, 10, 15 µl from the standards. The mobile phase was formic acid-acetic acid-water-ethyl acetate (7.5:7.5:18:67). The developed plates were dried under an intense air-stream in a fume cupboard for 10 min. The plates were then dipped into a 1% methanolic solution of aminoethyl diphenylborinate and heated at 40°C in an oven for 10 min. The dried plates were subsequently dipped in a 5% solution of PEG 400 in methanol and then heated in the same way as above. The densitometric measurement was made between 10 and 40 min after visualization. The investigations were performed at 395 nm and filter no. III was chosen. The coefficients of variation were 1.90% for CM, 0.88% for FB, and 1.57% for

VE. In the following additional information are described in connection with the method by each investigation.

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

#### 4.3.2.1. Materials and methods: plant material and TLC-densitometry

In this investigation we wanted to obtain information on possible temporal fluctuations of PPs in all plant organs of *B. nigra* L. except roots (leaves, stems, shoots, flowers/reproductive parts). Plant material was collected in 2007 on every week from 23rd May to 29th October from the experimental field (Vácrátót). The recently developed TLC-densitometric method was used to test the PPs content in the *B. nigra* during the vegetative period. The studied compounds were VE, FB and CM. The applied volumes of solutions were 5, 10 and 15 µL (test compounds); 5 µL (roots and leaves); 10 µL (shoots and flowers) and 20 µL (stems). Two parallel were applied in the case of plant organs. (Fig.17.).

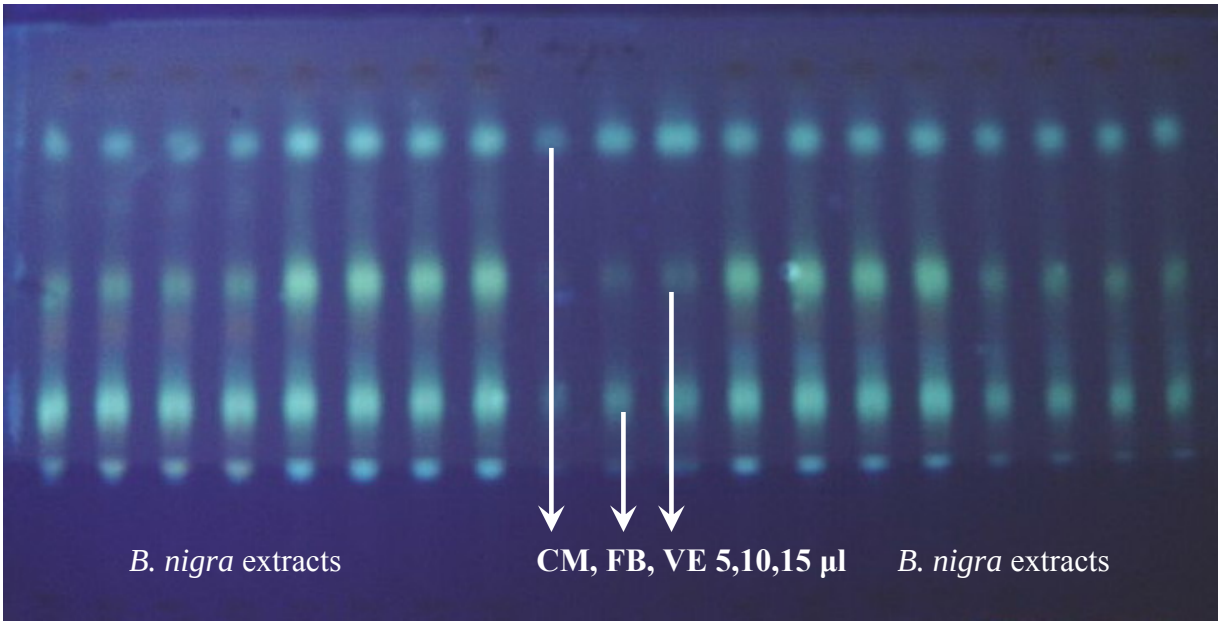



Fig.17. A developed plate detected in UV (395 nm) after visualization

### 4.3.2.2. Discussion

For all of the three investigated compounds, we observed similar tendencies in every single plant organ. The plant produced the three compounds in the biggest quantity at the beginning of our experiment (May), the maximum being reached at the beginning of summer (June), when the reproductive parts had been well developed, the flowering period had started (Fig.18, 19, 20.). With the increase in flowering intensity, the levels of the compounds started to decrease. This trend remained until the emergence of fruits. In the following six-seven weeks the measured quantities did not show significant changes. However, from the beginning of September, the amounts of the investigated phenylpropanoid glycosides and CM showed a moderate increasing trend. This observation was coincided with the appearance of fresh shoots. The rising trend was broken at the end of September because of slower plant development, which can be attributed to the decreasing average temperature.

The production of PPs reached the smallest quantity during October, when the first freeze appeared.

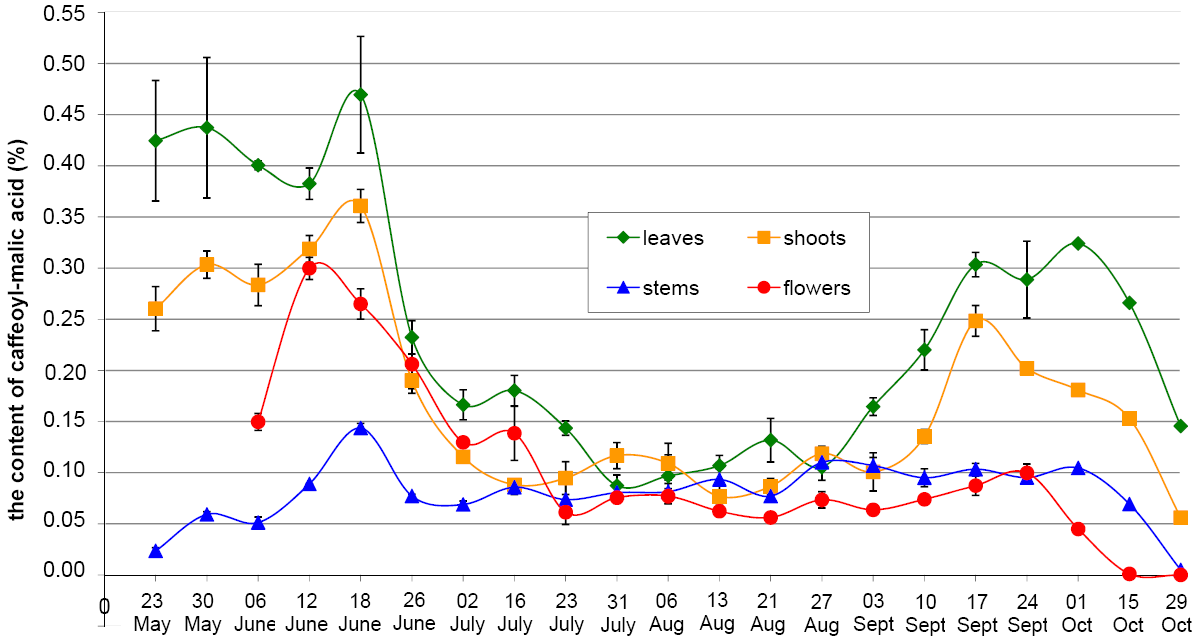



Fig.18. Variation of the content of CM in *B. nigra* in the vegetative period

Increases were observed in June and September, but that in June was much more pronounced. The levels of CM and VE changed more intensively than those of FB. 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 %. The highest values in the case of FB and CM were measured in the leaves of the young shoots (May-June), and of VE in the reproductive parts at the beginning of flowering.

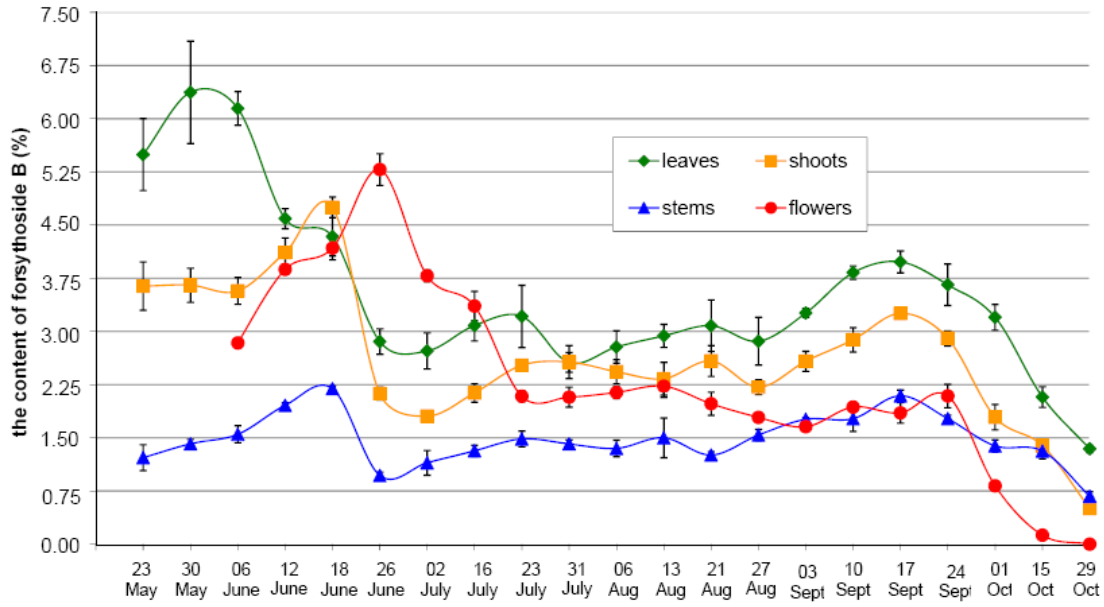



Fig.19. Variation of the content of FB in *B. nigra* in the vegetative period

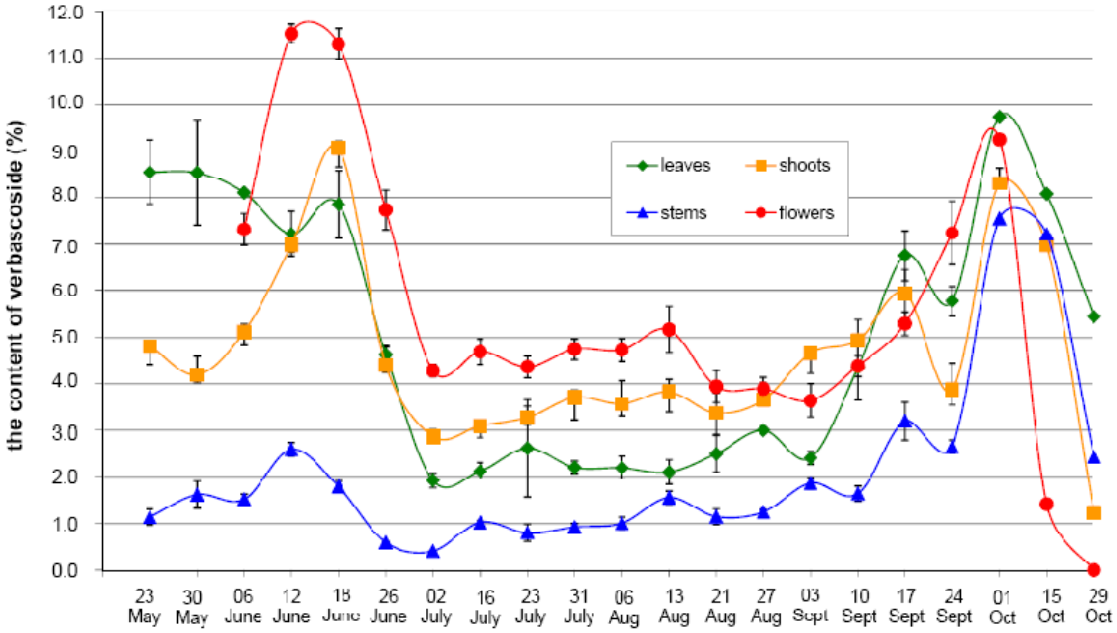



Fig.20. Variation of the content of VE in *B. nigra* in the vegetative period

Our findings indicate that the stems produced the compounds in the lowest (FB, VE: ≤ 2%, CM: ≤ 0.1%) concentrations in comparison with the other investigated plant organs and the variations in concentration during the vegetative period were not as intense in the stems (FB: max.: 2.19%, min.: 0.96%) as in the other organs (leaves, max.: 6.37%, min.: 2.56%).

### 4.3.3. Comparative analysis of *B. nigra* collected from different locations

The plant material was collected in the summer of 2007 from 11 locations. Table 4 shows the date and the place of *B. nigra* collection and the plant parts, which were available. The extraction and the TLC-densitometric investigation were carried out as described earlier by the methods.

| place of collection | date of collection (2007) | leaves | flowers | stems | shoots | roots |
|---|---|---|---|---|---|---|
| **Szomolya** | **9th June** | + | + | + | + | - |
| **Fót** | **17th June** | + | + | + | + | - |
| **Fót** | **25th June** | + | + | + | + | - |
| **Gyöngyössolymos** | **30th June** | + | + | + | + | - |
| **Fót** | **2nd July** | + | + | + | + | - |
| **Ópályi** | **8th July** | + | + | + | + | + |
| **Kerecsend** | **11th July** | - | - | - | + | + |
| **Brasov** | **14th July** | + | + | + | + | + |
| **Nagyvázsony** | **26th July** | + | + | + | + | + |
| **Gyenesdiás** | **18th August** | - | - | - | + | + |
| **Gyenesdiás** | **19th August** | - | - | - | + | - |
| **Rezi** | **19th August** | - | - | - | + | - |
| **Sződliget** | **21st August** | + | - | - | + | + |
| **Szeged** | **23rd August** | - | - | - | + | - |

Table 4. The date, the place of *B. nigra* collection and the collected plant parts

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. CM was not present in measurable quantities in the roots of the Gyenesdiás and Kerecsend samples, but values under 0.05 % were obtained for the roots of plants from Nagyvázsony, Ópályi, Sződliget and Brasov, respectively. The FB content ranged between

0.1-3 % (flowers: 2-3%). The values of VE were the highest in the flowers (7-10 %). The exception was the plants collected from Gyöngyössolymos, the flowers of which yielded only 2.5%. In the roots, VE was dominant, as had been observed by us previously by the investigation of *B. nigra.*


Fig.21. The locations of *B. nigra* collection (marked with red)

The *B. nigra* samples from Szomolya confirmed our former investigations in which it was shown that PPs production reached its highest level at the beginning of June. VE was the most significant, with a content of 5.9 % in the leaves, 10.0 % in the flowers and 4.4 % in the shoots. The FB contents were lower (1.2 % in the leaves, 2.2 % in the flowers). CM levels were less than 0.5 %, which was expected. Two collections were made from Fót in June and the results from both were very similar. VE production in the flowers was high (7.6 % and 8.2 %). These values were not remarkable, showed the former tendencies of the vegetative analysis.

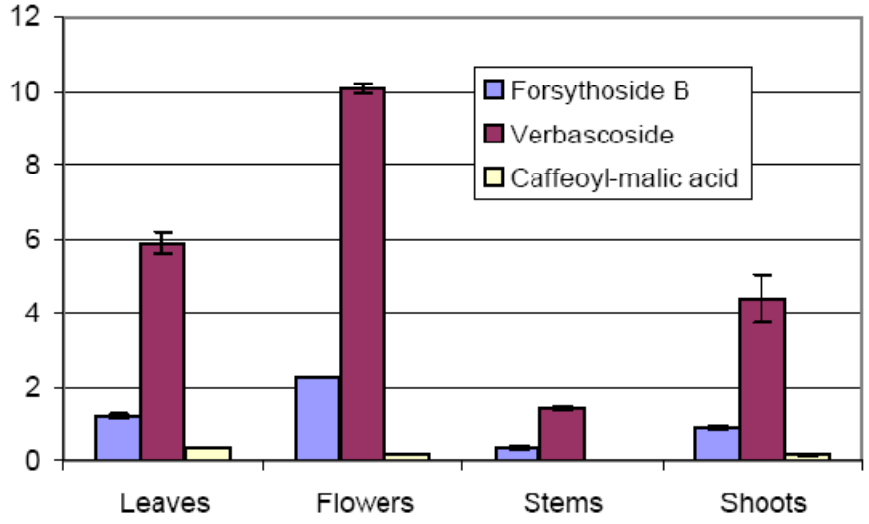



Fig.22. The PPs content (%) of *B. nigra* collected from Szomolya (9th June)

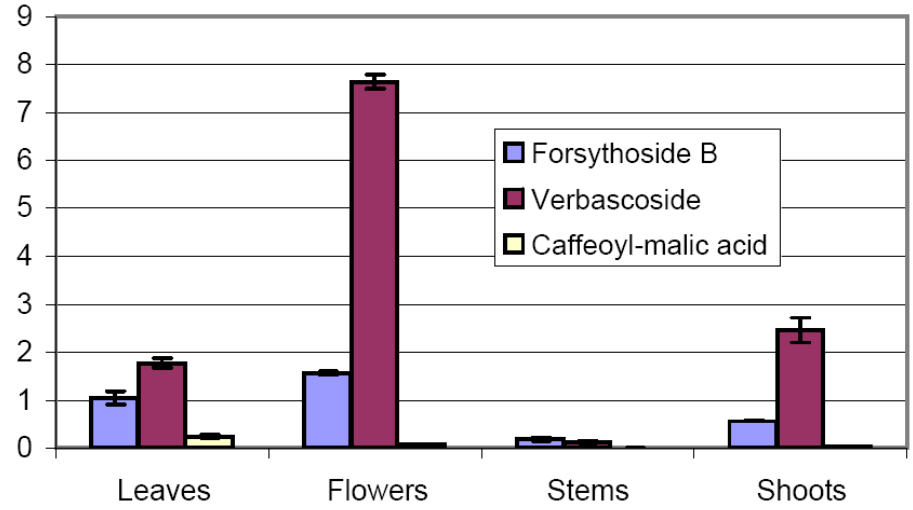



Fig.23. The PPs content (%) of *B. nigra* collected from Fót (17th June)

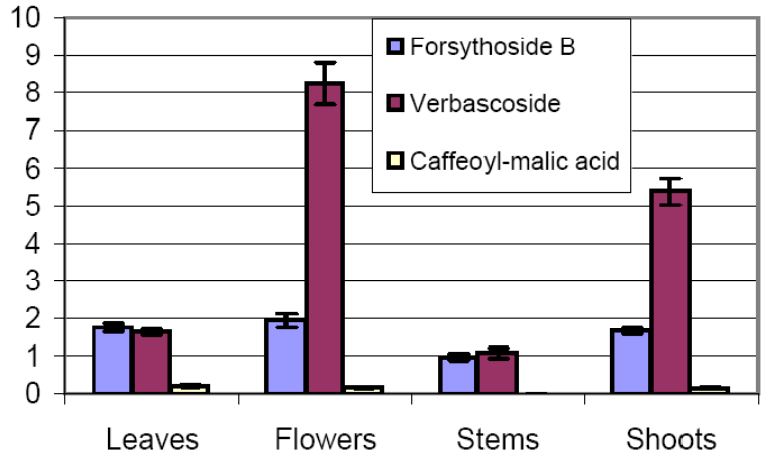



Fig.24. The PPs content (%) of *B. nigra* collected from Fót (25th June)

However, FB production was lower than that detected by us in the samples from the experimental field in Vácrátót. FB content was less than 2 % in the samples from Fót in comparison with 5-6 % from the Vácrátót samples.

The collection from Gyöngyössolymos made at the end of June showed a decreasing trend in PPs production. VE was under 2.5 % in the flowers, while VE and FB contents were 1 % in both the leaves and shoots.

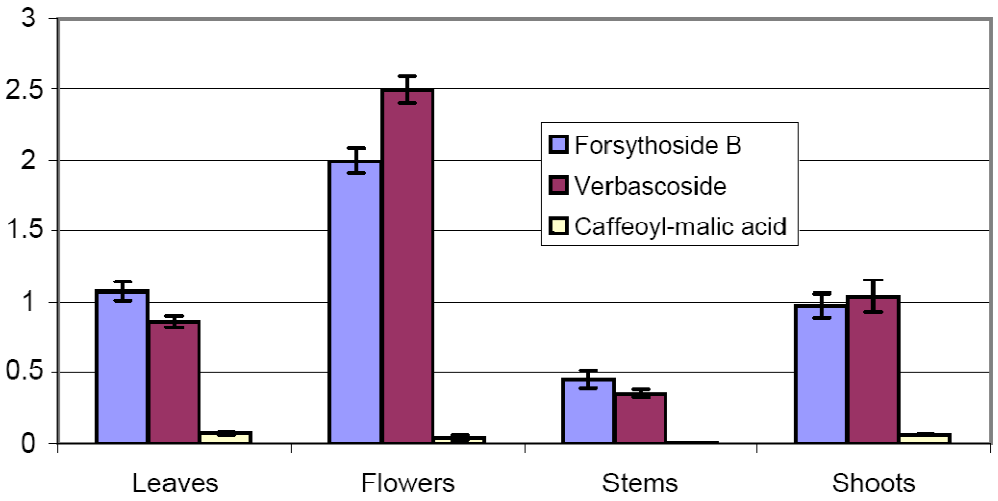



Fig.25. The PPs content (%) of *B. nigra* collected from Gyöngyössolymos (30th June)

At the beginning of July, PPs content decreased, with the exception of VE in the flowers (7.3%) from Fót. The FB content of the leaves was 0.8 %, VE 0.2 % and CM 0.2 %. These values were similar to those for the stems.

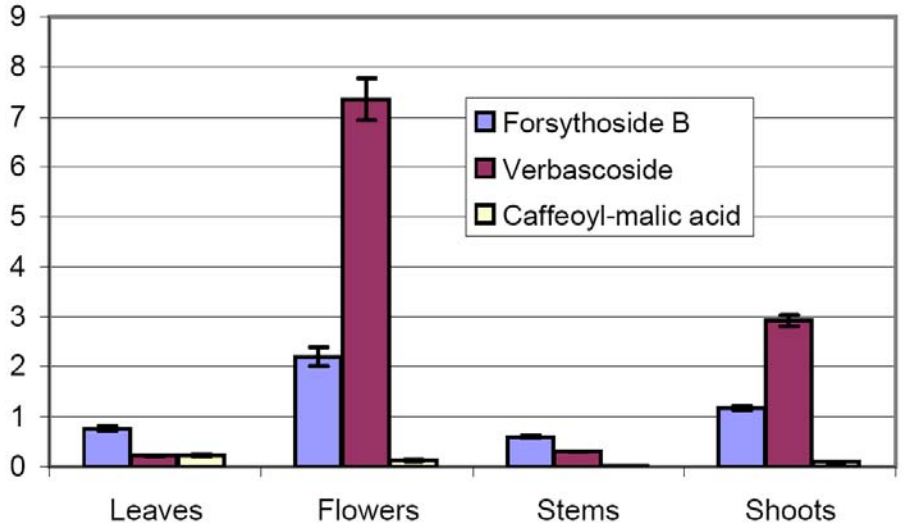



Fig.26. The PPs content (%) of *B. nigra* collected from Fót (2nd July)

The leaves of *B. nigra* from Ópályi, collected in early July, contained 2.0 % FB, 2.6 % VE and 0.2% CM. These data were similar to former trends, but VE production in the flowers was above 10 %. In the roots, VE was dominant (6.6 %). VE levels in the Brasov samples were also high (flowers: 9.3 %; shoots: 10.4 %; roots: 11.3 %). In the leaves and shoots, CM content reached 1 %, which was the highest level recorded in our investigations. The VE quantity of the flowers (9.7%) and roots (15.2%) was also prominent in the samples from Nagyvázsony.

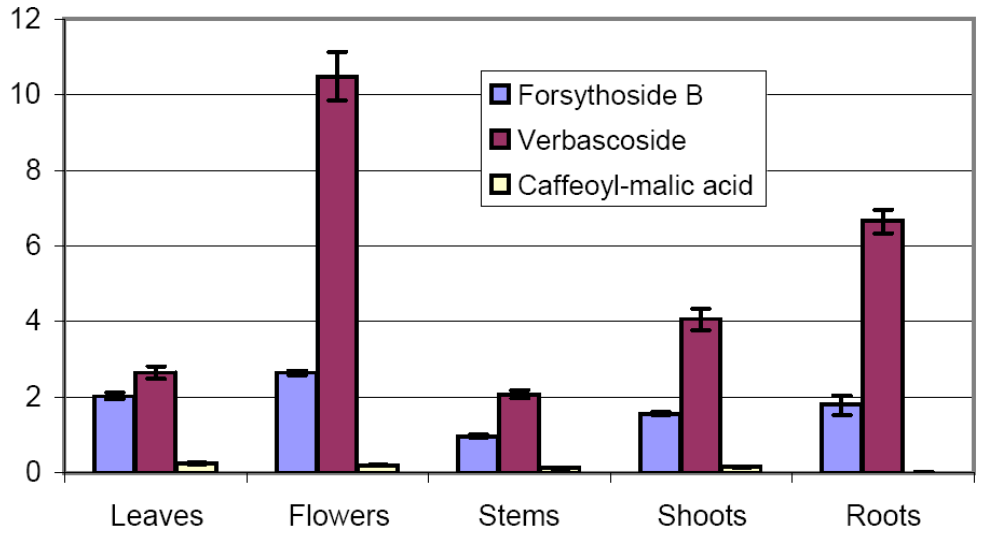



Fig.27. The PPs content (%) of *B. nigra* collected from Ópályi (8th July)

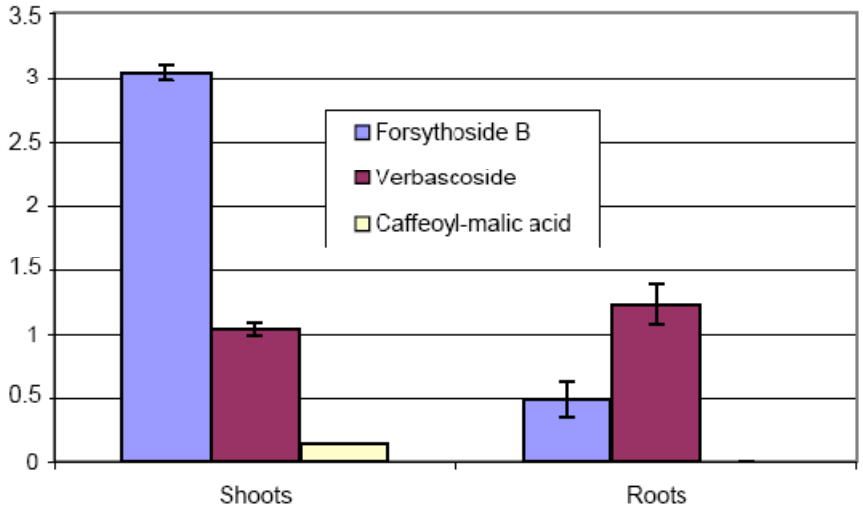



Fig.28. The PPs content (%) of *B. nigra* collected from Kerecsend (11st July)

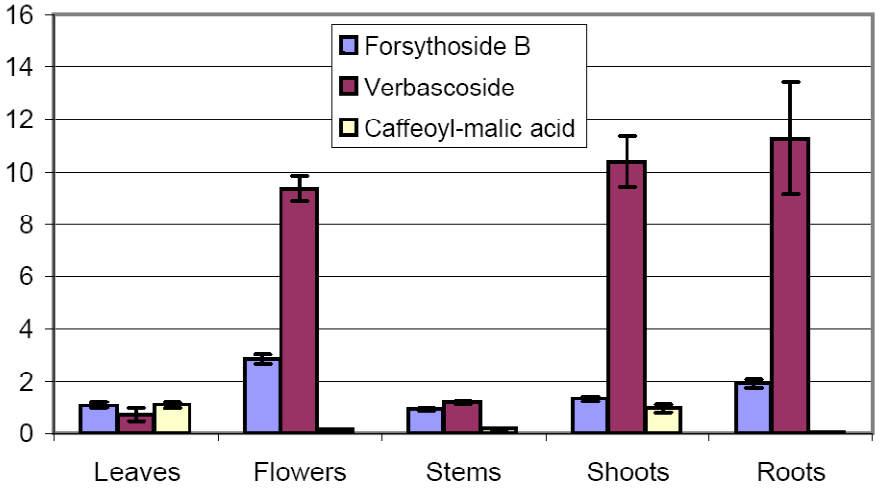



Fig.29. The PPs content (%) of *B. nigra* collected from Brasov (14th July)

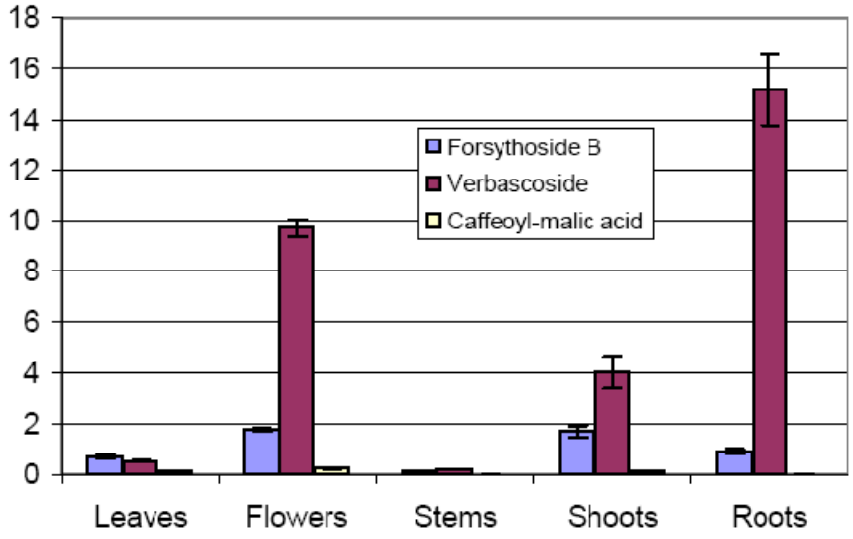



Fig.30. The PPs content (%) of *B. nigra* collected from Nagyvázsony (26th July)

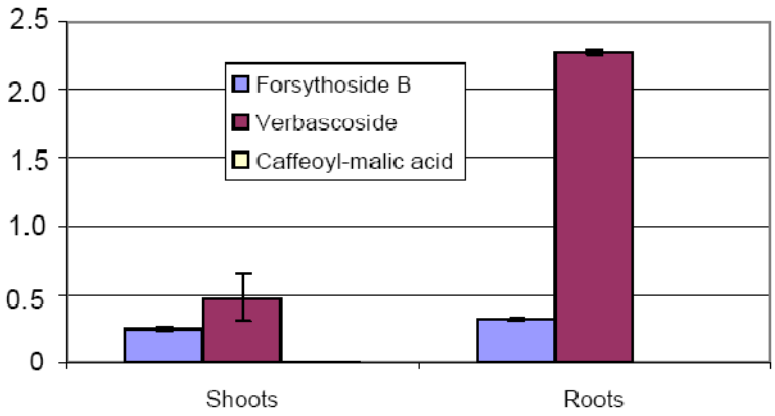



Fig.31. The PPs content (%) of *B. nigra* collected from Gyenesdiás (18th Aug)

The *B. nigra* samples gathered in August contained between 1-2 % of PPs. The VE content was not as high as in earlier samples. Roots were only available from the Gyenesdiás and Sződliget collections, in which VE was predominant.

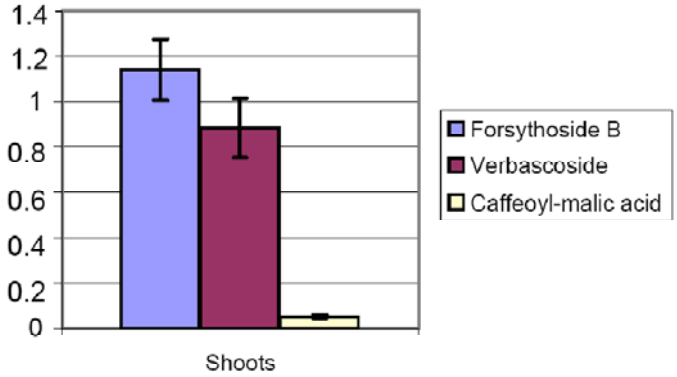



Fig.32. The PPs content (%) of *B. nigra* collected from Rezi (19th Aug)

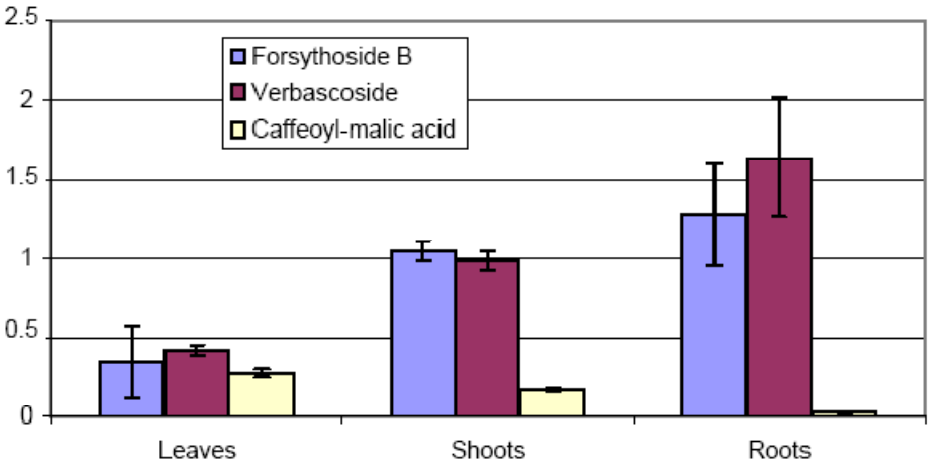



Fig.33. The PPs content (%) of *B. nigra* collected from Sződliget (21st Aug)

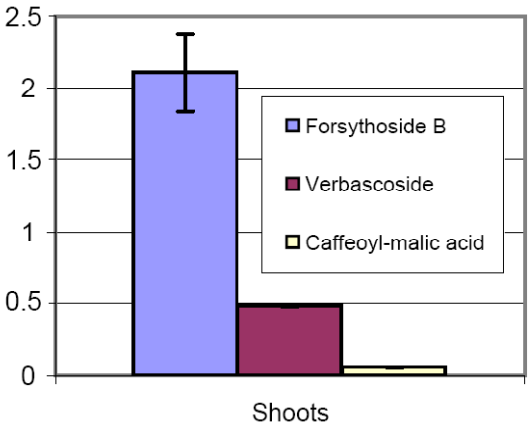



Fig.34. The PPs content (%) of *B. nigra* collected from Szeged (23rd Aug)

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

### 4.3.4.1. Materials and methods

For the comparative study, fresh material of *B. hirsuta* and *B. rupestris* was collected in July 2007 (at full flowering stage) from the experimental field (Vácrátót). Voucher specimens of each species have been deposited in the herbarium of the same institute (*B. nigra* no. L2111; *B. hirsuta* no. L2055 and *B. rupestris* no. L2115). *B. nigra* collected in July for vegetative analysis was used in the comaparative analysis also. The extraction, thin layer chromatography and densitometry were carried out as described earlier.

### 4.3.4.2. Discussion

First of all the three PPs (FB, VE, CM) were averrable in all the investigated *Ballota* species. This is the first report for *B. hirsuta* (native in Spain) and *B. rupestris* (native in Italy).The most significant observations always concerned with CM, which always produced the lowest absolute values (0.0%-0.31%), and which was not found in the roots of any of the three *Ballota* species, but showed maximum levels in the leaves of each plant (*B. rupestris*: 0.31%, *B. nigra*: 0.23%, *B. hirsuta*: 0.08%). Other similarities concerning the three species and the PPs were not apparent.

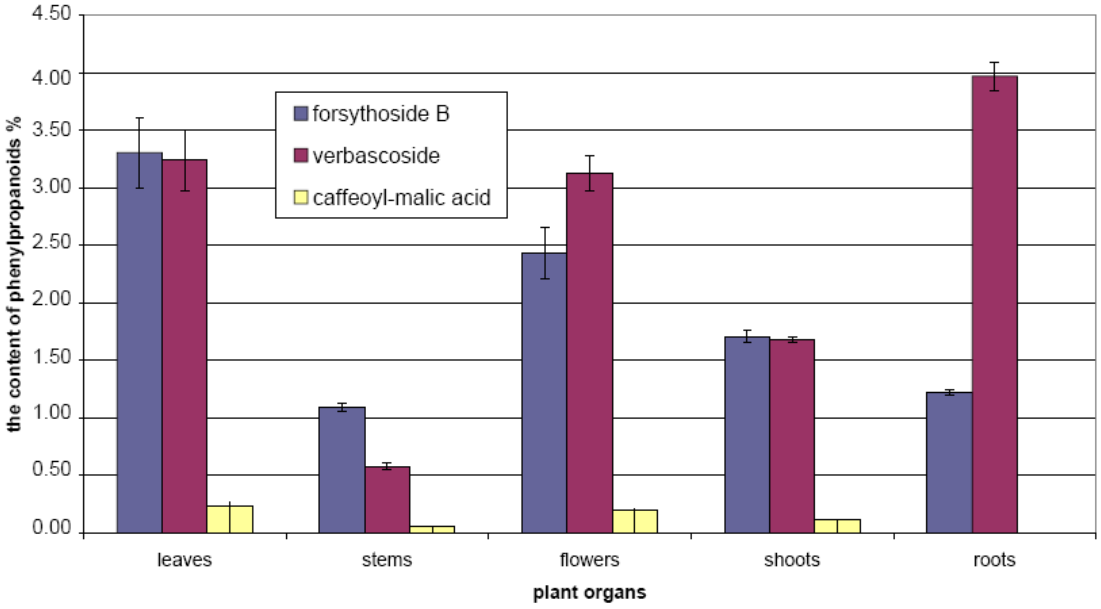



Fig.35.The content of PPs in *B. nigra*

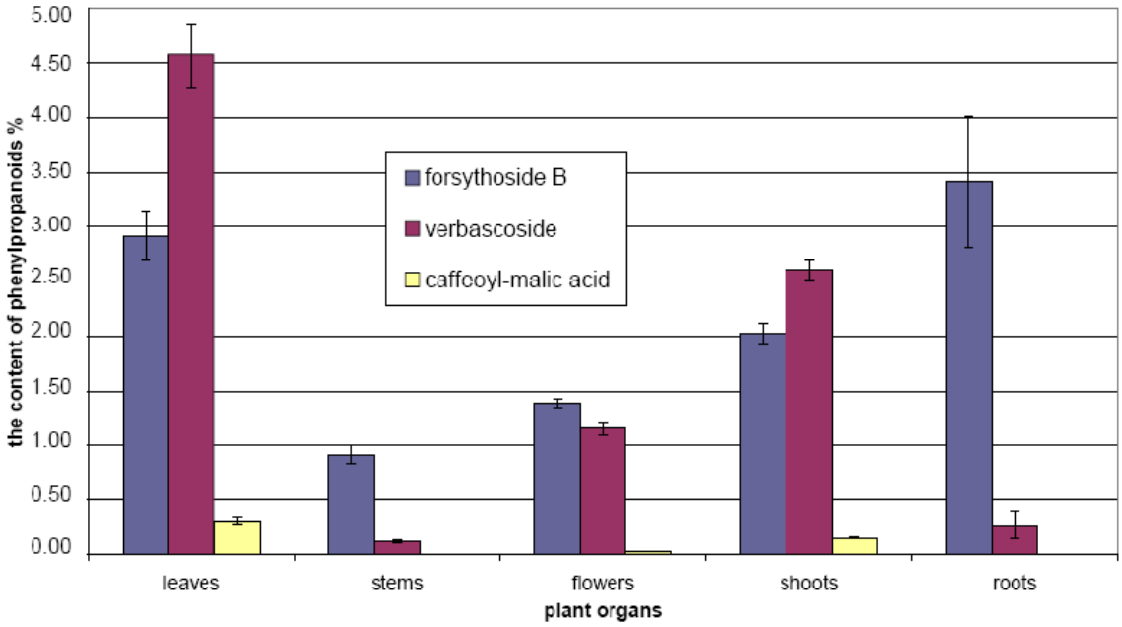



Fig.36. The content of PPs in *B. rupestris*

*B. rupestris* showed the highest amounts of the examined components: CM (0.3%) and VE (4.58%) in leaves and FB (3.0 %) in roots. *B. nigra* had 5-20% while *B. hirsuta* had 30-50% less maximum yields.

In the leaves of ***B. nigra*** (Fig.35.) the amount of FB and VE reached 3.30 %. CM was 0.23 %. The stems comprised in 1.09 % FB and in 0.57 % VE. The flowers contained the components almost equally with the leaves, but in smaller quantities (FB 2.43 %, VE 3.13 %, CM 0.2 %). In the roots we could measure high amounts of VE (3.96 %), but CM was not present in detectable quantities. The leaves of ***B. hirsuta*** (Fig.37.) produced the compounds in the following quantities: FB 0.91 %, VE 1.64 %. The flowers showed something higher amounts of FB and VE than the leaves. In the roots we could find 2.44 % FB production and 0.69 % VE content. ***B. rupestris*** (Fig.36.) showed the highest amounts of the examined components in the following cases. In the leaves of *B. rupestris* the VE quantity was 4.57 % and the FB was 2.91 %. The concentrations of the compounds in the flowers were much lower than in the leaves (1.38 % FB, 1.15 % VE). In the roots we could experience high levels of FB (3.41 %).

In the following cases the roots produced FB and VE in higher values than the leaves. FB in *B. hirsuta*: 0.9 % (leaves) and 2.4 % (roots) and in *B. rupestris*: 2.91% (leaves),

3.41% (roots); VE in *B. nigra*: 3.2 % (leaves) and 4.0 % (roots). In conclusion the roots of *B. nigra* contained VE in higher amounts, but in all the other species FB was dominant.

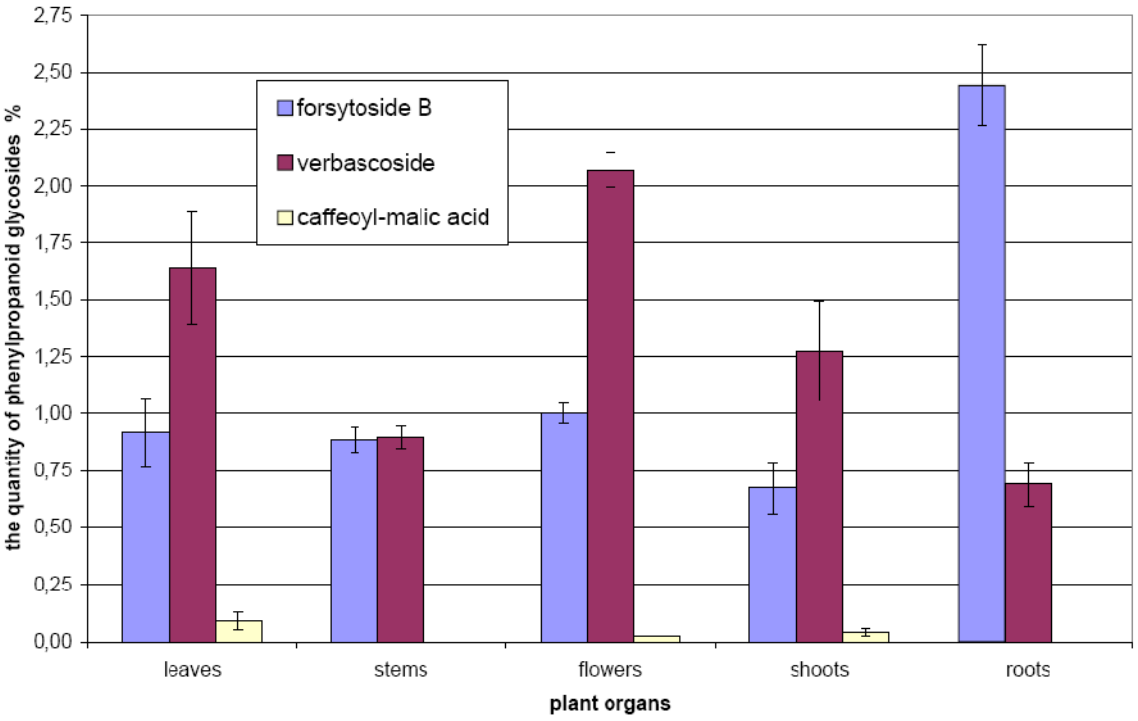



Fig.37. The content of PPs in *B. hirsuta*

In contrast with *B. rupestris* (FB: 1.38%, VE: 1.15% (flowers); FB: 2.91%, VE: 4.57% (leaves)), the flowers of *B. nigra* (FB: 2.43%; VE: 3.13%) and *B. hirsuta* (FB: 1.00%; VE: 2.06%) produced similar or even higher amounts of FB and VE than their leaves (*B. nigra* FB: 3.30%, VE: 3.23%) (*B. hirsuta* FB: 0.91%, VE: 1.64%). In comparison with other parts of *B. nigra* and *B. rupestris*, the stems showed significantly the lowest FB and VE values.

## 4.4. Antioxidant investigations

Former researches attribute most of the biological activities to the phenylpropanoids. The antioxidant activity was proved by us also with two methods (assay of lipid peroxidation and DPPH radical scavenging assay). The investigated compounds were verbascoside, forsythoside B, caffeoyl-malic acid and martynoside.

The enzyme-independent lipid peroxidation (LPO) was assayed on a standard rat-brain homogenate by estimating the thiobarbituric acid-reactive substances by using the standard

method with minor modifications. In brief, different concentration of extracts was added to 5 ml brain homogenate. After 60 min, 2 ml of 28% trichloroacetic acid (TCA) was taken in the tubes. After centrifugation the supernatant was separated and mixed with 1 ml of 1% thiobarbituric acid (TBA). The mixture was heated in a 95 ˚C water bath for 15 min. The production of malonyldialdehyde (MDA) was measured at 532 nm. The percentage inhibition of lipid peroxidation was calculated by comparing the results with those of controls not treated with the extracts.

Radical scavenging activity of plant extracts against stable 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) was determined spectrophotometrically. DPPH is not a biologically important free radical. It is a stable free radical and its free radical character is neutralized in presence of molecules having ability to donate a hydrogen atom . When DPPH· reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep-violet to light-yellow) were measured at 519 nm on a UV/VIS light spectrophotometer. Inhibition of free radical by DPPH in percent was calculated. Extract concentration providing 50% inhibition ($IC_{50}$) was calculated from the plot of inhibition percentage against concentration. The compounds showed antioxidant activity as shown in the Table 5 and 6.

| **Compound** | Inhibition of LPO | Inhibition of scavenging activity |
|---|---|---|
| forsythoside B | 57.71 | 38.91 |
| verbascoside | 84.36 | 84.95 |
| caffeoyl-malic acid | 91.04 | 93.16 |
| martynoside | 14.92 | 65.05 |

**Table 5.** Inhibitory effect of the pure compounds at 60 μg/ml

| Compound | $IC_{50}$ (μg/ml) | |
|---|---|---|
| | Inhibition of LPO | Inhibition of scavenging activity |
| forsythoside B | 47.25 | >100 (104.1) |
| verbascoside | 19.01 | 14.53 |
| caffeoyl-malic acid | 9.244 | 1.786 |
| martynoside | >100 (383.7) | 17.86 |

**Table 6.** Inhibitory effect of the pure compounds on LPO and DPPH radical.

# 5. CONCLUSIONS, RESULTS

## 5.1. Isolation from *B. nigra*

Phenylethanoid glycosides, including FB (Seidel et al., 1999), 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 (Seidel et al., 1999; Ahmad et al., 2004), but 7a-acetoxyroyleanone is the first quinoid type diterpene to be reported for the genus. Ladanein has been identified in *Ballota* species, for example in *B. saxatilis* C. Presl. (Citoglu et al., 1999) and *B. hirsuta* Benth. (Seidel et al., 1999), as well as from a species of the closely related genus *Marrubium* [from *M. trachyticum* Bois. (Citoglu et al., 2002)], but this is the first finding in *B. nigra*. 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 system) (Cantino et al., 1986) to which both ganera; *Ballota* and *Marrubium* belong. Sporadic data justify their presence in the Subfam. Nepetoideae, but martynoside was only found in *Salvia officinalis* (Hohmann et al., 2003). Our observations support the chemotaxonomic similarities and differences suggested by Erdtman's, and Cantino, Harley and Wagstaff's classifications (Cantino et al., 1992).

## 5.2. 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 attributed to its PPs content. There are numerous literature data on the isolation of PPs from *Ballota* species, but apart from one LC-MS investigation (Plaza et al., 2005) no publications deal with the distribution of these compounds have been found. A rapid TLC-densitometric method with acceptable accuracy has been elaborated for quantitative routine analysis of the most characteristic components of *B. nigra*. Quantitative data of these compounds, i.e. VE, FB, and CM were not available in the scientific literature

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

To get the maximum of the investigated PPs we tried two methods of extraction. The amount of the dried, powdered plant material was 0.5 g. The extraction with reflux condenser was more efficient. In our investigations we used this method. The mobile phase was formic acid-acetic acid-water-ethyl acetate (7.5:7.5:18:67) (the official mobile phase in the Ph.Hg. VIII.). In the optimization of the densitometric method we determined the mode of detection, in which Naturstoff and PEG 400 reagent gave the best results. 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 measurements. With the calibration lineal of each investigated PPs we determined the linear relationships. To establish the sensitivity of the method, the smallest detectable amounts of the three PPs were determined. The reproducibility of densitometric evaluation was examined by tenfold replicate scanning of a mixture of authentic compounds on a plate. 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 stored in the dark at either 5°C or at 25°C for 14 days. We suggest the use of the method in the Pharmacopoeia.

## 5.3. 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 variation 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.

For all of the three 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 %. The VE content reached the 12 %. The

highest quantity of FB was 7 %. Our results give important evidences of the utilization of the plant.

## 5.4. 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*. The content of CM was also the smallest in this investigation, but in one case it reached 1 %. The samples gathered in June produced the PPs in advanced quantity. In July we could measure high VE values in Fót, Ópályi, Brasov, and Nagyvázsony. The *B. nigra* samples gathered in August from Gyenesdiás, Rezi, Sződliget and Szeged produced the PPs in 1-2 %. We could establish that the PPs production of *B. nigra* from different places was rather similar. By our investigations we could summarize the results that for collection and cultivation of *B. nigra* any part of Hungary is suitable. From the point of view of utilization we suggest the collection in the flowering period because of the high phenylpropanoid content.

## 5.5. 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 a newly developed TLC-densitometric method. The studied compounds were VE, FB and CM. Similar amounts were detected in *B. rupestris* and *B. nigra*, with quantitative differences in the plant organs, while *B. hirsuta* had the least amounts. The lack of CM was a characteristic only of the roots. In conclusion the roots of *B. nigra* contained VE in higher amounts, but in all the other species FB was dominant.

# 6. SUMMARY

*B. nigra* is a very common plant in Europe and in the Mediterranean region. The main components of the plant are diterpenes, phenylpropanoid glycosides, flavonoids. Most of the terpenoids isolated from the genus *Ballota* are labdane diterpenoids. The royleanone type diterpenes are characteristic only of *Salvia* species in the Subfamily Nepetoideae. That's why the presence of 7-a-acetoxyroyleanone in *B. nigra* (in the Subfamily Lamioideae) is of great chemotaxonomic importance from a theoretical viewpoint. The other isolated compound; ladenein is also new in the *Ballota nigra.*

This work mainly deals with the phenylpropanoid content, the supposed active ingredients of the plant. As our investigations (isolation and NMR analysis) proved, this group of compounds is present in the plant in the highest quantity.

The Hungarian Pharmacopoeia VIII does not contain quantitative information about the main phenylpropanoid glycosides. (After a spectrometric investigation it calculates the content of total ortho-dihydroxycinnamic acid derivatives (Pharmacopoeia).) So the elaborated TLC-densitometric method proved to be quick and efficient for the quantitation of the phenylpropanoids. We suggest its use in the Pharmacopoeia. Our TLC-densitometric method gives accurate (CV= 2 %), quick, straight information about the CM, FB, and VE content of less than 0.5 g dried drug samples.

The newly elaborated analytical method for the quantitative measurement of PPs made it possible to study the variation of the three compounds: VE, FB and CM in plant organs and in the vegetation period. The comparison of *B. nigra* samples gathered from various regions of Hungary and also the comparative study of other two *Ballota* species could have been performed with the help of this method. The main conclusions are as follows:

- The PPs content proved to be the highest in inflorescence and leaf and between them the shoots which were followed by root and stem.

- The highest content of PPs was in the first and second flowering stages of development (in June and in September). The variation of the three compounds shows similar, more or less parallel changes in all the organs.

- *Ballota nigra* samples gathered from ten locations, from hilly and flat areas of various regions of Hungary do not show significant differences. The slight differences can be attributed more to the differences in phenological stages (the samples were not harvested exactly at the same time) than to the geographical origin of the plants. 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.

-VE, FB, CM contents of *B. nigra, B. rupestris* and *B. hirsuta* plants growing under similar conditions and harvested at the same time were determined. All the three compounds; VE, FB, CM were present in all organs (except CM in roots). The highest PPs concentration was in all cases in the inflorescence, in the composition of compounds, however some, not significant differences were observed. *B. rupestris* produced somewhat higher PPs values than *B. nigra* in the leaves and roots. *Consequently,* this species should also be suggested, beside B. *nigra*, for further studies as a drug candidate.

In all *B. nigra* seems to be a plant that can be cultivated well in Hungary and have advantageously high PPs content. Its cultivation and collection for producing drug can be suggested.

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**ACKNOWLEDGEMENTS**

I would like to express my grateful thanks to my supervisor

**Professor Dr. Imre Máthé**,

for the continuous support, encouragement, thanks for his inexhaustible optimism, constructive thoughts during my researching years and for his help finishing my PhD thesis. I thank to Professor Mathe also, that I could do my work in the beautiful surroundings of the botanical garden of Vácrátót as a member of his research group.

I would like to express my thanks to **Prof. Dr. Judit Hohmann**, the head of the Department of Pharmacognosy in the University of Szeged and to **Dr. Katalin Török** the director of the Institute of Ecology and Botany of the Hungarian Academy of Sciences, Vácrátót, who made me possible to do the work in those institutes.

I express my thanks to **Dr. Gábor Janicsák** for the direct coordination of my work, for his exactness, critical remarks, and help in the TLC-densitometric investigations.

Special thanks to **Professor Gábor Tóth** for the NMR investigations.

I would also like to express my thanks to **Professor Dr. Gerald Blunden** for his support and remarks hereby perfecting my thesis.

I would like to thank the help in the plant collection for **Krisztina Szabó**, and for **Mrs János Juhász** for the technical help in the laboratory.

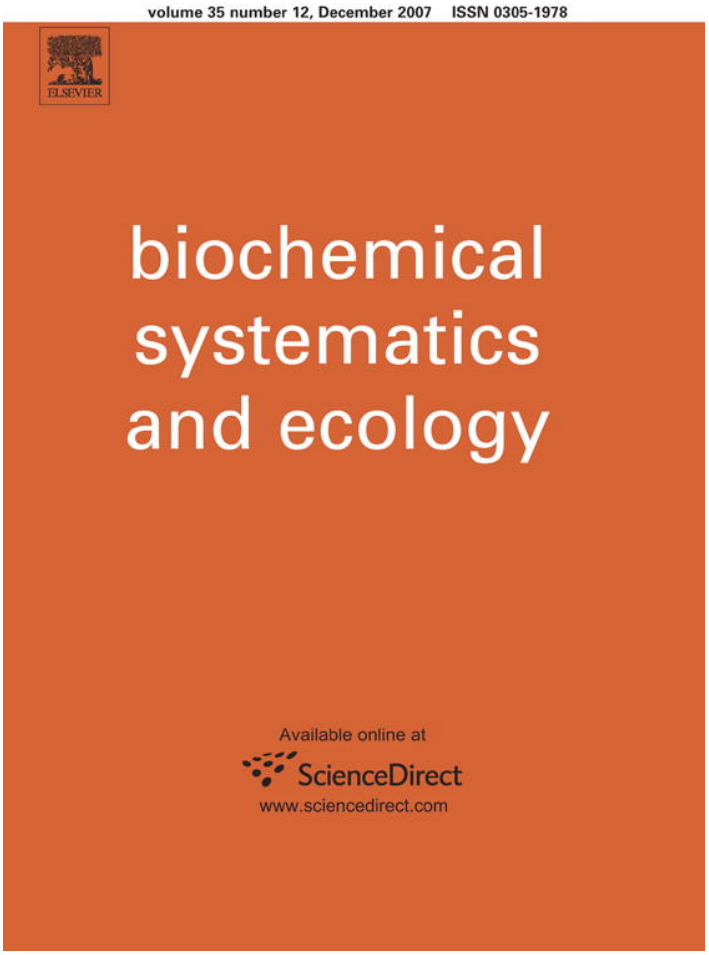
I thank to **Borbála Réthy** for the antioxidant studies.

We acknowledge financial support from the **Hungarian National Foundation** (grant OTKA T043148)

At last, but not least I thank very much for my husband's self-sacrificing help in the technical finish. And I would like to express my grateful thank to my husband's, my parents' and my brothers' continuous encouragement and loving support in reaching my aims.

# ANNEX

# I.



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Biochemical Systematics and Ecology 35 (2007) 894–897
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# Martynoside, forsythoside B, ladanein and 7a-acetoxyroyleanone from *Ballota nigra* L.

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Received 23 March 2007; accepted 21 April 2007

*Keywords:* Lamiaceae; *Ballota nigra* L.; Martynoside; Forsythoside B; Phenylethanoid glycosides; Ladanein; 7a-Acetoxyroyleanone

## 1. Subject and source

*Ballota nigra* L. (Lamiaceae) is widely distributed in Europe. In Hungary, this species is used as a medicinal plant, and in the new Hungarian Pharmacopoeia (VIII Edition), it has become official. We have isolated β-sitosterol; two phenylethanoid glycosides, martynoside (Fig. 3) and forsythoside B (Fig. 1); a flavonoid, ladanein (Fig. 2); and a diterpene, 7a-acetoxyroyleanone (Fig. 4) from *B. nigra* collected at the Institute of Ecology and Botany of the Hungarian Academy of Sciences at Vácrátót (30 km north of Budapest). A voucher specimen was deposited in the herbarium of the same institute (Index Herbariorum code VBI).

## 2. Previous work

Previous investigations of *B. nigra* revealed neurosedative, antioxidant (Daels-Rakotoarison et al., 2000), spasmolytic and antibacterial effects (Didry et al., 1999). The main constituents reported are phenylpropanoid glycosides, diterpenes, flavonoids (Seidel et al., 1999; Siciliano et al., 2005) and betaines (Blunden et al., 1996).

## 3. Present study

The dried aerial parts of *B. nigra* (500 g) were extracted three times with MeOH (3 l) at room temperature. The extracts were evaporated to dryness and extracted successively with diethyl ether, $CHCl_3$, EtOAc, and *n*-BuOH. The diethyl ether and $CHCl_3$ extracts were combined (2.5 g) and chromatographed on a silica gel (Kieselgel 60)

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0305-1978/$ - see front matter 
doi:10.1016/j.bse.2007.04.009

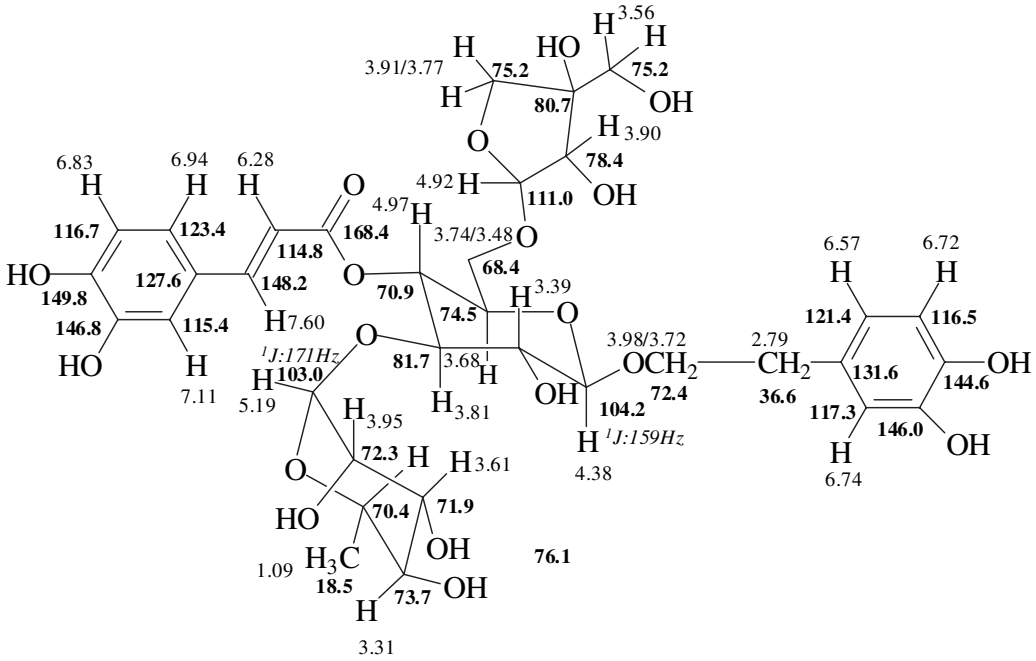

Fig. 1. $^{1}H$ and $^{13}C$ chemical shifts of compound forsythoside B.

open column, eluting with mixtures of *n*-hexane, $CHCl_3$ and MeOH of increasing polarity, which resulted in 10 fractions. The fractions were submitted to repeated gel filtration chromatography on Sephadex LH-20, eluting with $CHCl_3$–MeOH (1:1). Fractions containing ladanein (6-hydroxyapigenin 7,4′-dimethyl ether) (52 mg), 7a-acetoxyroyleanone (4.3 mg) and β-sitosterol (24.6 mg) were chromatographed on Merck Kieselgel 60 $F_{254}$ layers using $CHCl_3$–MeOH (19:1) as the development solvent and 50% methanol–sulphuric acid as the locating reagent. The phenylethanoid glycosides, martynoside (34 mg) and forsythoside B were obtained from the EtOAc fraction (2.8 g) of the plant extract. During gel filtration, the purity of martynoside and forsythoside B was controlled by TLC using formic acid–acetic acid–$H_2O$–EtOAc (7.5:7.5:18:67) as the development solvent system. The structures of the isolated compounds were elucidated by NMR spectroscopy using $^{1}H$, $^{13}C$, DEPT-135, 1-D selective TOCSY, 1-D selective NOESY, 2-D $^{1}H$–$^{13}C$ HSQC, $^{1}H$–$^{13}C$ HMBC, $^{1}H$–$^{1}H$ COSY and $^{1}H$–$^{1}H$ NOESY techniques (Pretsch et al., 2002; Duddeck et al., 1998). The UV, $^{1}H$ NMR, and $^{13}C$ NMR spectroscopic data obtained were identical with those previously described for these compounds in the literature (Citoglu et al., 1999; Skrzypek et al., 1999) (Figs. 1–4).

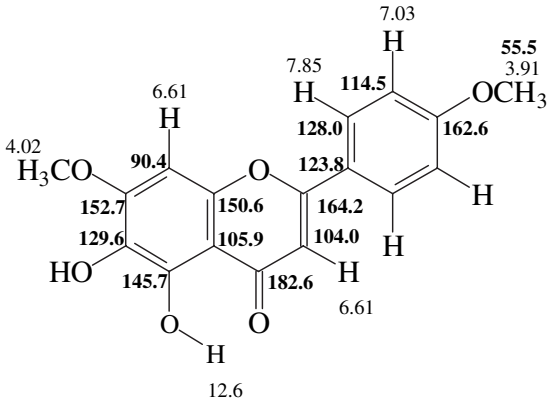

Fig. 2. $^{1}H$ and $^{13}C$ chemical shifts of compound ladanein.

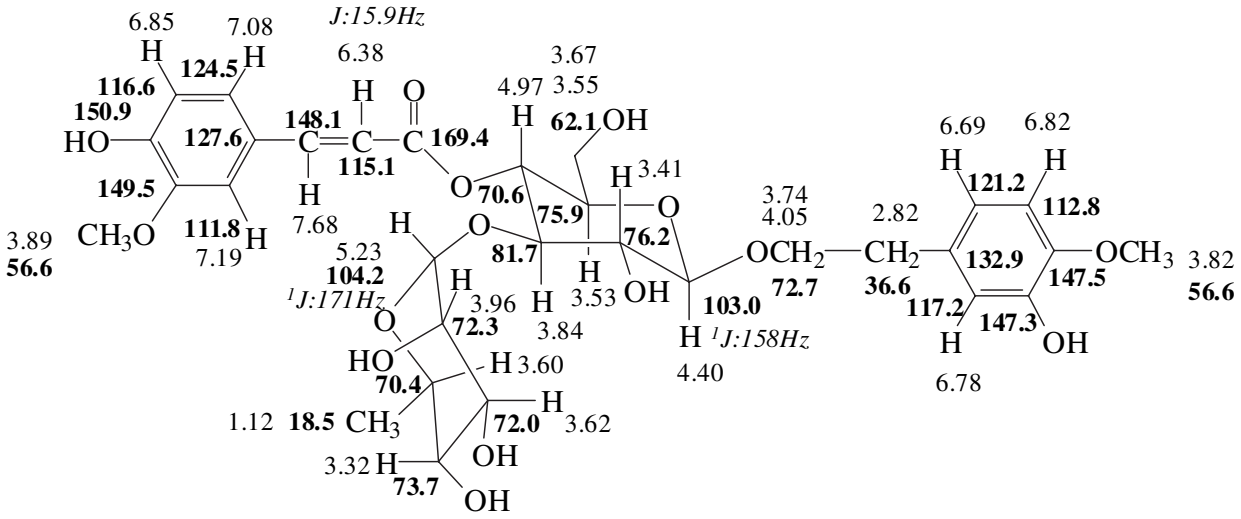


Fig. 3. $^{1}H$ and $^{13}C$ chemical shifts of compound martynoside.

## 4. Chemotaxonomic significance

Martynoside, ladanein and 7a-acetoxyroyleanone have not previously been reported for *B. nigra*. Phenylethanoid glycosides, including forsythoside B (Seidel et al., 1999), have been found in other *Ballota* species, but this is the first record of martynoside for the genus. Labdane and clerodane type diterpenoids have been isolated from *Ballota* species (Seidel et al., 1999; Ahmad et al., 2004), but 7a-acetoxyroyleanone is the first quinonoid type diterpene to be reported for the genus. Ladanein has been identified in *Ballota* species, for example *Ballota saxatilis* C. Presl. (Citoglu et al., 1999) and *Ballota hirsuta* Benth. (Seidel et al., 1999), as well as from a species of the closely related genus *Marrubium* [from *Marrubium trachyticum* Bois. (Citoglu and Aksit, 2002)]. 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's system) (Cantino and Sanders, 1986). Sporadic data justify their presence in the Subfam. Nepetoideae, but martynoside has only been found in *Salvia officinalis* (Hohmann et al., 2003). Our observations support the chemotaxonomic similarities and differences suggested by Erdtman's, and Cantino, Harley and Wagstaff's classifications (Cantino et al., 1992).

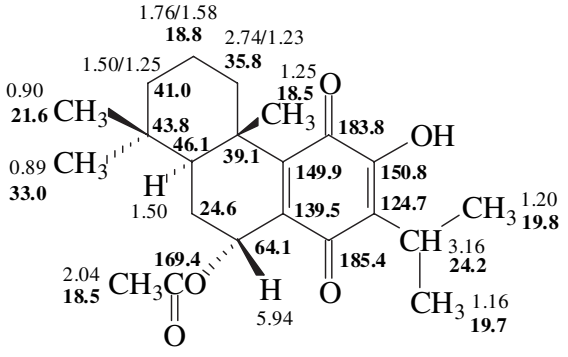


Fig. 4. $^{1}H$ and $^{13}C$ chemical shifts of compound 7a-acetoxyroyleanone.

## Acknowledgements

The authors express their thanks for the financial support of the Hungarian Science Foundation (OTKA, T43148, OTKA TO46127) and to Dr. V.V. Miklóssi for the botanical identification.

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# II.

# TLC–Densitometric Investigations of Phenylpropanoid Glycosides in Black Horehound (*Ballota nigra* L.)

Gábor Janicsák*, Enikő Tóth, and Imre Máthé

**Key Words**

*Ballota nigra*
TLC–densitometry
Phenylpropanoid glycosides
Caffeoylmalic acid
Forsythoside B
Verbascoside

Journal of Planar Chromatography

2007

MODERN TLC

*Vol. 20*

**SEPARATUM**



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# Journal of Planar Chromatography

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# TLC–Densitometric Investigations of Phenylpropanoid Glycosides in Black Horehound (*Ballota nigra* L.)

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Caffeoylmalic acid
Forsythoside B
Verbascoside



## Summary

**Most of the beneficial biological effects (e.g. neurosedative, antioxidant, and antimicrobial) of black horehound (*Ballota nigra* L.) are because of its phenylpropanoid glycoside content. A rapid TLC–densitometric method with acceptable accuracy has been established for quantitative analysis of the most characteristic components of black horehound. Experiments were performed to establish the optimum conditions for densitometric measurement and for storage of extracts. Practical application of the elaborated method is reported for five plant extracts.**


## 1 Introduction

Black horehound (*Ballota nigra* L. (Lamiaceae)) is a perennial herb widely distributed in Europe as a native or naturalized alien [1]. Large populations can be found near rubbish-tips and cemeteries. The plants reach almost one meter in height. *Ballota* species are used in folk medicine as anti-ulcer, diuretic, and choleretic agents, and for the treatment of wounds and upper respiratory inflammation [2]. Aqueous and water–alcohol extracts of *B. nigra* are used to treat influenza and coughs and for their neurosedative effect [3]. In Hungary, *B. nigra* was rarely used to treat diseases, but since the adoption of the European Pharmacopoeia it has become an official medicinal plant [4].

The main chemical components of *B. nigra* are phenylpropanoid glycosides (PGs) [5], which have been reported to be responsible for the neurosedative activity [6]. They also have antioxidant and, occasionally, antimicrobial effects [7].

PGs have also been found in *Plantago*, *Scrophularia*, *Teucrium*, *Phlomis*, *Pedicularis*, *Stachys*, and *Salvia* species [8–11]. There are numerous literature data on the isolation of PGs from *Ballota* species [5], but apart from one LC–MS investigation [12] there have been no publications dealing with the distribution of these compounds. Accordingly, we set out to develop a TLC–densitometric method for quantitative analysis of the most characteristic PGs (caffeoylmalic acid (CM), forsythoside B (FB) and verbascoside (VE)) in *B. nigra* (**Figure 1**). Densitometry, with its high speed and accuracy, is a possible means of a rapid chemotaxonomic screening of the *Ballota* genus.

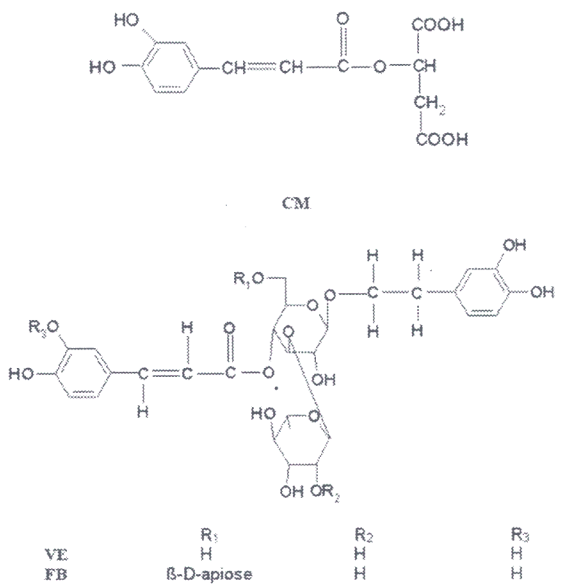


**Figure 1**

**The chemical structures of caffeoylmalic acid (CM), forsythoside B (FB), and verbascoside (VE).**

## 2 Experimental

During the optimization procedure, authentic samples of CM, FB, and VE were used. CM was purchased from PhytoLab, Germany. FB and VE were isolated by us from *B. nigra* (voucher specimen no.: BN0607). The purities of both were monitored by TLC and NMR. The standard solution contained 0.305 mg CM,

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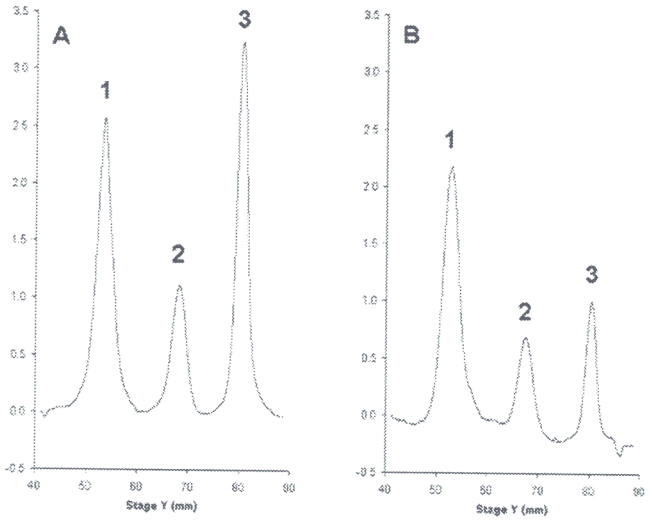


**Figure 2**

**Densitograms obtained from standards (A) and from an extract of *Ballota nigra* L. shoots (B). Forsythoside B (1), verbascoside (2), caffeoylmalic acid (3).**

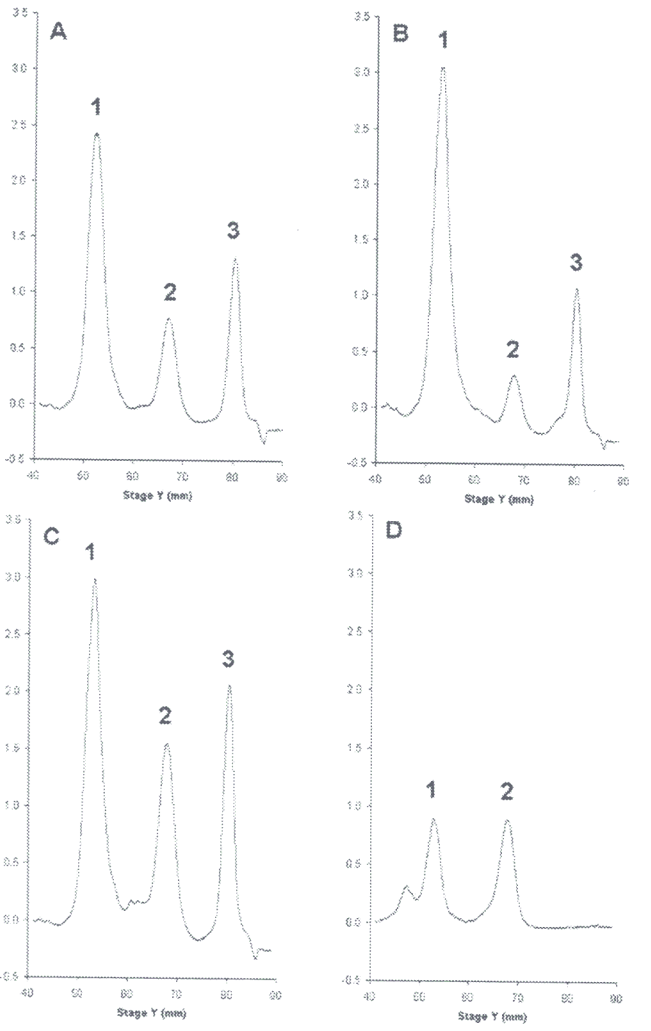


**Figure 3**

**Densitograms obtained from extracts of *Ballota nigra* L. leaves (A), stems (B), flowers (C), and roots (D). Forsythoside B (1), verbascoside (2), caffeoylmalic acid (3).**

1.785 mg FB, and 2.015 mg VE in 5 mL methanol. The volumes applied to TLC plates were 5, 10, and 15 µL.

All solvents used were commercial products of analytical grade (Merck, Germany). TLC was performed on precoated glass plates (silica gel 60, 20 cm × 10 cm, Merck) in an unsaturated chamber (Desaga, Germany), by using the ascending technique at room temperature. Each sample was applied as a spot 15 mm from the edge of the plates. The volumes applied to the plates were 5 µL from root and leaf extracts, 10 µL from shoot and flower extracts, and 20 µL stem extracts. The mobile phase was formic acid–acetic acid–water–ethyl acetate 7.5:7.5:18:67 [4]. The development distance was always 90 mm. The developed plates were dried under an intense air-stream in a fume cupboard for 10 min. The plates were then dipped into a 1% methanolic solution of aminoethyl diphenylborinate and heated at 40°C in an oven for 10 min. The dried plates were subsequently dipped in a 5% solution of PEG 400 in methanol and then heated in the same way as above [4].

Densitograms of the PGs 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 derivatized PGs. The conditions used for densitometric determination were: scan mode, linear; beam size, 10 mm × 0.5 mm; zero set mode at start. Densitograms obtained by use of these conditions are shown in **Figures 2** and **3**.

To determine the optimum wavelength for excitation, i.e. that resulting in maximum emission, densitometric measurements on the spots were made between 330 and 430 nm in 5-nm steps. During this procedure, the appropriate emission filter was also selected, according to the wavelengths to be cut off.

The period of color stability is the time during which the intensity of emission of the spots undergoes only negligible changes. For this purpose, we used plates developed, dried, and treated with the two visualization reagents in the same mode as reported above. After the derivatization, densitometric evaluation of the three components was started immediately. The determination was repeated every 5 min for 30 min, then at 10-min intervals, from the moment of insertion of the plate into the instrument up to 2.5 h.

To determine the ranges of the quantities of the compounds for which there was a linear correlation with fluorescence intensity, calibration plots were drawn. The ranges were 0–2.75 µg for CM, and 0–12.70 µg for FB and VE. Every spotting was repeated twice. The conditions used for densitometric determination were as listed above.

To establish the sensitivity of the method, the smallest detectable amounts of the three PGs were determined via the calibration study.

The reproducibility of densitometric evaluation was examined by tenfold replicate scanning of a mixture of authentic compounds on a plate prepared as described above.

Practical application of the method was demonstrated by analysis of samples of five different parts of the same *B. nigra* plant (voucher specimen no. L2111). The plant material was gathered in July 2007 from the experimental field of the botanic garden in Vácrátót, Hungary, and dried at room temperature. The powdered samples (0.5 g) in 25 mL methanol were heated under reflux on a water bath for 30 min. The filtered extracts were co-

**Table 1**

**Forsythoside B (FB), verbascoside (VE) and caffeoylmalic acid (CM) content of different tissues of *Ballota nigra*, determined by use of the TLC–densitometric method described above.**

| Tissue | Content [% dry weight] | | |
|---|---|---|---|
| | FB | VE | CM |
| Shoots | 1.71 | 1.68 | 0.11 |
| Leaves | 3.30 | 3.24 | 0.24 |
| Flowers | 2.43 | 3.13 | 0.20 |
| Stems | 1.09 | 0.58 | 0.05 |
| Roots | 1.23 | 3.97 | 0 |

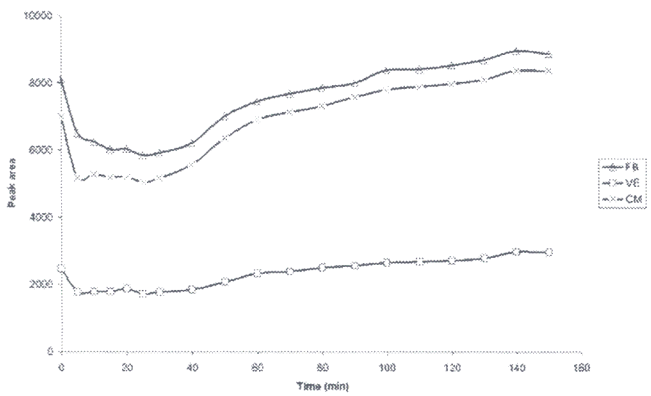


**Figure 4**

**Changes in the emission intensities of caffeoylmalic acid (CM), forsythoside B (FB), and verbascoside (VE).**

chromatographed with authentic samples of CM, FB, and VE. The PG contents of the samples are listed in **Table 1**.

The best conditions for storage of PG-containing methanolic extracts of *B. nigra* were also established. For this purpose, an extract of 1.0 g herb (collected in May 2007 in the same botanic garden) in 50 mL of methanol was prepared as described above. After filtration and dilution to 50 mL, six 5-mL samples were separately exposed to three different storage conditions: in the dark under refrigeration at 5°C, in the dark at room temperature (25 ± 3 °C), and in the light at room temperature. The amounts of CM, FB, and VE in the solutions were measured after 0, 1, 3, 6, 7, 10, and 14 days.

## 3 Results and Discussion

The mobile phase and spray reagent for the TLC analysis of PGs in *B. nigra* are official in the present Hungarian Pharmacopoeia [4]. In the article on this plant, the TLC method described is used for qualitative control only, but the good separation and derivatization enable densitometric quantification of the spots of the main components. The differences between the $R_F$ values of the three PGs were substantial (FB 0.53, VE 0.70, and CM 0.87) (Figures 2 and 3). Compared with UV absorption the sensitivity of detection could be increased threefold by derivatization. In studies relating to the distribution of components or their variation during growth, TLC enables the simultaneous separation

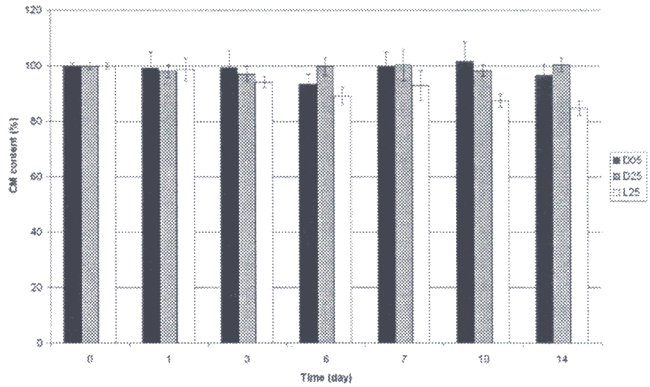


**Figure 5**

**Variation of the caffeoylmalic acid (CM) content of methanolic extracts of *B. nigra* stored in the dark at 5°C (D05), in the dark at room temperature (D25), or in the light at room temperature (L25).**

and densitometric evaluation of many samples, without prior purification of the extracts.

In the wavelength range 330–430 nm, it was found that peak areas for the emission were maximum at 395 nm. Densitometric evaluations of these PGs were therefore performed at 395 nm. Selection of the emission filter was based on the requirement that it should cut off the exciting light but allow through as much of the fluorescent emission as possible. Accordingly, filter no. III was chosen, which cuts off excitation wavelengths below 440 nm.

Color stability for the three compounds was very similar (**Figure 4**). During the 150-min observation period the change in emission intensity passed through three stages. In the first 10 min there was a steep decrease. This was followed by a period of negligible change for approximately 30 min and, finally, an increase for the remainder of the time. The trends were more marked for CM and FB than for VE.

The changes during the 30-min period were not large, as reflected by coefficients of variation of 3.43% for CM, 2.67% for FB, and 3.23% for VE. Accordingly, we suggest densitometric measurement between 10 and 40 min after derivatization.

The calibration plots demonstrate the relationship between the amount spotted and measured peak area. Linear relationships were found in the ranges 0–1.65 µg for CM, 0–13.00 µg for FB, and 0.60–10.50 µg for VE. The correlation coefficients ranged between 0.992 and 0.999.

The fluorescence of the components enabled exact quantification of even small amounts. The lower limits of detection were 0.05 µg for CM, 0.3 µg for FB, and 0.6 µg for VE.

Reproducibility was good – tenfold replicate densitometric estimation of the same spot yielded coefficients of variation of 1.90% for CM, 0.88% for FB, and 1.57% for VE. Thus, differences observed on replicate instrumental measurement were negligible.

Results from densitometric determinations of these PGs in different parts of *B. nigra* are listed in Table 1. The roots contained the highest amount of VE, but no CM. The leaves contained the highest levels of FB and CM, with a quite high content of VE. All three compounds were found in the stems, but in very low quantities. It was interesting that the flowers produced amounts reasonably similar to those in the leaves.

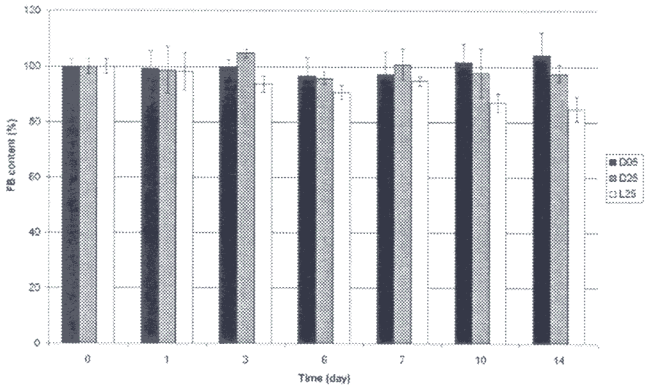


**Figure 6**

**Variation of the forsythoside B (FB) content of methanolic extracts of *B. nigra* stored in the dark at 5°C (D05), in the dark at room temperature (D25), or in the light at room temperature (L25).**

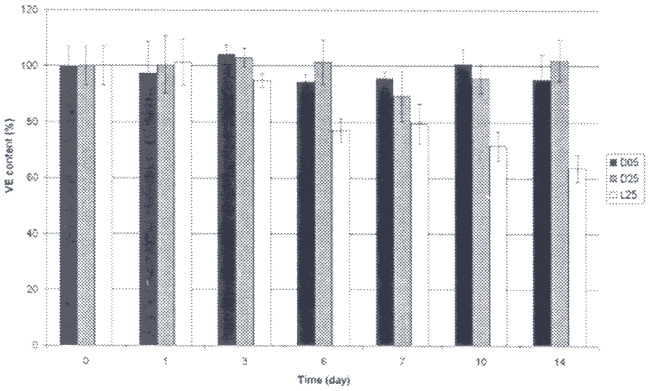


**Figure 7**

**Variation of the verbascoside (VE) content of methanolic extracts of *B. nigra* stored in the dark at 5 °C (D05), in the dark at room temperature (D25), or in the light at room temperature (L25).**

The storage experiments proved that the PG content of the methanolic extracts of *B. nigra* were more stable in the dark than in the light (**Figures 5–7**). The extracts proved stable when stored in the dark at either 5°C or at 25°C for 14 days; there were no losses whereas losses in the light were substantial – approximately 15% for both CM and FB and approximately 37% for VE in 14 days.

## Acknowledgment

We acknowledge financial support from the Hungarian National Scientific Research Foundation (grant OTKA T043148).

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Ms received: August 30, 2007
Accepted: November 13, 2007

2007

***Journal of Planar Chromatography* is published in yearly volumes of six issues by**

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# III.

# Determination of phenylpropanoids in three *Ballota* species

**Enikő Tóth*, Gábor Janicsák, Imre Máthé,** and **Gerald Blunden**

## Key Words

***Ballota nigra***

***Ballota rupestris***

***Ballota hirsuta***

**TLC-densitometry**

**Verbascoside**

**Forsythoside B**

**Caffeoyl-malic acid**

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## Summary

**Different parts of three *Ballota* species (*B. nigra*, *B. hirsuta* and *B. rupestris*) were analysed for phenylpropanoids content using a simple method of extraction and a newly developed TLC-densitometric method. The studied compounds were verbascoside (VE), forsythoside B (FB) and caffeoyl-malic acid (CM). The highest contents were detected in *B. rupestris*, CM (0.30%) and VE (4.58%) in leaves and FB (3.0 %) in roots, followed by *B. nigra*, while *B. hirsuta* had the least amounts; CM (0.08%), VE (1.64%), FB (0.91%) in leaves. The lack of caffeoyl-malic acid was a characteristic only of the roots. *B. nigra*, the common representative of the genus in Hungary, was chosen for thorough study of phenylpropanoids variation during vegetative period. Increased amounts of phenypropanoids were found during the main and secondary flowering periods in June (FB: 6.00%, VE: 8.50%, CM: 0.45% in leaves) and September (FB: 3.80%, VE: 7.00%, CM: 0.35% in leaves).**

## 1 Introduction

*Ballota* belongs to the Lamioideae subfamily [1]. Patzak divided *Ballota* genus into ten sections [2]. The Lamioideae is characterized by a lack of rosmarinic acid [3] and low amounts of other components, such as volatile oils, caffeic, ursolic and oleanolic acids [3] [4] [5] [6], but high levels of betaines [7]. Rosmarinic acid, which is common in the Nepetoideae subfamily, may be replaced by phenylethanoid glycosides in the Lamioideae [8]. Therefore, phenylpropanoid glycosides, compounds with a similar structure and frequently reported for *Ballota* species [9] [10] [11] [12] [13] [14] may be valuable as taxonomic markers. Other components reported from *Ballota* genus are flavonoids [15] [16] [17] [14] and diterpenes [18] [19] [20] [21] [22] [23] [14].

*Ballota* species are used in folk medicine as antiulcer, diuretic and choleretic agents, and for the treatment of wounds and upper respiratory inflammation [24]. Further investigations revealed antifungal activity of the diterpenoids and flavonoids [25].

The most common species of the genus in Hungary is *B. nigra* L., a perennial herb widely distributed in Europe [26]. *B. nigra* was little used in Hungary, but since the adoption of the European Pharmacopeia ( IVth Edition ) it has become official in the Hungarian Pharmacopeia as well [27]. Aqueous and hydroalcoholic extracts of *B. nigra* are used to treat influenza and coughs and for their neurosedative effect [28]. Phenylpropanoid glycosides have been reported to be responsible for this neurosedative activity [28].

Regarding the biological and taxonomical importance of the phenylpropanoid glycosides, three different European *Ballota* species (*B. nigra* L., *B. hirsuta* Benth., and *B. rupestris* (Biv.) Vis.) were compared for their forsythoside B (FB), verbascoside

(VE) and caffeoyl-malic acid (CM), contents. *B. nigra* was chosen for more detailed examination of these phenylpropanoids during the vegetative period of the plant.

## 2 Experimental

### 2.1. Plant material

Plant material was collected in 2007 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 climate of the field is continental, with a mean annual temperature of 10.3 °C, and a mean annual precipitation of 525 mm. The plants were mainly grown from seeds obtained either via a botanical garden seed exchange programme or collected in Hungary.

The fresh plants were separated into organs (shoots, leaves, flowers/generative parts, stems, roots), then dried at 40°C. For the comparative study, fresh material of *B. hirsuta* and *B. rupestris* was collected in July (at full flowering stage). For study of the variation during the vegetative period, parts of *B. nigra* (except roots) were gathered every week (from 23th May to 24th September). In the case of *B. nigra* the appropriate samples (with roots) from July were used for comparison. Voucher specimens of each species have been deposited in the herbarium of the same institute (*B. nigra* no. L2111; *B. hirsuta* no. L2055 and *B. rupestris* no. L2115).

### 2.2. Extraction and analysis

The dried and powdered samples (0.5 g) in 25 ml of methanol were heated under reflux on a water bath for 30 min. After cooling, the extracts were filtered and made up to 25 ml.

Each extract was examined, along with CM, FB and VE reference samples, on Kieselgel 60 layers (10 x 20 cm, Merck, Germany) using formic acid-acetic acid-water-ethyl acetate (7.5:7.5:18:67) as the development system. CM was purchased from PhytoLab, Germany. FB and VE were isolated by us from the herb of *B. nigra* (voucher specimen no.: BN0607) [14]. Both of their purities were controlled by TLC and NMR studies. The standard solution contained 0.305 mg CM, 1.785 mg FB and 2.015 mg VE in methanol. The applied volumes of solutions were 5, 10 and 15 µL (test compounds); 5 µL (roots and leaves); 10 µL (shoots and flowers) and 20 µL (stems). The chromatograms, after development, were dried for 20 min. at room temperature. Densitograms were obtained using a Shimadzu CS-9301PC (Japan) densitometer. The fluorescences of derivatized spots of CM, FB and VE were measured at 395 nm. The parameters of the derivatization and densitometric measurement have been described by Janicsák et al. (2007) [29].

## 3 Results and Discussion

### 3.1. Comparison of PG profiles of *B. nigra, B. rupestris* and *B. hirsuta*

All the selected phenylpropanoids (CM, FB and VE) could be detected in the three investigated *Ballota* species. This is the first report for *B. hirsuta* (native in Spain) and *B. rupestris* (native in Italy). The most significant observations always concerned with CM, which always produced the lowest absolute values (0.0%-0.31%), and which

was not found in the roots of any of the three *Ballota* species, but showed maximum levels (*B. rupestris*: 0.31%, *B. nigra*: 0.23%, *B. hirsuta*: 0.08%) in the leaves of each plant. Other similarities concerning the three species and the two PGs were not apparent.

*B. rupestris* showed the highest amounts of the examined components: CM (0.3%) and VE (4.58%) in leaves and FB (3.0 %) in roots (Fig.1.). *B. nigra* (Fig.2.) had 5-20% while *B. hirsuta* had 30-50% less maximum yields (Fig.3.).

In the following cases the roots produced FB and VE in higher values than the leaves. FB in *B. hirsuta*: 0.9 % (leaves) and 2.4 % (roots) and in *B. rupestris*: 2.91% (leaves), 3.41% (roots); VE in *B. nigra*: 3.2 % (leaves) and 4.0 % (roots). In contrast with *B. rupestris* (FB: 1.38%, VE: 1.15% (flowers); FB: 2.91%, VE: 4.57% (leaves)), the flowers of *B. nigra* (FB: 2.43%; VE: 3.13%) and *B. hirsuta* (FB: 1.00%; VE: 2.06%) produced similar or even higher amounts of FB and VE than their leaves (*B. nigra* FB: 3.30%, VE: 3.23%) (*B. hirsuta* FB: 0.91%, VE: 1.64%). In comparison with other parts of *B. nigra* and *B. rupestris*, the stems showed significantly the lowest FB and VE values.

**Place of figures 1, 2, 3.**

### 3.2. Variation of phenylpropanoids in *Ballota nigra* during the vegetative period

For all of the three investigated compounds, we observed similar tendencies in every single plant organ. The plant produced the three compounds in highest quantity at

early flowering period (May). With the increase in flowering intensity, the levels of the compounds started to down to 50 %. This trend remained until the emergence of fruits. In the following six-seven weeks the measured quantities did not show significant changes. However, from the beginning of September, the amounts of the investigated phenylpropanoid glycosides and CM showed a moderate increasing trend. This observation was coincided with the appearance of fresh shoots. The rising trend was broken at the end of September because of slower plant development, which can be attributed to the decreasing average temperature.

Figures 4, 5 and 6 show the variations in PG and CM levels of *B. nigra* during the vegetative period. Increases were observed in June and September, but that in June was much more pronounced. The levels of CM and VE changed more intensively than those of FB. The values for CM were lower by an order of magnitude than those of FB and VE. The highest values in the case of FB and CM were measured in the leaves of the young shoots (May-June), and of VE in the reproductive parts at the beginning of flowering. Our findings indicate that the stems produced the compounds in the lowest (FB, VE: $\leq$ 2%, CM: $\leq$ 0.1%) concentrations in comparison with the other investigated plant organs and the variations in concentration during the vegetative period were not as intense in the stems (FB: max.: 2.19%, min.: 0.96%) as in the other organs (leaves, max.: 6.37%, min.: 2.56%).

**Place of figures 4, 5 and 6.**

## Acknowledgements

We acknowledge the plant material to Krisztina Szabó, and the late Vilmos Miklóssy Vári and financial support of the Hungarian National Scientific Research Foundation (OTKA): grant number: T043148.

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List of figure captions

Figure 1 The content of phenylpropanoid- glycosides in the organs of *Ballota rupestris* in % of dry weight

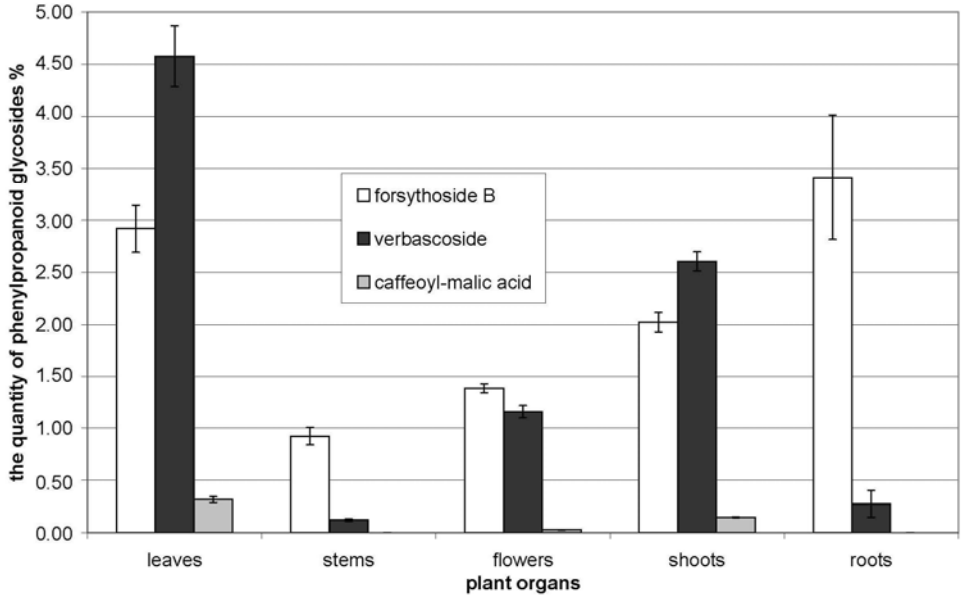


Figure 2 The content of phenylpropanoid- glycosides in the organs of *Ballota nigra* in % of dry weight

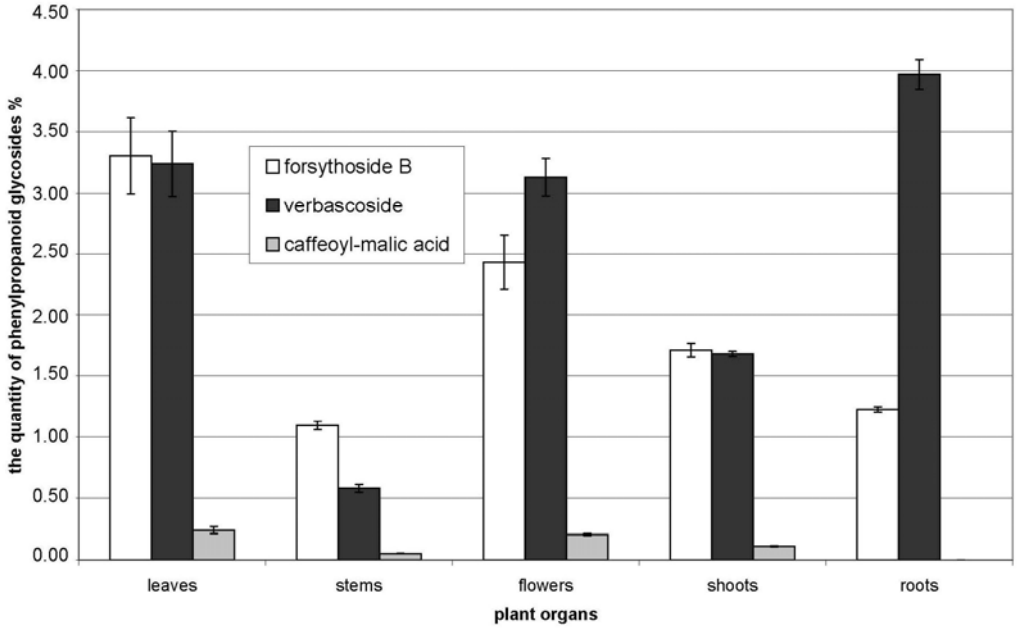

Figure 3 The content of phenylpropanoid- glycosides in the organs of *Ballota hirsuta* in % of dry weight

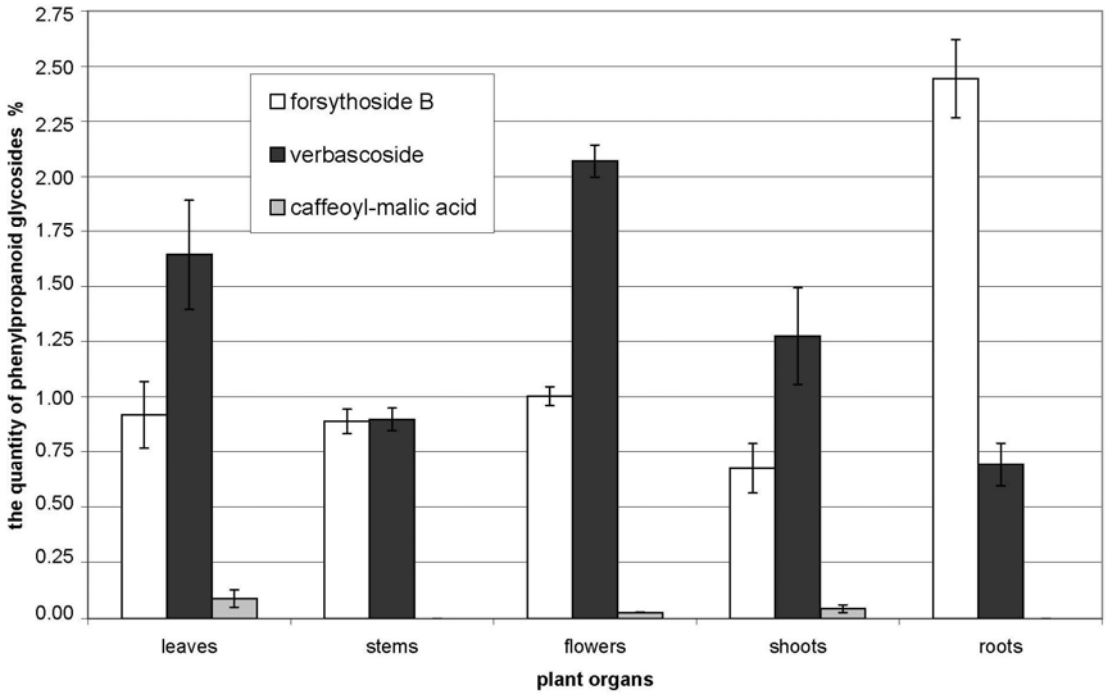


Figure 4 Variation of caffeoyl-malic acid content in the organs of *Ballota nigra* during a vegetative period in % of dry weight

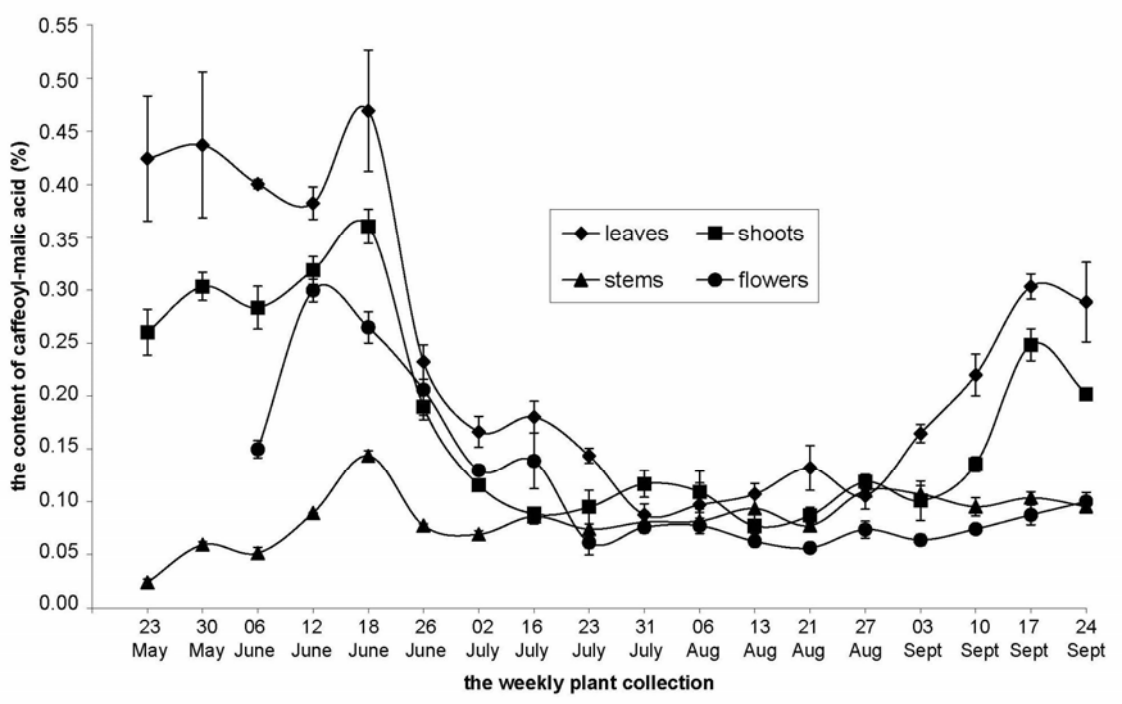

Figure 5 Variation of forsythoside B content in the organs of *Ballota nigra* during a vegetative period in % of dry weight

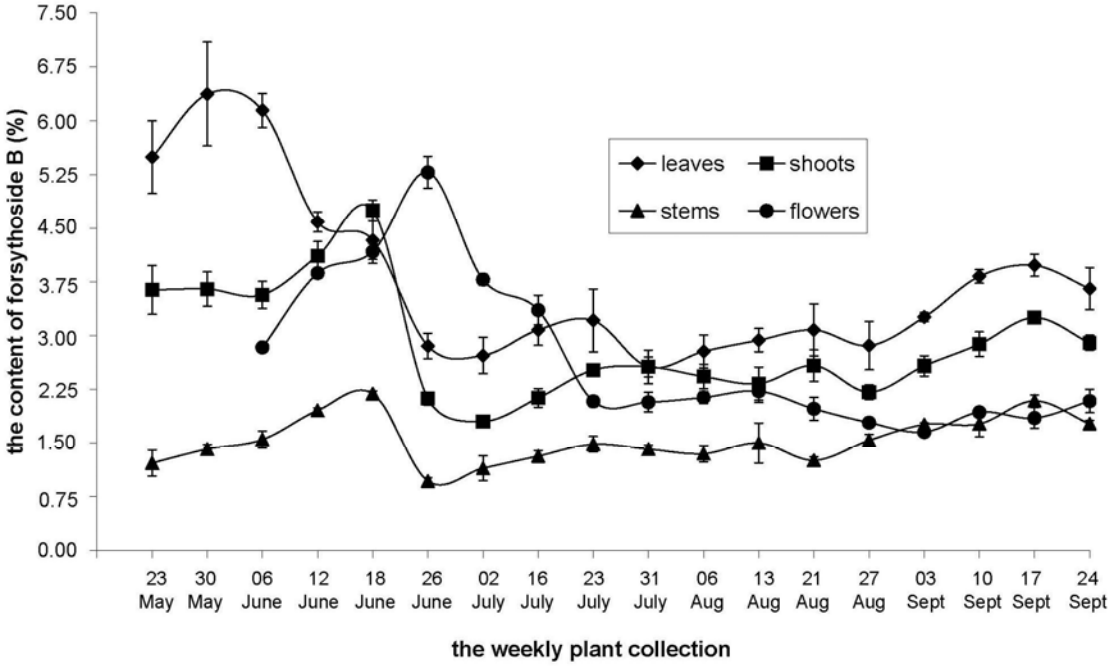


Figure 6 Variation of verbascoside content in the organs of *Ballota nigra* during a vegetative period in % of dry weight