University of Szeged
Department of Pharmacognosy

**Ph.D. Thesis**

# Examination of the volatile and non-volatile components of Hungarian *Stachys* species

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List of publications related to the thesis

**I.** **E. Radnai**, Á. Dobos, K. Veres, L. Tóth, I. Máthé, G. Janicsák, G. Blunden: Essential Oils in Some *Stachys* species Growing in Hungary, *Acta Horticulturae*, 597 (2003), 137-142

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

**III.** **E. Háznagy-Radnai**, Sz. Czigle, G. Janicsák and I. Máthé : Iridoids of *Stachys* Species Growing in Hungary, *JPC (Journal of Planar Chromatography)*, 19 (2006) 187-190

**IV.** **E. Háznagy-Radnai**, Sz. Czigle, I. Zupkó, Gy. Falkay, I. Máthé: Comparison of antioxidant activity in enzyme-independent system of six *Stachys* species, *Fitoterapia,* 77 (2006) 521-524

**V.** **E. Háznagy-Radnai**, Sz. Czigle, I. Máthé: TLC and GC Analysis of the Essential Oils of *Stachys* species, *JPC* (*Journal of Planar Chromatography).* 20 (2007) 189-196

**VI.** **E. Háznagy-Radnai**, Sz. Czigle, I. Máthé, : Analysis of the essential oil of downy woundwort (*Stachys germanica* L.). *Acta Facult. Pharm. Univ. Comenianae* (*Acta Facultatis Pharmaceuticae Universitatis Comenianae),* 54, 2007

**VII.** **E. Háznagy-Radnai**, B. Réthy, Sz. Czigle, I. Zupkó, E. Wéber, T. Martinek, Gy. Falkay, I. Máthé: Cytotoxic effect study of *Stachys* species and their iridoids, *Fitoterapia* (accepted)

**Abbreviations**

| | |
|---|---|
| 1D | one-dimensional |
| 2D | two-dimensional |
| AA | arachidon acid |
| COSY | correlated spectroscopy |
| COX | ciklooxigenase |
| GC | HP 5890 Series II. gas chromatograph (FID) |
| GC-MS | Finnigen GCQ ion-trap bench-top mass spectrometer |
| HMBC | heteronuclear multiple-bond correlation |
| HMQC | heteronuclear multiple-quantum coherence |
| HPLC | high-performance liquid chromatography |
| LT | leucotriene |
| MTT | 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide |
| NMR | nuclear magnetic resonance |
| NOESY | nuclear *Overhauser* effect spectroscopy |
| NP | normal-phase |
| PG | prostaglandine |
| RP | reverse-phase |
| SRAE | *St.riederi* var. Japonica |
| St. | *Stachys* |
| TLC | thin-layer chromatography |
| TX | tromboxane |
| VLC | vacuum-liquid chromatography |

*„The Lord has brought medicines into existence from the earth,*
*and the sensible man will not despise them"*

*Ecclesiasticus 38,4*

## 1. Introduction

The history of the peoples of the world cannot be imagined without studying the plants used as medicines, as the use of medicinal herbs is as ancient as man. The first written proofs of the plants used in medicine are to be found in mythology, in the Bible itself. *Stachys* is a word of Greek origin, it means spike, which refers to its genus, to its typical inflorescence. It first appeared in Dioscorides's work. Plinius also mentions it. Roman gladiators wore it as an amulet as mystic power was attributed to the plant. [1]

The family *Lamiaceae* consists of approximately 200 genera of 3500 species. The woundwort (*Stachys*) genus consists of 300 species. This is the third largest relationship group of Lamiaceae plants. It grows everywhere in the world with the exception of Australia, New Zealand and the Arctic regions. The number of species is particularly high in the Mediterranean region, in Eastern Europe, in Cape Province and in Chile. 10 species live in Central Europe. The flowers of these annual or perennial herbs are light purple, dark pink, yellow or white. [2, 3]

Some species grow in Hungary, *St. officinalis* L. is found in Europe, so in Hungary as well. *St. alpina* L. likes shady places, it is found in fresh hornbeam-beech forests. In Hungary it grows in the Bükk and Mecsek mountains. *St. germanica* L. also native in Hungary, it is quite frequent in dry grasslands and pastures. *St. byzantina* L. is found as an ornamental plant, *St. grandiflora* L. and *St. macrantha* (Koch) Stearn in botanical gardens. *St. sylvatica* L. can be found in hilly and mountainous zones along shrubs and forest paths, in moist, leafy forests, groves, scrubs and by forest springs. It lives on moist and wet clay and adobe soils which are rich in nutrients and have a neutral pH. *St. palustris* L. is widespread in the greater part of Europe, it is common in Hungary, especially along marshes and bogs. *St. recta* L. is frequent on dry, stony grasses, steppe slopes. *St. annua* L. is found in most of Southern and Central Europe, it is native to Northern Europe, in Hungary it is an ordinary plant. It can be found in plough-lands, stubble fields, mainly on hard soils.

### 1.1. Botanics of *Stachys* genus

In the course of the systematic chemical and chemotaxonomic evaluation of the species of the Lamiaceae family it is important to study the *Stachys* genus. This is justified not only by the great number of species in the genus and by the relatively smaller extent of its chemical examination but also by the taxonomic uncertainty according to which – contrary to Bentham's and Erdtman's system – the genus is classified by Briquet into a subfamily which should have morphological and chemical features characteristic of both the Lamioideae and the Nepetoideae subfamilies in Erdtman's view. In Erdtman's opinion the two natural subfamilies differ with respect to pollens. One has pollens of tricolpate nature, which he called Lamioideae, the other of hexacolpate nature, which he named Nepetoideae subfamily. It is clear from Cantino's analysis of the two subfamilies that the Lamioideae subfamily has a non-volatile oil nature but other secondary substance groups such as e.g. iridoids are present while rosmarinic acid is absent. This is in contrast with the Nepetoideae subfamily, which lacks in iridoids but has a considerable volatile oil and rosmarinic acid content. [4, 5, 6]

#### 1.1.1. Classification of the *Stachys* genus

The species of the *Stachys* genus native to or cultivated in Hungary are the following: [2]
Order (*Ordo*): Lamiales, Family (*Familia*): Lamiaceae, Subfamily (*Subfamilia*): Lamioideae, Genus: *Stachys* (The names *Stachys* and *Betonica* still represent a controversial issue today. Having studied various literature sources, I decided to use the nomenclature described in Flora Europa in my thesis. I also found contradictions as to the judgement of *St. grandiflora* and *St. macrantha*. They are used as synonyms for one another but are also described as separate species.)

Species: *Stachys officinalis* L
(subspecies): -betonica
*Stachys alpina* L
*Stachys germanica* L
*Stachys byzantina* C. Koch
*Stachys grandiflora* L.
*Stachys macrantha* (Koch) Stearn
*Stachys sylvatica* L
*Stachys palustris* L.
*Stachys recta* L.
(subspecies): recta, -subcrenata, - labiosa
*Stachys annua* L

### 1.1.2. The botanical description of the *Stachys* species examined

*Stachys (Betonica) officinalis* L. (Common hedgenettle):
It is an erect, perennial plant ranging from almost bare to densely hirsute, 15-100 cm. Its leaves can be oblong or ovate. It occurs in woodlands, clearings, mountain fields. [2, 3]

*Stachys alpina* L. (Mountain woundwort):
The stems are 30-100 cm, it is green, hirsute-tomentose with glandular hair at least above. The leaves are ovate or ovate-lanceolate. It occurs in shady places, especially in the mountains (Bükk, Mecsek), in fresh hornbeam-beech forests. It is in flower from July to August. [2, 3]

*Stachys germanica* L. (German hedgenettle):
The stems are whitish tomentose, eglandular. It is an erect plant with a stiff, 4-edged stem. The leaves are from oblong to ovate. It frequently occurs in dry grasslands, pastures, along paths. It is in flower from July until September. [2,3]

*Stachys byzantina* C. Koch. (Woolly hedgenettle):
The stems are 15-80 cm, densely tomentose. The lower leaves are oblong-spathulate, the upper ones elliptical, at the base white-sericeous-lanate. It is a garden ornamental plant originating from Southwest Asia. It is widely cultivated and locally naturalized. Flowering: July-August. [2, 3]

*Stachys grandiflora* L (Bigsage):
It is a 30-50 cm, erect, sparsely hairy, glandular perennial. The leaves are lanceolate or oval. The calyx is glandular and sparsely pubescent. The corolla is white/pinkish/reddish brown. [3]

*Stachys macrantha* (Koch) Stearn:
It is a 30-50 cm, hairy perennial. Its leaves are oval. The calyx is pubescent, the corolla is purplish-violet. [3]

*Stachys sylvatica* L. (Hedge woundwort):

It is an erect, hirsute and glandular or pubescent perennial, it is 30-120 cm high. The leaves are ovate and all petiolate. It frequently occurs in fresh, leafy forests, especially in hornbeam-beech forests. It is in flower from June until September. [3]

*Stachys palustris* L. (Marsh woundwort):

It is a perennial, 30-120 cm, sparsely to densely hairy, usually eglandular, with stolons. Its stem is erect, four-edged. The leaves are oblong or oblong-lanceolate. It occurs all over the territory of Hungary, it is frequent in damp places, such as in marshes, reeds, bogs and it is a common weed in gardens, ditches and fields. Flowering: June - September. [2, 3]

*Stachys recta* L. (Seaport hedgenettle):

It is an erect or ascending, subglabrous to sparsely hirsute, usually eglandular perennial, it is 15-100 cm. It is in flower from May until October. This species is prone to variations, including a great number of inferior taxa, but local populations are also encountered. [2, 3]

*Stachys annua* L. (Annual hedgenettle):

It is an erect, pubescent and sometimes glandular annual, it is 10-40 cm. The leaves are lanceolate, cuneate, oblong-ovate, blunt, petiolated, crenate-dentate. In Hungary it is common mainly in fields, especially in stubble fields, semi-hard or hard soils. It is of particular importance from bee-keeping aspects, it is a good melliferous plant. Flowering: June – November. [2, 3]

## 1.2. Chemical overview of the *Stachys* species

### 1.2.1. *Volatile oil components of Stachys species*

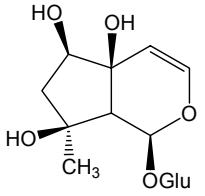
*St. annua* [7, 8, 9, 10] is the species the volatile oil components of which were examined the earliest but during the past ten years the examination of the volatile oil content of *Stachys* species was continued. Thus the volatile oil components of *St. alpina* [10-12] and those of *St. byzantina*, which is a common ornamental plant and known as “lambs' ears”, were determined by several research teams independently of each other. [13-17] The volatile oil

components of *St. germanica* were examined by Hungarian, Greek and Serbian researchers as well. [12, 18, 19] The volatile oil components of *St. grandiflora* [13, 18] and *St. macrantha* were first studied by Háznagy-Radnai et al. [13] The examination of *St. officinalis* was started as early as in 1965. In 2004 Greek researchers determined not only its volatile oil composition, together with other Stachys species, but their antibacterial effect was also studied. [8, 10, 19, 13, 20, 21] Several researchers carried out investigations of the volatile oil components of *St. palustris* [8, 10, 11], *St. recta* [8, 10, 18, 22, 23] and *St. sylvatica*. [11, 13, 19, 24]

In addition to the determination of the volatile oil components of *Stachys* species which are or can be introduced to Hungary, the Turkish [25-30], Egyptian [31], Italian [32], French [33, 34], Greek [35, 36], Iranian [37-52] and Serbian-Montenegrian [53, 54] *Stachys* flora was also examined.

### 1.2.2. Iridoids from *Stachys* species

Iridoids are a class of secondary metabolites found in a wide variety of plants. They are monoterpenes biosynthesized from isoprene and they are often intermedies in the biosynthesis of alkaloids. Iridoids are typically found in plants as glycosides, most often bound to glucose. The first investigation of the iridoid content of *Stachys* species dates back to the beginning of the 70s, when Adema detected iridoid glycoside in *St. palustris* [55], and when Ukrainian researchers determined harpagide(1) and 8-O-acetylharpagide(2) in 17 *Stachys* species. [56]

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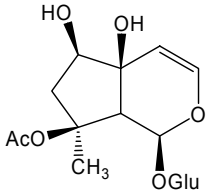
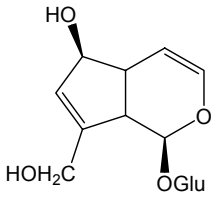



(2)

The main iridoid components in the *Stachys* species studied by E. N. Gritsenko et al. in 1977 were harpagide(1) and 8-O-acetyharpagide(2). The presence of aucubin(3) was also detected in some *Stachys* species. [57] Ajugol(4), ajugoside(5), harpagide(1) and 8-O-acetylharpagide(2) were detected in *St. atherocalyx, St. inflata* and *St. iberica* by Komissarenko et al. in 1976 and 1979. [58, 59]

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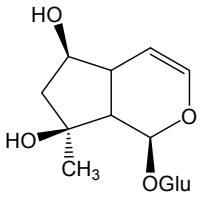


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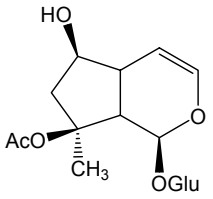
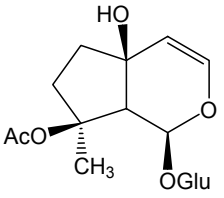



(5)

In 1980 the research team of D. A. Pakaln started an examination concerning 20 different *Stachys* species, which supplemented the previous results: beside harpagide(1) and acetylharpagide(2) as the main components, reptoside(6) and diacetyl-reptoside(7) were also found. [60]

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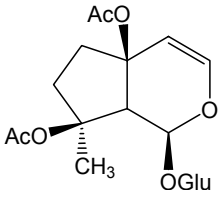


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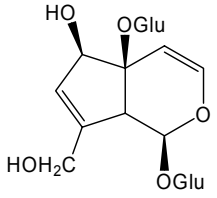
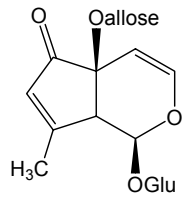



(8)

In 1984 Lenherr et al. performed the RP-HPLC analysis of 10 species belonging to the *St. recta* group and melittoside(8), harpagide(1), acetylharpagide(2), ajugoside(5) and ajugol(4) were identified. [61]

The data relating to the iridoids isolated until 1980 are summarized by El Naggar. [62]

Phytochemical investigations were performed with *Stachys* (*Betonica) officinalis* in 1986. Ukrainian researchers found two iridoids harpagide(1) and acetylharpagide(2), in the aqueous decoction of the above-ground parts. [63] In addition to harpagide and acetylharpagide as the main components, ajugol(4) and ajugoside(5) were also detected by Russian researchers. [64] Jeker et al. made experiments with this plant, too, in 1989. They could also detect acetylharpagide(2) and reptoside(6), and they also isolated two new compounds, allobetonicoside(9) (an allose-containing iridoid diglycoside) and 6-0-acetylmioporoside(10). [65]

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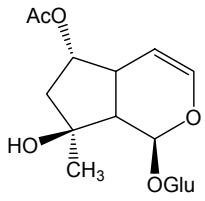


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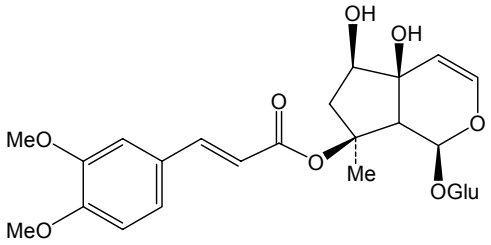



(11)

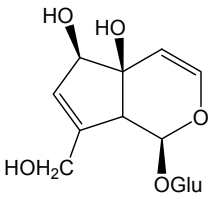
Calis et al. examined the iridoid components of *St. macrantha*. A new iridoid, macranthoside(11) and 6 already known iridoid glycosides such as harpagide(1), allobetonicoside(9), ajugol(4), 8-O-acetylharpagide(2), ajugoside(5) and reptoside(6) were isolated. [66]

El Naggar's summary was followed by the work by Boros et al., which contains the summary of iridoids isolated between 1980-1990. [67, 68]

Russian researchers continued to investigate the chemical components of the *Stachys* species growing in Russia. [69, 70]

Japanese researchers also conducted research into the iridoids of *Stachys* species. [71]

Monomelittoside(12), melittoside(8), 8-O-acetylharpagide(2), harpagide(1), ajugol(4), catalpol(13), 7-O-acetyl-8-epi-loganic acid(14), aucubin(3) and 5-allosyloxy-aucubin(15) were isolated by research made by Montenegrian, Greek, Hungarian and Italian scientists. [13, 72-78] The isolated iridoid components of *Stachys* species found in Hungary are included in Table 1.

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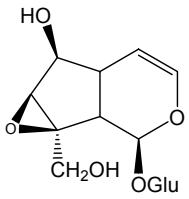


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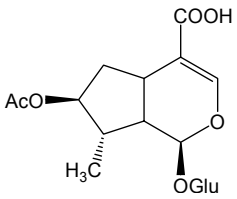


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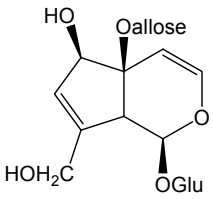



(15)

**Table 1** Iridoids of *Stachys* species from Hungary

| ***Stachys species*** | **Iridoids** | **Reference** |
|---|---|---|
| *St. officinalis* | 1,2,4,5,6,9,10, | [63,64,65, 78] |
| *St. alpina* | 1,2 | [78] |
| *St. germanica* | 1,2 | [78] |
| *St. byzantina* | 1,2 | [13,78] |
| *St grandiflora* | 1,2,8 | [13,78] |
| *St. macrantha* | 1,2,4,5,6,9,11 | [66,78] |
| *St. sylvatica* | 1,2 | [12,78] |
| *St. palustris* | 1,2,3 | [55,78] |
| *St. recta* | 1,2,3,4,5,,8 | [61, 78] |
| *St.annua* | 8 | [61] |

### 1.2.3. Other chemical constituents

#### 1.2.3.1 Diterpenes, triterpenes and pseudoalkaloids

During the examination of the diterpenoids of the *Stachys* species Popa, Miyase, Adinolfi extracted diterpenes from the dry, above-ground parts of *St. annua*, *St. officinalis*, *St. sylvatica* and *St. recta*. [79-83] Derkach et al. studied the *Stachys* species growing in the Ukraine. [84] Piozzi et al. examined the diterpene components of the *Stachys* species found in the Italian flora. Their examinations revealed the occurrence of mainly labdane- and kaurane-type diterpenes. [85] Maleki and Fazio extracted diterpenes from the above-ground parts of *St. inflata* and *St. mucronata.* [86, 87]

The presence of α-amyrine and β-sitosterol was detected in the plant during the examination of the triterpene and sterol content of *St. palustris.* [88] Eight new oleanane-type triterpene saponines were identified in *St. riederi*. [89] Oleanolic and ursolic acid were also detected in Hungarian *Stachys* species. [90]

The presence of the alkaloid-like stachydrine was confirmed in *St. officinali, St. palustris* and *St. recta.* [91, 92]

#### 1.2.3.2. Phenolic components

Phenylethanoid glycosides, flavonoid glycosides and phenolic diterpenes were isolated and identified from *St. officinalis, St. germanica* and *St. sylvatica* herbs and root. [93] In 1996 Japanese researchers examined *St. officinalis* and isolated 6 new (betonicoside A-F) and 6 already known phenylethanoid glycosides. [94] Megastigmane glycosides were isolated by Japanese researchers from *St. byzantina.* [95] Ukrainian researchers examined the phenolic

components of *St. officinalis* and *St. palustris*. Caffeic acid, chlorogenic acid, neo- and isochlorogenic acid and p-coumaric acid were obtained. [96, 97] Buchwald et al. compared the phenolic components of *St. officinalis, St. alpina, St. germanica, St. sylvatica, St. palustris, St. recta* and *St. annua*. Caffeic, protocatechu- and chlorogenic acids were identified in all the species, rosmarinic acid in all the species with the exception of *St. annua*, while p-coumaric acid was found only in *St. sylvatica* and *St. recta.* [98] Croatian researchers examined not only the phenolic components but also the flavonoids in *St. officinalis*, *St. alpina*, *St. sylvatica, St. palustris*, *St. recta subsp. recta* and *subcrenata* and *St. salviifolia*. [99] The experiments performed with *St. macrantha* confirmed that, in addition to iridoids, phenylpropane derivatives also occur in the plant. [66]

Flavonoids are present practically in all higher plants, thus they are typical of the *Lamiaceae* family [100], and within this of the *Stachys* genus as well. In 1971 Ukrainian researches examined the flavonol content of *St. annua*. Stachannin, stachannoside and stachannoaciside were isolated. They are all methoxyflavone derivatives. [101] This research group isolated an aglycone, 4'-methoxyscutellarein from this species. [102] The further examination of *St. annua* revealed a new flavonol glycoside, stachyflaside [103]. Isoscutellarein, scutellarein, 4'-methoxyscutellarein and baikalein derivatives were also identified in *St. palustris* by Ukrainian researchers. [104, 105, 106] Three flavonoid-glycosides obtained from *St. recta* were identified as 7-0-(2"-0-6"'-0-acetyl-β-D-allopyranosyl-β-D-glucopiranosides) of 4'-0-methyl-isoscutellarein, isoscutellarein and 3'-hydroxy-4'-0-methyl-isoscutellarein [107]. In 1987 allose was detected in TFA hydrolysed samples of the flavonoid-glycoside from *Stachys recta*, isoscutellarein 7-0-(2"-0-6"'-0-acetyl-β-D-allopyranosyl-β-D-glucopyranoside). [108] The occurrence of flavonoid p-cumaroyl-glycosides and 8-hydroxyflavone 7-allosylglucosides is limited to a few genera of the Lamioideae subfamily. 8-hydroxyflavone 7-allosylglucosides are accumulated in *Stachys* species. [109] Ukrainian researchers examined the flavonoid content of *Stachys (Betonica.) officinalis* in 1986. [110] Uzbeg researchers examined the coumarin content of their own national flora. Thus coumarins were identified in *St. hissaric*. [111]

### 1.3. Uses of *Stachys* species in folk medicine and their justification

*St. officinalis* is recommended against coughing due to its expectorant and mucolytic effect, and in the case of earaches externally. Its over-ground parts have choleretic and antiphlogistic effects. [112]

*St. sylvatica* is antiphlogistic, haemostatic and stimulant. [112]

*St. palustris* is a medicinal herb used for a long time. It is used mainly for the treatment of wounds. The crushed leaves are placed on the wound where its haemostatic effect can be exerted. [113] The antiphlogistic and antiseptic effects together promote the healing of wounds. Its excellence is also proved by the synonyms given to it by people living in the countryside, such as All-Heal and Woundwort. Moreover, it is also used because of its spasmolytic and articular analgesic effects. It alleviates the symptoms of gout. The syrup made from the leaves of the plant stops haemorrhage internally and alleviates dysentery. [113] The hypotensive effect of the plant is confirmed by literature data. [114] *St. sieboldi* Miq is cultivated in Japan because of its edible tubers a vegetable dish is prepared from it. Its relative is marsh woundwort (*St. palustris*), the thickened stolons of which are also edible. In the course of the 19th century its cultivation was also experimented with but it was soon given up. [115]

In folk medicine the aqueous or alcoholic extract of *St. recta* was used for hysteria, colic, adenomas and difficulties of urination. It is still used for kidney and bladder diseases, by asthmatic patients. It is also used in homeopathy. [112]

The over-ground parts of *St. annua,* annual hedgenettle, are collected for therapeutic purposes at the time of flowering. The tea prepared from the drug is used for the treatment of respiratory diseases and previously in epileptic diseases and in common cold as well. [116]

The antibacterial effect of the volatile oil components of *St. candida* and *St. chrysantha* could be confirmed on two Gram positive and four Gram negative bacteria. [31]

The effect of the aqueous extract of *St. riedri var. japonica* Miq (*Labiatae*) (SRAE) on immediate-type allergic reactions was examined. SRAE manifests an inhibitory effect in mice on systemic anaphylaxis induced by the so-called material 48/48. SRAE inhibited plasma histamine release caused by 48/48 in mice. Furthermore, it also inhibited the passive cutaneous anaphylaxis reaction induced by IgE/anti-IgE in mice.[117]

It was revealed that *Stachys* species are not only antiphlogistic but they also possess antibacterial and antifungal effects due to aucubigenin, the aglycone of one of their iridoid components, namely aucubin. [117,118, 119] Besides, iridoids also have an antiphlogistic effect. A stronger effect can be expected topically, and a milder one when it is used orally. (1 mg of aucubin applied for the treatment of ear oedema in mice is almost as effective as 0.5 mg of indomethacine). [117] The purgative effect of iridoids was also described in literature, in the case of aucubin and catalpol it is manifested more than 6 hours later. [118]

It is well-known that prostaglandins (PG), prostacyclines and thromboxanes (TX) formed from arachidon acid (AA) upon the effect of the cyclo-oxygenase enzyme (COX), as well as leukotrienes (LT) formed upon the effect of the 5-lipoxygenase (5-LOX) enzyme play a major role in inflammatory processes. Spanish researchers examined the effect of iridoid glycosides extracted from *Scrophularia scorodonia* L. on COX and 5-LOX enzymes. The release of $PGE_2$ was inhibited by none of the seven iridoid glycosides examined, the release of leukotriene $C_4$ ($LTC_4$) only by aucubin, while that of thromboxane $B_2$ ($TXB_2$) by bartsioside, aucubin, 8-O-acetylharpagide and harpagoside as well. The experiment proved that the majority of the iridoid glycosides examined inhibited the TX-synthase enzyme selectively. [120]

The raw extract of *St. chrysantha* inhibited the release of leucotriene $C_4$ ($LTC_4$) by peritoneal macrophages stimulated by calcium-ionofore in mice. However, the majority of the studied samples showed a considerable effect concerning the release of thromboxane $B_2$ ($TXB_2$) from thrombocytes stimulated by calcium-ionofore. The percentage of the inhibition was slightly lower than in the case of the reference drug, ibuprofen. These results indicate that their antiphlogistic effect is exerted through the selective inhibition of the TX-synthase enzyme. [121]

## 2. Aims of the study

The experts' opinions differ as concerns the classification of the genera belonging to the Lamiaceae family (approx. 200 genera, 3500 species), which is extremely rich in medicinal plants. The chemical examination of the *Stachys* genus and the comparison of the extraction of the species with the similar data of other taxa belonging to the Lamiaceae family contribute to the elucidation of disputed taxonomical issues. The assessment of the active agents of the species examined also reveals information on the potential use of the species. My task in the Department of Pharmacognosy of the Faculty of Pharmacy of the University of Szeged is the examination of the secondary metabolic products of the *Stachys* species which are native to or can be introduced to Hungary, which is a project supported by OTKA (National Scientific Research Fund).

**My major aims included:**

- to examine the volatile and non-volatile components of *Stachys* species
- to isolate iridoids from *Stachys* species
- to work out a simple routine method for the detecting of the iridoids
- to identify the iridoid content, composition and the quantity of each component in Hungarian *Stachys* species (TLC/densitometry)
- to perform their biological effect study (antioxidant and cytotoxic effects)
- to draw conclusions as to their taxonomy and potential use as a medicinal plant

## 3. Materials and methods

### 3.1. Plant material

For the examination of the volatile – and non-volatile components of *Stachys* species the plants were cultivated in the experimental field of the Research Institute of Ecology and Botany of the Hungarian Academy of Sciences or gathered in the vicinity of Lake Balaton and Szeged as well as at the Őrbottyán turn-off in 2000, 2001, 2002 and 2004. The plants were identified by † Dr. Vilmos Váry -Miklóssy. The plants were under cold storage until use. Voucher specimens are deposited in the Research Institute of Ecology and Botany of the Hungarian Academy of Sciences and the Department of Pharmacognosy University of Szeged, Hungary.

### 3.2. Identification of volatile oil components:

Volatile oil was obtained from the plants with steam distillation according to Section J/c 15 of the VII Hungarian Pharmacopoeia.

The volatile oils were examined with TLC chromatography and with the GC/FID, GC/MS gas chromatographic methods.

Prior to the detailed gas chromatographic evaluation, preliminary examinations were carried out to determine whether terpenoid components could be found in the samples during the NP-TLC (Merck) examination following *n*-hexane extraction. 1 g of fresh plant was extracted with 3x20 mL *n*-hexane. Then it was concentrated to 5 mL and in NP-TLC was used with the following solvent system: benzol:ethylacetate/9:1. Vanillin- sulphuric acid reagent was used as developer.

As the oil content was low, it was distilled using a Clevenger type apparatus for 4 h into *n*-hexane as an auxiliary phase. The weighed drug was 30 g. Distillation time was 4 hours with the exception of 2 species. (A lower temperature was used in the case of *St. byzantina* and *St. germanica* because of intensive foaming, but distillation time was increased by 1 hour.) The oil composition was determined both by GC (HP 5890 Series II gas chromatograph (FID) using a 30 m x 0.35 mm x 0.25 μm HP-5 fused silica capillary column. The oven temperature was programmed at $3^{o}$ C/min from $60^{o}$ C to $210^{o}$ C and then at $5^{o}$ C/min to $250^{o}$ C; which was held for 2 min. The detectore and injector temperatures were $250^{o}$ C the carrier gas: $N_2$) and by GC/MS (ion trap instrument: Finnigan GCQ, MS detector, column: DB-5MS, 30 m; the other main parameters were as above; carrier gas: He).

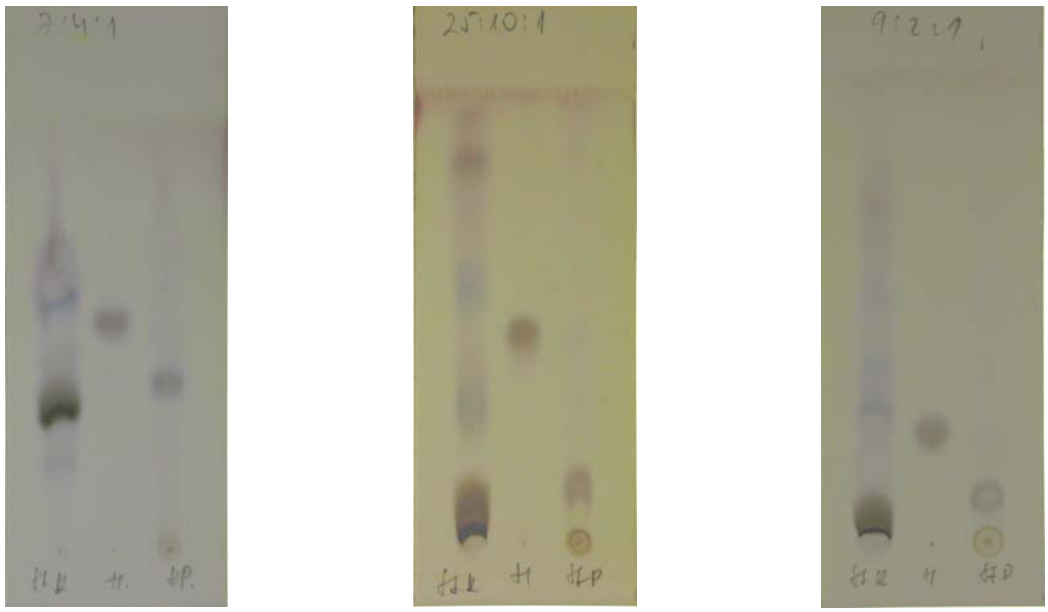
### 3.3 Extraction and isolation of iridoids

#### 3.3.1. Extraction

The first samples to be examined were the *Stachys palustris* specimens collected in Fonyód and the *Stachys recta* specimens collected at Őrbottyán. First orienting examinations were performed with the use of the NP-TLC (silica gel $60F_{254}$ Merck) method.

5 g of herb of the *Stachys* species examined were extracted with 2x30 mL of metanol. After filtration the dry residue was 0.56 g for *St. palustris* and 0.62 g for *St. recta*. Extraction was carried out for 2x15 minutes with an ultrasonic shaker. By using several developing solvents the TLC method was applied to choose the one which ensured the best separation for the iridoid components of all the species. Harpagide was used as the authentic standard.

1. EtOAc-HCOOH-$H_2O$ / 7:4:1
2. $CHCl_3$-MeOH-$H_2O$ / 32:10:1.6
3. $CHCl_3$-MeOH-$H_2O$ / 25:10:1
4. EtOAc-MeOH-$H_2O$ / 9:2:1



**1** **3** **4**

**Figure 1.** Typical TLC chromatograms obtained from extracts of *St. palustris* and *St. recta* (StP., StR.,) and authentic standard of the iridoid harpagide (H)

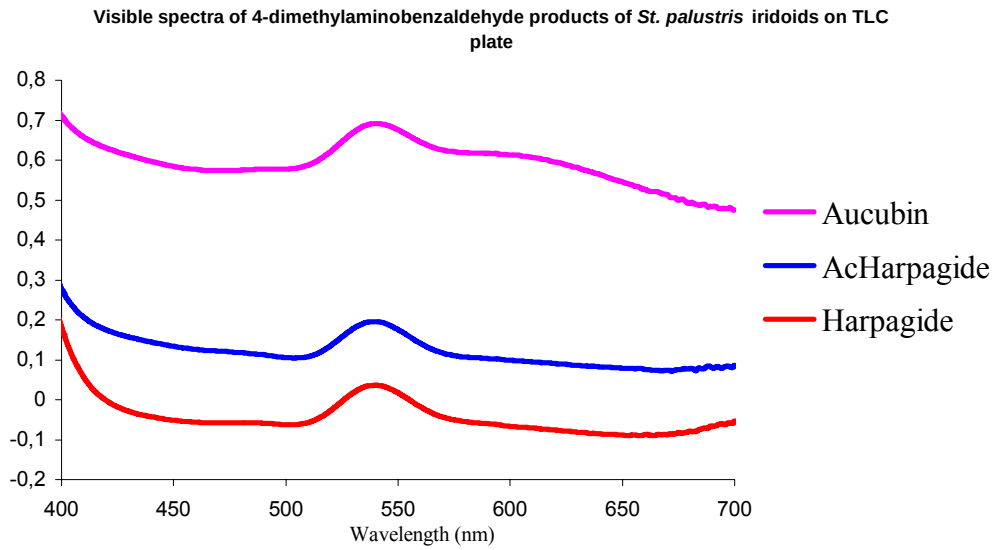



**Figura 2.** Visible spectra of 4-dimethylaminobenzaldehyde products of *St. palustris* iridoids on TLC plate

With respect to the chromatograms of **Figure 1**., solvent system No. 3 proved to be the best as this resulted in the best separation of the components. In the course of subsequent examinations this system was used to check the fractions taken.

Part of the examination was to choose the proper developing reagent as the iridoids we examined are not visible under (either long-wave or short-wave) UV. Several developing reagents were tested: vanillin-sulphuric acid, Trim Hill reagent and the 1 % concentrated hydrochloric acid solution of paradimethylaminobenzaldehyde. The latter one showed the greatest selectivity, that is the intensity of the colour obtained considerably exceeded that of the first two, therefore this reagent was chosen to be a developer. The iridoid spots appeared on the TLC plate was heated at 105$^{o}$ C for 5 min.

Examinations were also performed to assess the colour intensity change of two test materials, aucubin and catalpol. After development with dimetylaminobenzaldehyde, colour intensity stabilised 2-3 hours later in the case of catalpol. (**Figure 3**.)

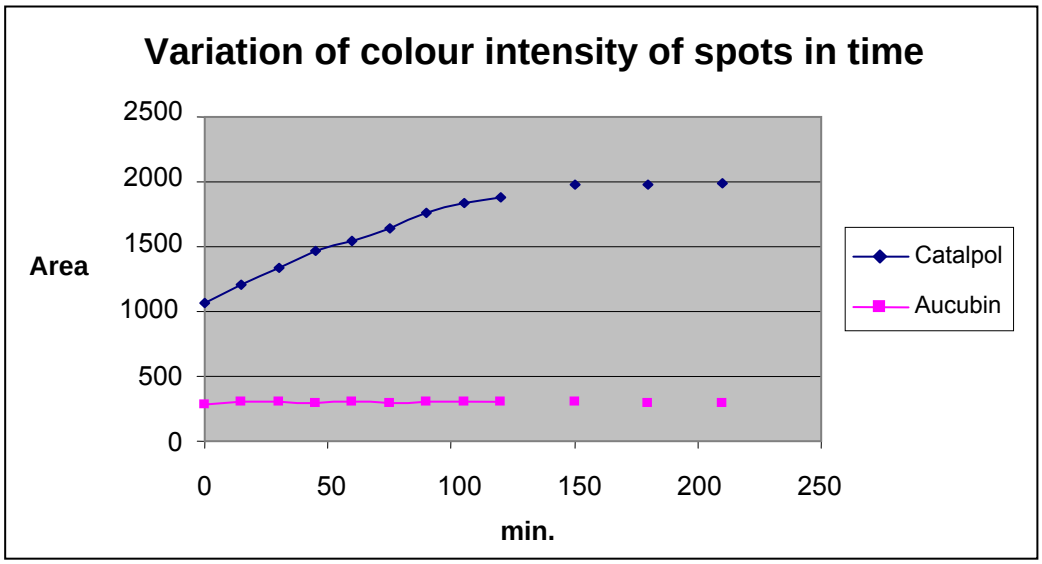



**Figure 3**. Variation of the intensity of spot color with time

*St. palustris* and *St. recta* were rubbed with $CaCO_3$ (the use of $CaCO_3$ is a simple method for the inhibition of the hydrolysing activity of the acids present) [122] and was extracted with methanol with the help of an ultrasonic shaker and Gerhardt shaker. The total methanolic extract was further purified on aluminium oxide (90 active neutral column 0.063-0.200 mm Merck (70-230 mesh ASTM)). Extracts were concentrated under vacuum with a Rotavapor RE (Büchi) rotary evaporation system. This was dissolved in water and a liquid – liquid distribution was performed with chloroform. Chlorophyll was removed with polyamide column chromatography.

### 3.3.2 Purification and isolation of components

TLC silica gel 60 G (mean particle size 15 μm) (Merck 11677) was used for VLC-vacuum-liquid chromatography. The concentrated extract was 3 g for *St. palustris* and 26 g for *St. recta*. After dissolution in water, VLC fractionation was carried out by using a water pump.

Column VLC chloroform – methanol – water [70:10:1 – 10:70:1] and
methanol – water [40:10 – 10:40]

The composition of the fractions was checked with thin layer chromatography in each case, the solvent system was $CHCl_3$-MeOH-$H_2O$ / 25:10:1 a mixture developed by us, while the developer was the 1% concentrated hydrochloric acid solution of dimethylaminobenzaldehyde (105$^oC$, 5min.).

*Isocratic high-performance liquid chromatography*. HPLC columns BST (250 mm, 4mm) SI-100 10 C-18, and LiChrospher RP-18 (5μm) reversed phase Merck. Instrument Shimadzu SPD 10 A/10 AV HPLC.

RP method

Method 1 eluent: water – acetonitrile [90:10]; flow rate: 0.5 mL/min.

Method 2 eluent: water – acetonitrile [98:2]; flow rate: 0.5 mL/min.

Method 3 eluent: water – methanol [80:20]; flow rate: 0.5 mL/min.

Method 4 eluent: water – methanol [90:10]; flow rate: 0.5 mL/min.

The fractions were concentrated under vacuum with a Rotavapor RE (Büchi) rotatory evaporation system.

### 3.4 Identification of isolated components

- Melting points were measured with a Boetus apparatus (Dresden, Germany)
- The UV spectra were recorded in MeOH and $H_2O$ with Shimadzu UV 2101 PC spectrophotometer
- $^{1}H$, $^{13}C$ and 2D (COSY, NOESY, HMBC and HMQC) NMR spectra were recorded in MeOH-*d*, $H_2O$-*d* and DMSO-*d* sample tubes at room temperature, with a Bruker Avance DRX 400 spectrometer, at 400 MHz ($^{1}H$) and 100 MHz ($^{13}C$))

### 3.5 TLC/densitometric examinations

5 g of fresh plant was extracted in the presence of $CaCO_3$ with 3x15mL of water for 15 minutes in ultrasonic water bath. The aqueous extract was filtered through a neutral $Al_2O_3$ column in order to remove phenolic substances. The extracts obtained in this way and the isolated components were used for the TLC/densitometric measurements.

The TLC/densitometric measurements were performed with a (Shimadzu CS-9301 PC, λ=540nm) densitometer.

### 3.6. Quantitative determination of secondary metabolic products

#### 3.6.1. Determination of hydroxycinnamic acid derivatives with Arnow's method [123]

1 gram of drug is boiled in a water bath with 20 mL of 50% ethanol for 30 minutes. Then it is cooled, filtered and made up to 100 mL with 50% ethanol (Solution A). 0.5 mL of Solution A is taken and 1 mL of 0.5 mol/l HCl, 1 mL of Arnow's reagent (1:1 mixture of the solution of 10g of $NaNO_2$ prepared with 100 mL of $H_2O$ and the solution of 10 g of Na-

molibdenate prepared with 100 mL of $H_2O$) and 1 mL of 1 mol/l NaOH solution are added to it. This solution is made up to 10 mL with distilled water. Absorbance is measured after 30 minutes at 510 nm with reference solution (the mixture of 1.0 mL of 0.5 mol/l HCL solution, 1.0 Arnow's reagent, 1.0 mL of 1mol/l NAOH made up to 10 mL with distilled water).
Evaluation is made with the help of the calibration curve of caffeic acid (k=3.62). [124, 125]

### 3.6.2. Determination of flavonoid content with Glasl's method [126]

0.600 g of drug (250) is boiled in a water bath with 20 mL of acetone, 2 mL of 25% HCl solution and 1 mL of 0.5 % methenamine for 30 minutes in a 100 mL flask under a cooling pipe. After cooling, it is filtered into a 100 mL volumetric flask. The drug is boiled again with 20 mL of acetone for 10 minutes. After cooling and filtering, it is added to the previous filtrate and the whole procedure is repeated again. Then it is made up to 100 mL (Solution A).
80 mL of distilled water is added to 40.0 mL of Solution A in a shaking funnel and shaken with 3 x 60 mL of cyclohexane. The cyclohexane phases are removed. The water-acetone phase is shaken with 3 x 60 mL of ethylacetate. The ethylacetate phase is washed with 4 x 50 mL of distilled water. The water-acetone phase is added to the aqueous phase and is made up to 500.0 mL with acetone in a volumetric flask (Solution B).
The combined ethylacetate phase are concentrated in vacuum to 50 mL, then it is made up to 100 mL with ethylacetate (Solution C).

#### 3.6.2.1. Determination of O-glycosides (apigenin and quercetin type)

1.0 mL of $AlCl_3$ 5% methanolic solution is added to 10.0 mL of Solution C with ethylacetate in a 25 mL volumetric flask, then it is made up with the mixture of methanol : 98% acetic acid (95:5).
*Reference solution:* 10.0 mL of Solution C is made up in a 25 mL volumetric flask with the mixture of methanol: 98% acetic acid (95:5).
Absorbance is measured after 30 minutes at 405 - 436 nm. (Hελios Range of UV Visible spectrophotometer)
The O-glycoside content [%] can be calculated with the help of the specific absorption coefficient of apigenin (Sigma-Aldrich, 405 nm) and quercetin (Merck, 436 nm).

*O*-glycoside content [%] = (A/$A^{1\%}_{1cm}$) . D

A= absorbance of the sample measured

$A^{1\%}_{1cm}$ (apigenin λ=405 nm) = 380

$A^{1\%}_{1cm}$ (quercetin λ=436 nm) = 772

1 = cuvette thickness 1 cm

D = dilution

### 3.6.2.2. Determination of C-glycoside content

50.0 mL of aqueous-acetonic Solution B is evaporated, then it is dissolved with the (1:10) mixture of 11 mL of methanol : 98% acetic acid. 10 mL of oxalborate reagent is added to it in a 25 mL volumetric flask and it is completed with 98% acetic acid.

*Reference solution:* 50.0 mL of Solution B is evaporated, then it is dissolved with the (1:10) mixture of 11 mL of methanol : 98% acetic acid. 10 mL of formic acid is added to it in a 25 mL volumetric flask and it is completed with 98% acetic acid. Absorbance is measured at 405 nm after 30 minutes.

The C-glycoside content can be measured with the help of the specific absorption coefficient of apigenin (405 nm).

*C*-glycoside content [%] = (A/$A^{1\%}_{1cm}$) . D

A= absorbance of the sample measured

$A^{1\%}_{1cm}$ (apigenin λ=405 nm) = 978

1 = cuvette thickness 1 cm

D = dilution

### 3.6.3. Quantitative determination of polyphenolic compounds [127]

### 3.6.3.1. Determination of tannin content

*Preparation of extract*: 150 mL of distilled water is added to 0.75 g of drug. After heating in water bath for 30 minutes and cooling, it is made up to 250 mL with distilled water. The first 50 mL of filtrate is removed and the remaining 200 mL is used.

*Determination of total polyphenol content*: 5 mL of basic filtrate is made up to 25 mL with distilled water in a volumetric flask. 2 mL of phosphowolframic acid is added to 5 mL of this (10 g of Na wolframate, 8 mL of 85% phosphoric acid, 75 mL of distilled water and in water bath for 3 hours using a cooling pipe. After cooling it is made up to 100 mL with distilled water) and it is made up to 50 mL with 15% $Na_2CO_3$ solution. After 3 minutes absorbance is measured at 715 nm against distilled water ($A_1$).

*Determination of polyphenol which was not absorbed with hide powder*: 0.2 g of hide powder is added to 20 mL of basic filtrate, then after shaking for 60 minutes and filtering 5 mL of it is made up to 25 mL with distilled water. 2 mL of phosphowolframic acid is added to

5 mL of this and it is made up to 50 mL with 15% $Na_2CO_3$ solution. After 3 minutes absorbance is measured at 715 against distilled water ($A_2$).

*Reference measurement*: 50 mg of pyrogallol is made up to 100 mL with distilled water. 5 mL of this is made up to 100 mL with distilled water. 5 mL part of this is diluted with distilled water to 25 mL. 2 mL of phosphowolframic acid is added to 5 mL of this solution and is made up to 50 mL with 15% $Na_2CO_3$ solution. After 3 minutes absorbance is measured at 715 nm against distilled water ($A_3$).

Tannin content [%] = $13.12.(A_1-A_2)/A_3.m$

where m= mass of drug [g]

### 3.6.3.2. Determination of total polyphenol content expressed in gallic acid

The determination of the total polyphenol content (*A1*) is the same as described in Chapter 3.6.3.1, but evaluation is made on the basis of the calibration curve of gallic acid (k= 23.41).

### 3.6.3.3. Determination of total polyphenol content expressed in tannin

The determination of the total polyphenol content (*A1*) is the same as described in Chapter 3.6.3.1, but evaluation is made on the basis of the calibration curve of tannin (k= 25.11)

## 3.7. Methods of biological effect studies

### 3.7.1. Extraction

2 gram of drug is extracted with 100 mL of methanol at room temperature with the use of an ultrasonic shaker for 2x15 minutes. Extracts were concentrated under vacuum. The dry residue is 12.2 % for *St. officinalis*, 14 % for *St. annua*, 16 % for *St. recta*, 11.6 % for *St. macrantha*, 13.2 % for *St. alpina*, 12.4 % for *St. sylvatica*.

### 3.7.2. Measurement of antioxidant activity

Enzyme-independent lipid-peroxidation was assayed on a standard ox-brain homogenate according to Stocks et al.. Seven different concentrations of each extract were tested for antioxidant activity, from 0.00033 mg/mL to 0.15 mg/mL). All the *in vitro* antioxidant experiments were performed in duplicate and means were calculated. No error was computed, as the differences were approximately 1%. Saturation curves were fitted to the measurement data and $IC_{50}$ values (the concentration at which 50% of the maximal lipid-peroxidation inhibition is exerted) were calculated by means of the computer program

GraphPad Prism 2.01. Linear regression was calculated between the concentration of the special substances and the antioxidant activity to evaluate the contribution of the given compound to the biological effect. Ascorbic acid and $\alpha$-tocoferol succinate were used as positive controls resulting in $IC_{50}$ values (the concentration of each component that exerts the expected antioxidant activity in 50 % of the samples examined) of 0.058 mg/mL and 0.202 mg/mL, respectively. Antioxidant activity was defined as $1/IC_{50}$ [128, 129].

### 3.7.3. Extraction and isolation

5 g of the organs of the *Stachys* species examined were extracted with the 8:2 mixture of 2x 30 mL of methanol:water with an ultrasonic shaker. The dry residue was 12.6% for *St. officinalis* L. (leaf, stem, inflorescence), 12.2% for *St. alpina* L. (leaf, stem, inflorescence), 13.2% for *St. grandiflora* L. (leaf, stem, inflorescence), 11.4% for *St. germanica* L. (leaf, stem, inflorescence), 12.2% for *St. byzantina* C. Koch ( leaf, stem, inflorescence), 13.4% for *St. macrantha* (Koch) Stearn (leaf, stem, inflorescence), 12.4% for *St. sylvatica* L. (leaf, stem, inflorescence), 15.5% for *St. palustris* L. (leaf, stem, inflorescence), 16% for *St. recta* L. (leaf, stem, inflorescence) and 14% for *St. annua* L. (leaf, stem, inflorescence). The iridoid components were isolated from *St. palustris* and from *St. recta* described in Chapter 3.3

### 3.7.4. Cytotoxicity determination using the MTT colorimetric assay

Cytotoxic effects were measured *in vitro* on three human cell lines: MCF-7 (breast adenocarcinoma), HeLa (cervix adenocarcinoma), and A431 (skin epidermoid carcinoma). The cells were cultivated in minimum essential medium (Gibco BRL, Paisley, UK) supplemented with 10% foetal bovine serum, 1% non-essential amino acids and antibiotic-antimycotic (10 000 units/mL penicillin G sodium, 10 000 μg/mL streptomycin sulfate and 25 μg/mL amphotericin B). The cells were grown in a humidified atmosphere of 5% $CO_2$ at 37 °C.

Near confluent cell were seeded into a 96-well plate at a rate of 5000 cells/well. After standing overnight the medium was removed, and 200 μL of the medium containing the tested substance was added. 30 mM and 10 mg/mL stock solutions of the tested pure compounds and extracts were prepared with dimethyl sulfoxide (DMSO), respectively. The highest DMSO concentration of the medium was 0.3%, which did not have any significant effect on cell proliferation.

After a 72-hour incubation period, the cytotoxicity was measured by the addition of 20 μL of 5 mL/mL MTT ([3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide]) assay (Sigma-Aldrich Ltd, Hungary).

After a 4-hour contact period, the medium was removed, and the precipitated formazan crystals were solubilized in 100 μL DMSO at 37°C by shaking. Finally, the absorbance was read at 545 nm with an ELISA reader (Mosmann, 1983).

Sigmoidal dose-response curves were fitted to the measured absorbance points, and the $IC_{50}$ values (the concentration at which the inhibition of the cell proliferation attained half of the maximum) were calculated with GraphPad Prism 4.03 software. Doxorubicin and cisplatin were used as positive control. [130]

## 4. Results and discussion

### 4.1. Determination of volatile components from *Stachys* species

Based on the results of the orienting thin layer chromatography, the volatile components were determined. By using the method described in Chapter 3.2, volatile oil was obtained from the fresh plants (500 mL of distilled water was added to 30 g of weighed plant) with steam distillation, with the use of the *n*-hexane auxiliary phase. (In this phase the use of the auxiliary phase was necessary because of the slight quantity of volatile oil.)

The volatile components of the following *Stachys* species were examined: *St. officinalis* L., *St. officinalis* subs. betonicaL. *St. alpina* L *St. germanica* L., *St. byzantina* C. Koch *St. grandiflora* Host., *St. macrantha* (C. Koch) Stearn, *St. palustris* L., *St. recta* L., *St. sylvatica* L., *St. annua* L.,) [9, 12]. The volatile oil obtained with distillation was assayed with the GC-FID, GC/MS method. The components were determined with the Kováts retention index calculation, with the data base of the GC/MS apparatus and with the help of the reference materials.

Kováts retention index:

$$I_x = 100 \times \frac{\log t'_{R,x} - \log t'_{R,n}}{\log t'_{R,n+1} - \log t'_{R,n}} + 100n$$

n: C atomic number
log$t'_R$: logarithm of reduced retention time

where

$$t'_{R,n} \langle t'_{R,x} \langle t'_{R,n+1}$$

### 4.1.2 Results of gas chromatography

Our results are the following:

- The thin layer chromatography of the extract prepared with *n*-hexane and the distillate was also performed. The authentic standards were: sabinene, limonene, linalool, *β*-caryophyllene.
- All the species we examined contain a very small quantity of volatile oil.
- An auxiliary phase was used during steam distillation because of the low volatile oil content of all the species examined.
- 160 components of the species examined were identified with the method used. 62 of them are monoterpene like components, 98 sesquiterpene components or components with a higher number of carbon atoms. We also identified 6 mono- and sesquiterpene components each of which occurs only in one species in traces.
- The values of the peaks under 0.1 area percent are not given numerically, their presence is only indicated.
- From among monoterpenes, linalool occurs in all the species examined by us, but sabinene, *β*-phelladrene, *cis*-ocimene are also present in several species. As regards sesquiterpenes, *β*-caryophyllene occurs in all the species examined, but *γ*-muurolene, germacrene-D, *δ*-cadinene, spatulenol, *α*-cadinol also occurs as a volatile oil component with the exception of only 1-2 species.
- The following monoterpenes occur in the greatest %: sabinene 12% (*St. byzantina*), 1-octen-3-ol 5.6% (*St. recta*), *β*-phellandrene 4.8% (*St. germanica*), linalool 3.2% (*St. recta*), *trans*-pinocamphone 4.8% (*St. byzantina*), while from among sesquiterpenes *β*-caryophyllene 16.5% (*St. officinalis* ssp. serotina), aromadendrene 10.6% (*St. grandiflora*), *γ*-muurolene 23.5% (*St. alpina*), *γ*-curcumene 16.9% (*St. grandiflora*), germacrene-D 33.1% (*St. sylvatica*), valencene 46.1% (*St. officinalis* sample I.). These results are presented in **Tables 1**. and **2**. in the **Appendix**.
- The greatest number of components could be identified in *St. officinalis* sample II.. In agreement with the literature data, the components identified in the species examined are poorer in monoterpene components. The components identified in the species examined amount to 51-89 % of the total volatile oil content (**Table 3** in the **Appendix**)
- With respect to the *Stachys* genus sections examined, the *Eriostomum* section is the one which contains the smallest number of monoterpenes, while the greatest number was observed in the *Stachys* and *Olisia* sections. The same is true for *St. recta* in the *Stachys* section both with respect to the number and quantity of the monoterpenes contained in it.

The *Betonica* section is the richest in sesquiterpenes, but the *Olisia* and *Stachys* sections are also worth mentioning both regarding the number of their components. Once again, *St. recta* should be mentioned here, as it contains the lowest % of sesquiterpenes.

- In the course of identifying volatile components alkanes, alkenes, aldehydes, ketones, fatty acids, monoterpene and sesquiterpene hydrocarbons and their oxidated forms, diterpenes were identified. The percentage proportion of these components in volatile oil is shown in **Table 4**. in the **Appendix**.
- The double sample of *St. officinalis* and *St. annua* is explained by the different places (Szeged, Vácrátót) and different times (2001 and 2004) of collection.
- With regard to *Stachys* species no literature data were found concerning the volatile oil examination of *St. germanica, St. grandiflora* and *St macranta*, therefore it is presumable that we are the first to publish data concerning the volatile components of these species.
- All the volatile components found during the examination of the *Stachys* species occurred in other Lamiaceae species.
- The above statements apply only to the species examined.

### 4.2 Extraction, isolation and identification of iridoids

The examination of the non-volatile components of *Stachys* species native to Hungary was started with their iridoid content. Examinations were performed for the major iridoid components of the following species: *St. officinalis*, *St. alpina, St. germanica, St. byzantina, St. grandiflora*, *St. macrantha, St. sylvatica*, *St. palustris, St. recta,* and *St. annua.*

#### 4.2.1 Comparative analysis of iridoids with thin layer chromatography

**Figure 4**. shows the TLC chromatogram of the preliminary examination of iridiods described in Chapter 3.3.

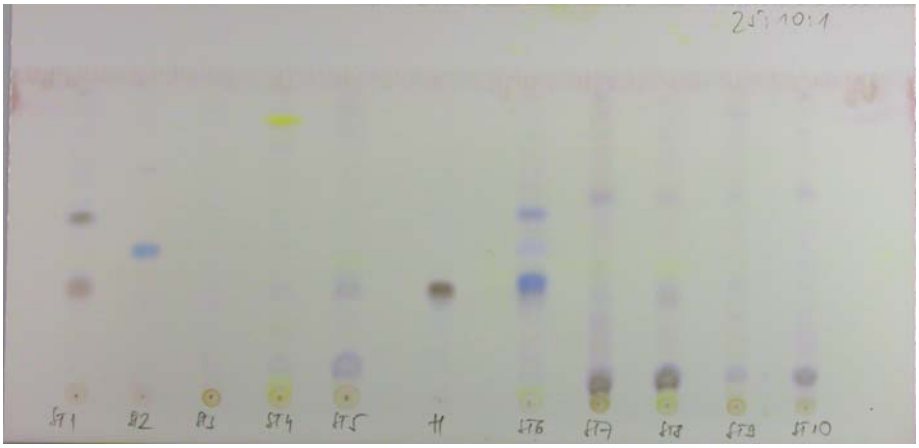


**Figure 4.** Typical TLC chromatograms obtained from extract of *St. officinalis* (St1), *St. sylvatica* (ST2), *St. grandiflora* (ST3), *St. macrantha* (ST4), *St. alpina* (ST5), *St. palustris* (ST6), *St. recta* (ST7), *St. byzantina* (ST8), *St. germanica* (ST9) and *St. annua* (ST10) and authentic standard of the iridoid , harpagide (H)

### 4.2.2 Isolation of the iridoid components of *Stachys palustris* and *Stachys recta*

250 g of *St. palustris* was rubbed with 10 g of $CaCO_3$ and extracted with 1600 mL of methanol with the use of an ultrasonic shaker and Gerhardt shaker. The total methanolic extract was further purified on a 35 g aluminium oxide (90 active neutral) column (*column 1*; *110x22mm*). The residue of dry evaporation was 5.14 g. This was dissolved in 500 mL of water and the liquid – liquid distribution was performed with 3x100 mL of chloroform. Chlorophyll was removed with the use of 5g (*column 2*; *80x12mm*) polyamide with column chromatography. The dry residue after concentration was 3 g.

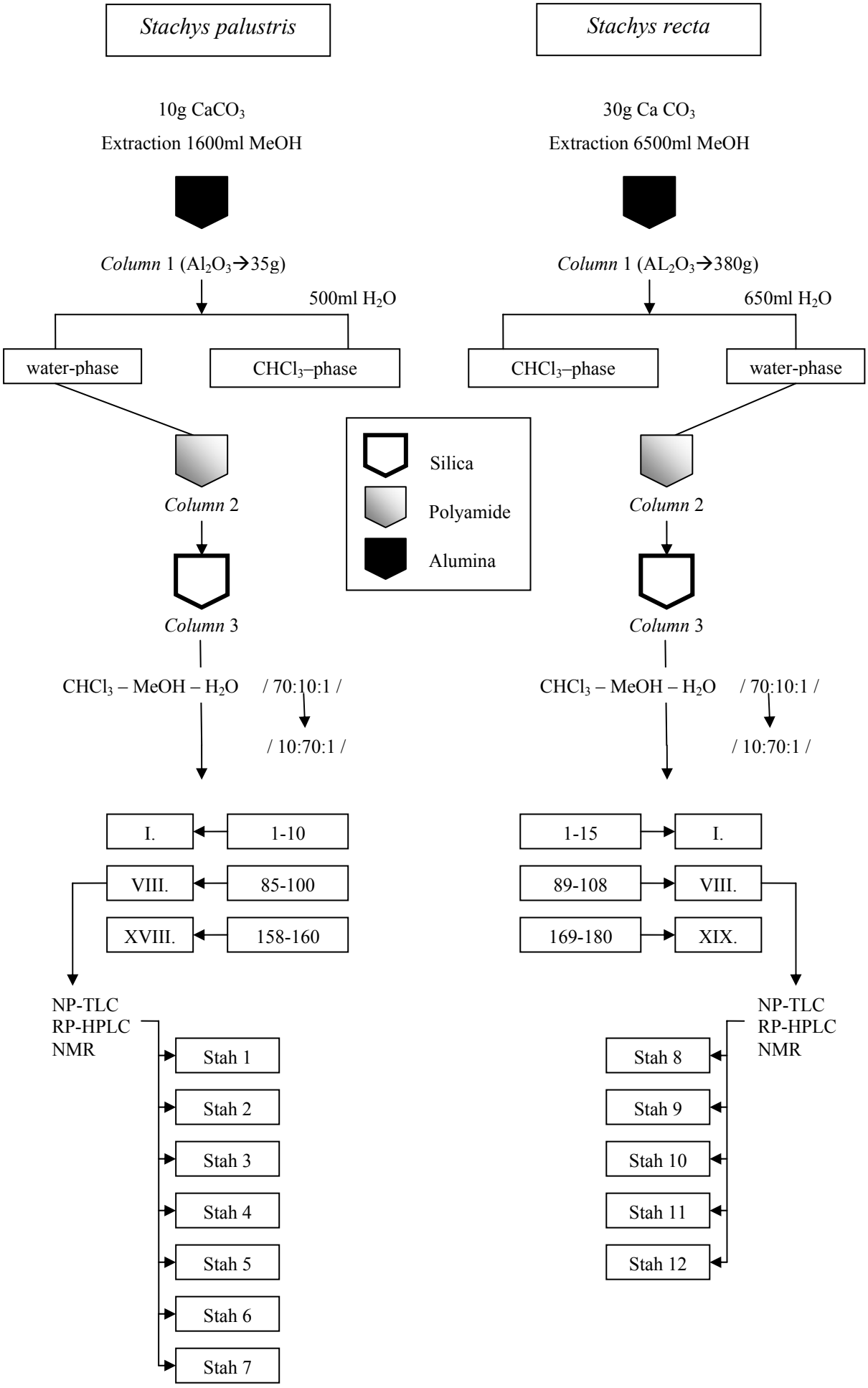
In the course of VLC fractionation 205 (100 mL) fractions were taken with various mixtures of chloroform : methanol : water (*column 3; 100x60mm*). The composition of the fractions was checked in each case with thin layer chromatography. After the evaluation of the chromatograms, the fractions containing the same materials were united, thus 18 fractions were obtained. The composition of the fractions was again checked in the above-described manner. The purification processes of *St. palustris* are summarized in **Figure 5.** Further unification and checking were carried out on the basis of the chromatograms, and it was found that the iridoids to be isolated were enriched in fraction VIII. The eighth fraction was concentrated under vacuum.The residue was 0.12 g. This multi-component fraction was purified with high performance liquid chromatography (RP-HPLC) according to Method 1 and Method 2. The peaks further purification was achieved in the course of re-injection, according to Method 1. Separation was carried out on a BST-type reversed-phase column.

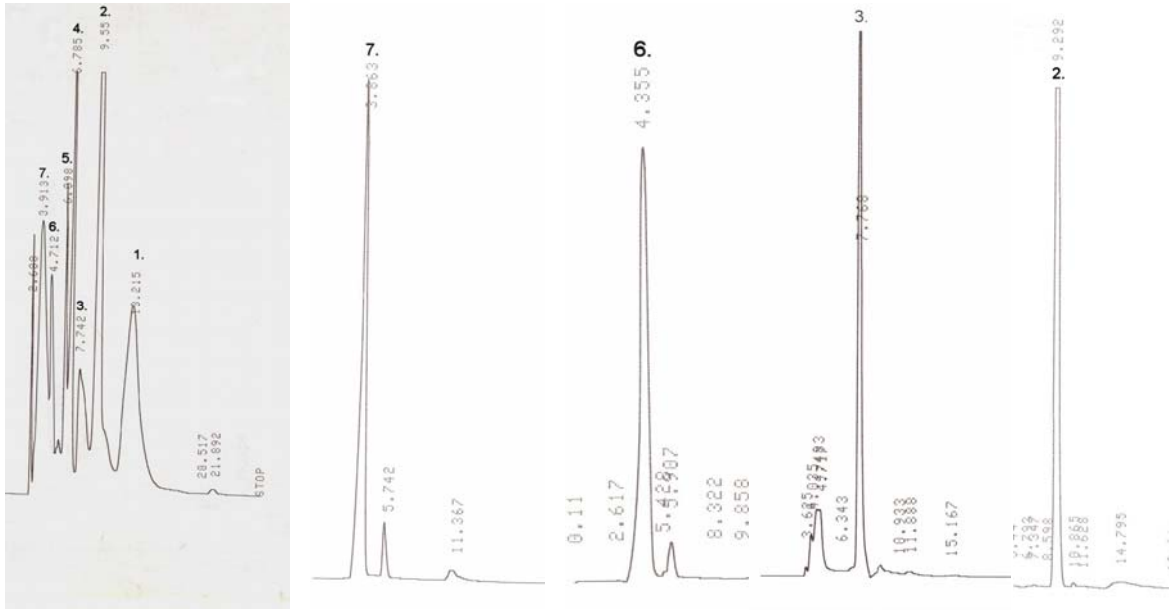
The RP-HPLC purification process is depicted in **Figure 6.** (The isolation of iridoids is made difficult by their similar structure, by their similar chromatographic behaviour and by their instability). 7 iridoids were isolated from *St. palustris* by applying combined chromatographic methods. The substances obtained during purification were stah-1, stah-2, stah-3, stah-4, stah-5 stah-6 and stah-7.

740 g of *St. recta* was rubbed with 30 g of $CaCO_3$ and extracted with 6500 mL of methanol by using an ultrasonic shaker and Gerhardt shaker. The total methanolic extract was further purified on a 200 g aluminium oxide (90 active neutral) column (*column 4*; 400x36mm). The residue of dry evaporation was 53.5 g. This was dissolved in 650 mL of water and the liquid – liquid distribution was performed with 3x250 mL of chloroform. Chlorophyll was removed with the use of 10 g (*column 5*; 160x12mm) polyamide with column chromatography. The dry residue after concentration was 26.61 g. The concentrated material was dissolved in 80 mL of water and suspended on 80 g of silica gel. A column was prepared from 900g of silica gel, then extract suspended with silica gel was layered on the top.

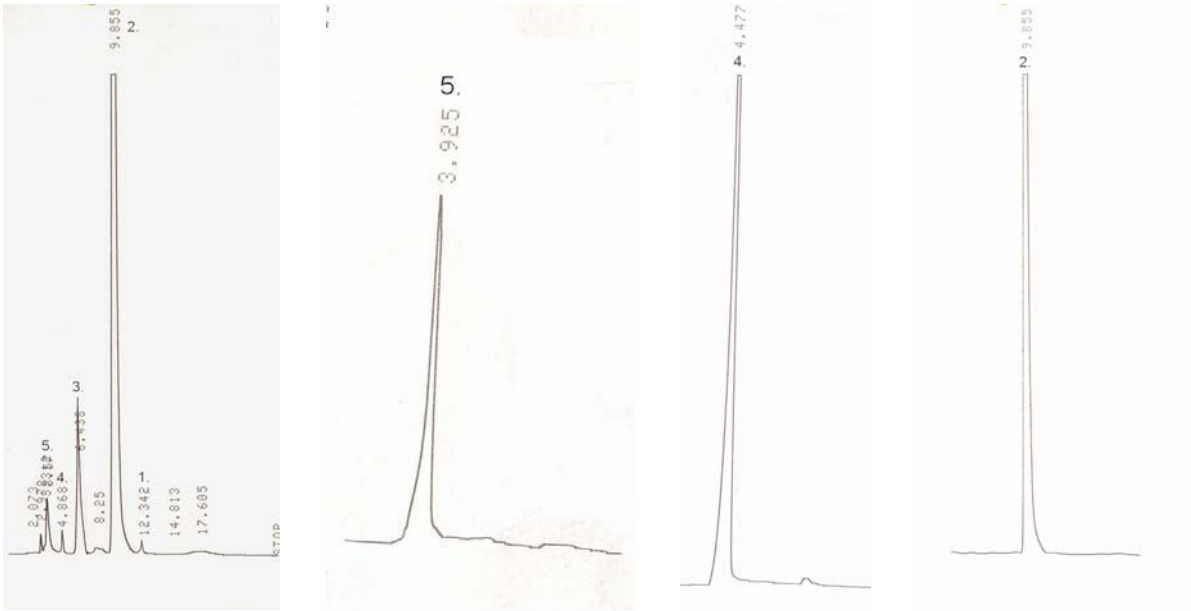
In the course of VLC fractionation 180 (100 mL) fractions were taken with various mixtures of chloroform : methanol : water (*column 6 120x75mm*). The composition of the fractions was checked in each case with thin layer chromatography. After the evaluation of the chromatograms, the fractions containing the same materials were united, thus 10 fractions were obtained. The composition of the fractions was again checked in the above-described manner. The purification processes of *St. recta* are summarized in **Figure 5.** From the chromatograms it was found that the iridoids to be isolated were enriched in fraction VIII. The residue of the eighth fraction after evaporation was 2.22 g. This multi-component fraction was purified with high performance liquid chromatography (RP-HPLC) according to Method 1, Method 2 and Method 3 described in Chapter 3.3.2. The peaks further purification was achieved in the course of re-injection, according to Method 2. Separation was carried out on a BST-type reversed-phase column and on a LiChrospher RP-18 (5µm) reversed-phase column. The RP-HPLC purification process is depicted in **Figure 6.** 5 iridoids were isolated from *St. recta.* The substances obtained during purification were stah-8, stah-9, stah-10, stah-11 and stah-12.

**Figura 5**. Purification processes of *St. palustris* and *St. recta*






RP-HPLC chromatogram of the *Stachys palustris* extract and the some isolated iridoids harpagide(2), acetyl-harpagide(3), ajugoside(6) and harpagoside(7)




RP-HPLC chromatogram of the *Stachys recta* extract and the some isolated iridoids harpagide(2), ajugoside (4) and harpagoside(5)

**Figure 6.** RP-HPLC chromatograms of the extract *St. palustris* and *St.recta* and the isolated compounds

### 4.2.3. Structure determination of the iridoids

The substances obtained with fractionation were identified on the basis of their physical and spectroscopic properties. The physical properties of the isolated components are shown in **Table 2**. The isolated compounds do not have a characteristic absorption spectrum so these data have less use for structure determination.

| Iridoids | M.P.($C^o$) | UV nm |
|---|---|---|
| Ajugoside | Amorph. powder | 206 (3.6) |
| Aucubin | 179-183$^o$ | 210 (3.4) |
| Acetylharpagide | 153-157$^o$ | 210 (3.7) |
| Harpagide | Amorph. powder | 210 (3.6) |
| Harpagoside | Amorph. powder | 216 (4.2) |
| 6 Epi-acetylharpagide | 150-156$^o$ | 204 (3.7) |
| Myoporoside | Amorph. powder | 209(3.2) |

**Table 2**. Physical and UV spectroscopic data on iridoids from *Stachys* species

The data obtained during identification were also compared with the data published in literature. The basic information concerning the structure of the compounds was provided by TLC, RP-HPLC and by one- and two-dimensional NMR measurements. The NMR measurements were carried out and evaluated with the guidance of colleagues in the Department of Pharmaceutical Chemistry. With the help of the HMBC spectrum, the interactions of protons and C atoms through 2 or 3 bonds can be observed. The position of the OH group within the molecule and the quaternary C atom was also determined during the evaluation of this spectrum. The interactions between the C atoms and the connecting protons were also determined with the HSQC spectrum. The H-H-COSY spectrum shows the interactions of neighbouring, coupled protons. The NOESY spectrum reveals the cross peaks of protons close in space. The entire proton and C spectrum was taken for each compound.

**Stah-1** The sugar part is indicated at $\delta_H$=3.22-4.67 ppm. The double bond between the $C_3$ and $C_4$ could be identified at $\delta_H$=5.05-6.3 ppm, the OH group on $C_6$ at $\delta_H$=4.43ppm (β configuration) and the $–CH_2OH$ group on $C_8$ (quaternary C atom) between $\delta_H$=4.16-4.34 ppm.

**Stah-2** The sugar part is indicated at $\delta_H$=3.21-4.58 ppm. The double bond between the $C_3$ and $C_4$ atoms was at $\delta_H$=4.95-6.31 ppm, an OH group is connected to the $C_5$ quaternary C atom. The OH group on $C_6$ could be identified at $\delta_H$=3.70ppm (β configuration), and on $C_8$ (quaternary C atom) an OH group and a $CH_3$ group at $\delta_H$=1.25 ppm could be identified.

**Stah-3** and **Stah-5** The sugar part is indicated at $\delta_H$=2.99-4.39 and $\delta_H$=2.99-4.38 ppm. The double bond between the $C_3$ and $C_4$ atoms was at $\delta_H$=4.88-6.36 and $\delta_H$=4.87-6.24 ppm, an OH

group is connected to the $C_5$ quaternary C atom. The OH group on $C_6$ could be identified at $\delta_H$=3.57 and at $\delta_H$=3.54 ppm, the $CH_3$ group on $C_8$ (quaternary C atom) at $\delta_H$=1.37 and at $\delta_H$=1.08 ppm and the $CH_3$ group of the acetyl-group at $\delta_H$=1.93 and at $\delta_H$=1.66 ppm. The two isolated compounds differ in their $C_6$ configuration. Stah-3 has β, while Stah-5 has α position.

**Stah-4** The sugar part is indicated at $\delta_H$=3.20-4.65 ppm. The double bond between the $C_3$ and $C_4$ atoms could be identified at $\delta_H$=4.71-6.21 ppm, the OH group on $C_6$ at $\delta_H$=4.01 ppm (β configuration), the $CH_3$ group on $C_8$ (quaternary C atom) at $\delta_H$=1.53 ppm and the $CH_3$ group of the acetyl group at $\delta_H$=2.00 ppm.

**Stah-6** The sugar part is indicated at $\delta_H$=3.19-4.66 ppm. The double bond between the $C_3$ and $C_4$ atoms could be identified at $\delta_H$=4.93-6.30, the OH group on $C_6$ at $\delta_H$=4.41ppm (α configuration), and on $C_8$ (quaternary C atom) an OH group and a $CH_3$ group at $\delta_H$=1.36 ppm could be identified.

**Stah-7** The sugar part is indicated at $\delta_H$=3.23-4.63 ppm. The double bond between the $C_3$ and $C_4$ atoms could be identified at $\delta_H$=4.94-6.42 ppm, an OH group is connected to the $C_5$ quaternary C atom. The OH group on $C_6$ was at $\delta_H$=3.77 ppm, the benzene ring of the cinnamoyl group on $C_8$ (quaternary C atom) between $\delta_H$=7.40 – 7.59 ppm and the $CH_3$ group at $\delta_H$=1.54 ppm.

The results of the physical and spectroscopic examinations show that the subsequently isolated **Stah-8** substance is identical to **Stah-2**, **Stah-9** to **Stah 1**, **Stah-10** to **Stah-3, Stah-11** to **Stah-4** and **Stah-12** to **Stah-7**.

**Table 5** in the **Appendix** contains the couplings determined with the help of the NMR spectrum.

### 4.2.4. Simpler method use for detecting the iridoids

In the following a simpler method was used for detecting the iridoid components. An aqueous extract was prepared from 5g of fresh plant of *St. palustris*. It was shaken in an ultrasonic shaker for 3x15 minutes in the presence of $CaCO_3$ with 25 mL of water. The extract was let through an $Al_2O_3$ column. The combined filtrates were concentrated under vacuum (0.15g) and dissolved in 2 mL of methanol : water/8:2, then TLC and HPLC examinations were carried out.

The materials obtained in this way were identified with the already used physical and spectroscopic methods. Aucubin was identified from the aqueous extract by using the above-mentioned methods.

#### 4.2.4.1. Detection of iridoids of further *Stachys* species growing in Hungary

After the isolation and identification of the main iridoid components of *St. palustris* and *St. recta*, the main iridoid components of *St. officinalis*, *St. alpina*, *St. germanica*, *St. byzantina*, *St. grandiflora*, *St. macrantha*, *St. sylvatica* and *St. annua* were detected. For the detection of the iridoid components of these species only aqueous extracts were prepared in accordance with the method worked out for *St. palustris*. Aqueous extracts of 10g of fresh plant (8 *Stachys* species) were prepared, by an ultrasonic shaker for 3x15 minutes in the presence of $CaCO_3$ with 50 mL of water. The extracts were let through 5g of $Al_2O_3$ column (*column 2*). The combined filtrates were evaporated (*St. officinalis* (0.35g), *St. alpina* (0.28g), *St. germanica* (0.19g), *St. byzantina* (024g), *St. grandiflora* (0.15g), *St. macrantha* (0.21g), *St. sylvatica* (0.31g) and *St. annua* (0.23g)) then the dry residues were dissolved in 5mL of methanol : water / 8:2 mixture. Their NP-TLC examination was performed in a $CHCl_3$ : MeOH : $H_2O$/ 25 : 10 : 1 solvent sytem.

The 1% concentrated hydrochloric acid solution of p-dimethylaminobenzaldehyde was used as a developing reagent [122]. Drying at 105°C for 5 minutes in a drying cabinet is necessary for the iridoid spots to appear on the plate. The NP-TLC chromatograms of 8 *Stachys* species are shown in **Figure 4**. Then the RP-HPLC examinations were performed, during which the methods described in Chapter 3.6 were applied. In each case the isolated components stored at -20$^{o}$C were also injected after the extracts.

### 4.2.5. Results of iridoids isolation

- Based on the results of the physical and spectroscopic examinations, the stah-1 substance is aucubin, stah-2 is harpagide, stah-3 is acetylharpagide, stah-4 is ajugoside, stah-5 is 6-epi 8-O-acetylharpagide, stah-6 is myoporoside and Stah-7 is harpagoside.
- We were the first to isolate aucubin, ajugoside, myoporoside and harpagoside from *St. palustris*. Moreover, harpagide, acetylharpagide and 6-epi 8-O-acetylharpagide were also isolated and identified.
- We were the first to isolate aucubin and harpagoside from *St. recta*. Besides, harpagide, acetylharpagide and ajugoside were also isolated and identified.
- In addition to harpagide, acetylharpagide and aucubin, ajugoside, and harpagoside can also be detected from *St. officinali*s (chapter 4.2.4.1.).
- From *St. sylvatica* acetylharpagide could not be detected, only ajugoside, harpagide and harpagoside (chapter 4.2.4.1.).

- Aucubin, harpagide, acetylharpagide, harpagoside and ajugoside were detected from *St. grandiflora*. This was the only *Stachys* species we examined in which the presence of a small amount of catalpol could be detected (chapter 4.2.4.1.).
- From *St. macranta* harpagide could be detected (chapter 4.2.4.1.).
- Aucubin and acetylharpagide can be seen on the NP-TLC and RP-HPLC chromatogram of *St. alpina* (chapter 4.2.4.1.).
- *St. byzantina* contains ajugoside, aucubin and harpagide (chapter 4.2.4.1.).
- Harpagide and harpagoside can be detected in *St germanica* (chapter 4.2.4.1.).
- The presence of iridoid could not be detected in *St. annua* (chapter 4.2.4.1.).
- We could develop a method which can be used for the identification and detection of the iridoids of *Stachys* species.

### 4.3.TLC/densitometric examination of iridoids

After the isolation, detection and identification of the main iridoids of the species examined, and their contents in percentage were determined reganding the organs separately. The method of TLC/densitometry proved to be suitable for this. In the course of this quantitative determination is made directly on the chromatogram on the basis of measuring the extinction of the substances. The extracts were prepared according to the method described in Chapter 3.5.

The results of our measurements refer to air-dry drugs. **Figure 7.** shows the densitograms obtained during the examination of the stem, leaf and inflorescence extracts of *St. officinalis*. Acetylharpagide, harpagide and harpagoside were used as reference materials.

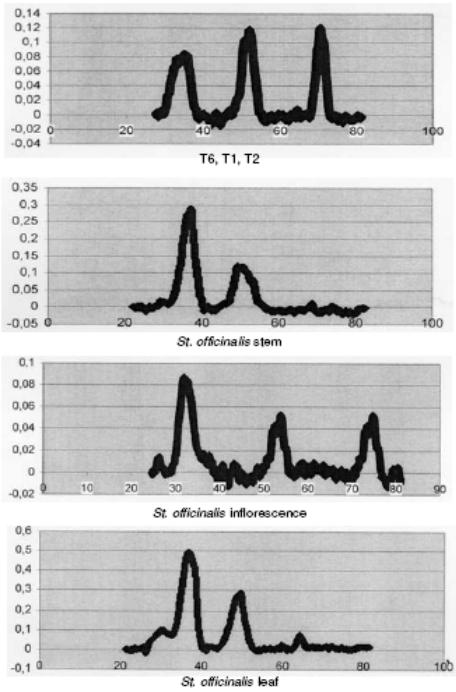



**Figure 7.** Densitograms obtained from a mixture of authentic samples of acetyharpagide (T1), harpagoside (T2) and harpagide (T6) and from extracts of the inflorescence, stem and leaf of *St. officinalis*

TLC/densitometry is based on the spot size and intensity of the components examined after development. The weighed mg quantities of the iridoids used as reference material were made up to 100 mL in each case. The exact amounts of 10, 15 and 20 µl were applied on the layer. A calibration curve was made from this and the quantity of the substance in the 10, 15 and 20 µl and in the 100 mL was calculated based on the area of the spot. Subsequently, the TLC chromatogram of the plant extracts was prepared. After developing, the size and intensity of the spots were measured and the quantity of the components for 100g of plant was determined. **Tables 3. 4.** and **5.** show the results of the densitometric measurements.

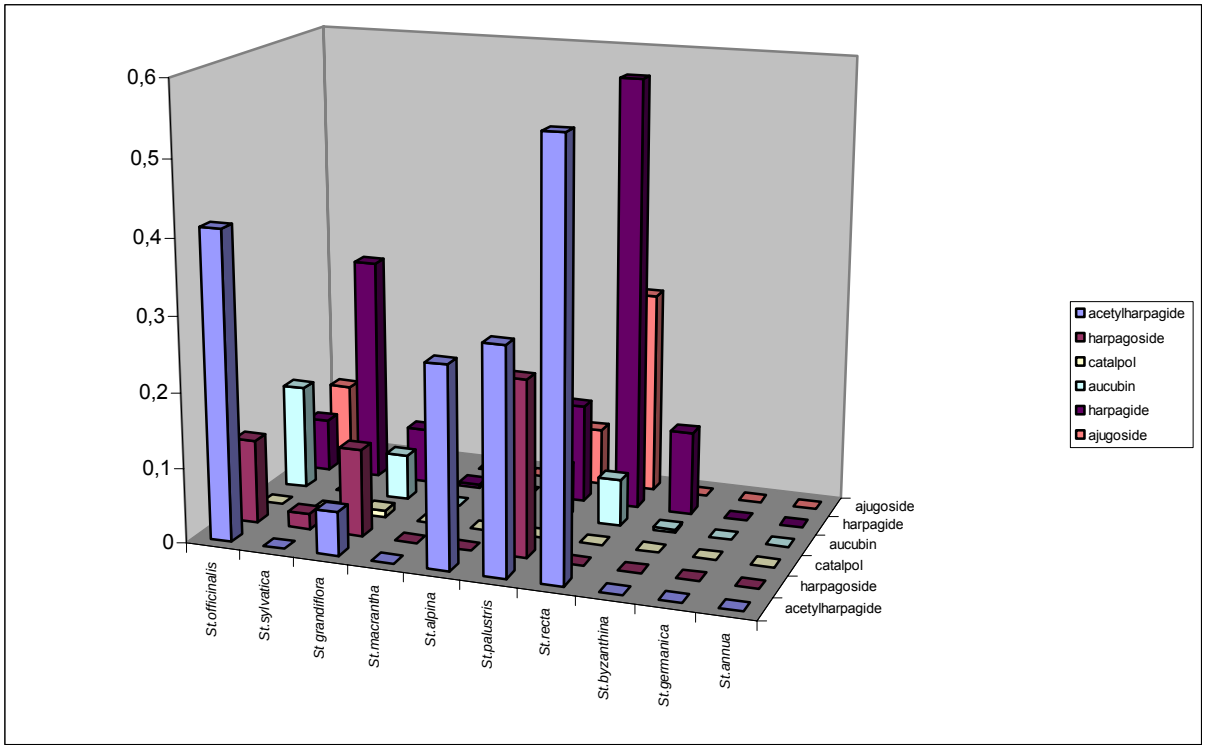



**Table 3.** TLC/densitometric measurement of isolated iridoids in the stem of *Stachys* species

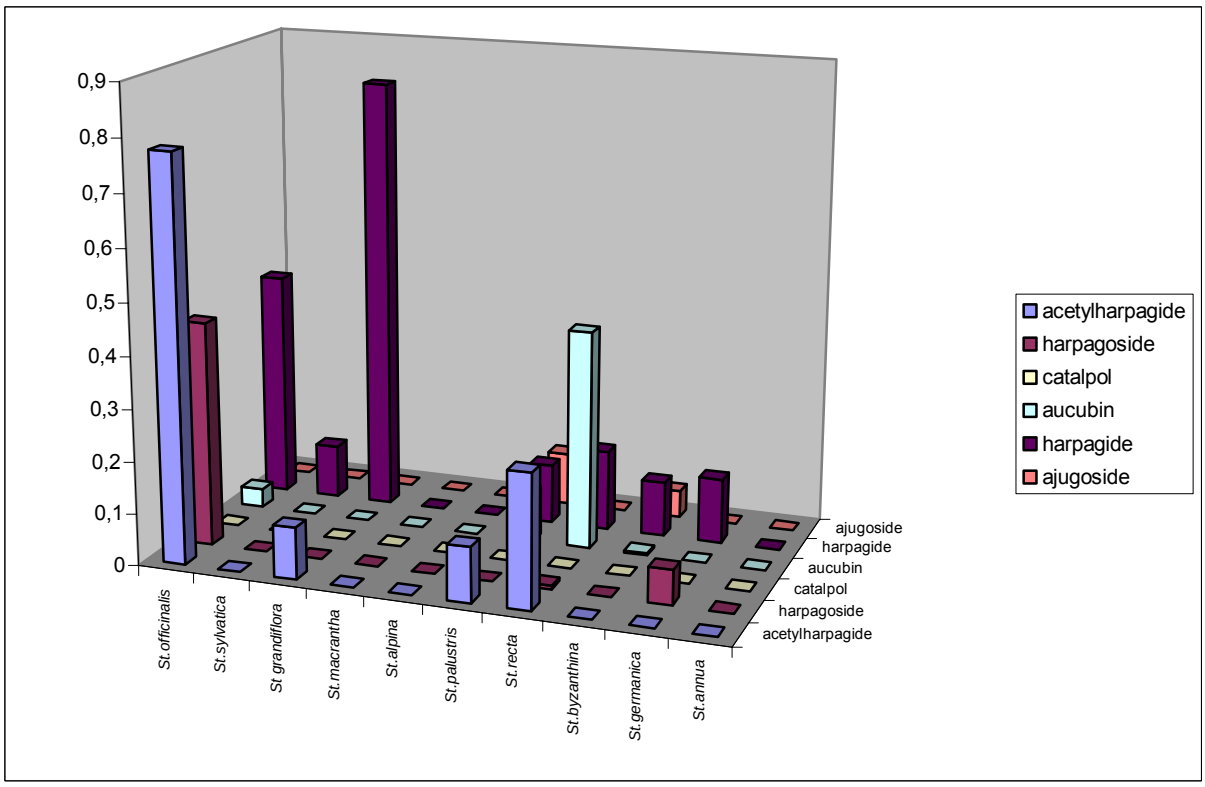



**Table 4.** TLC/densitometric measurement of isolated iridoids in the inflorescence of *Stachys* species

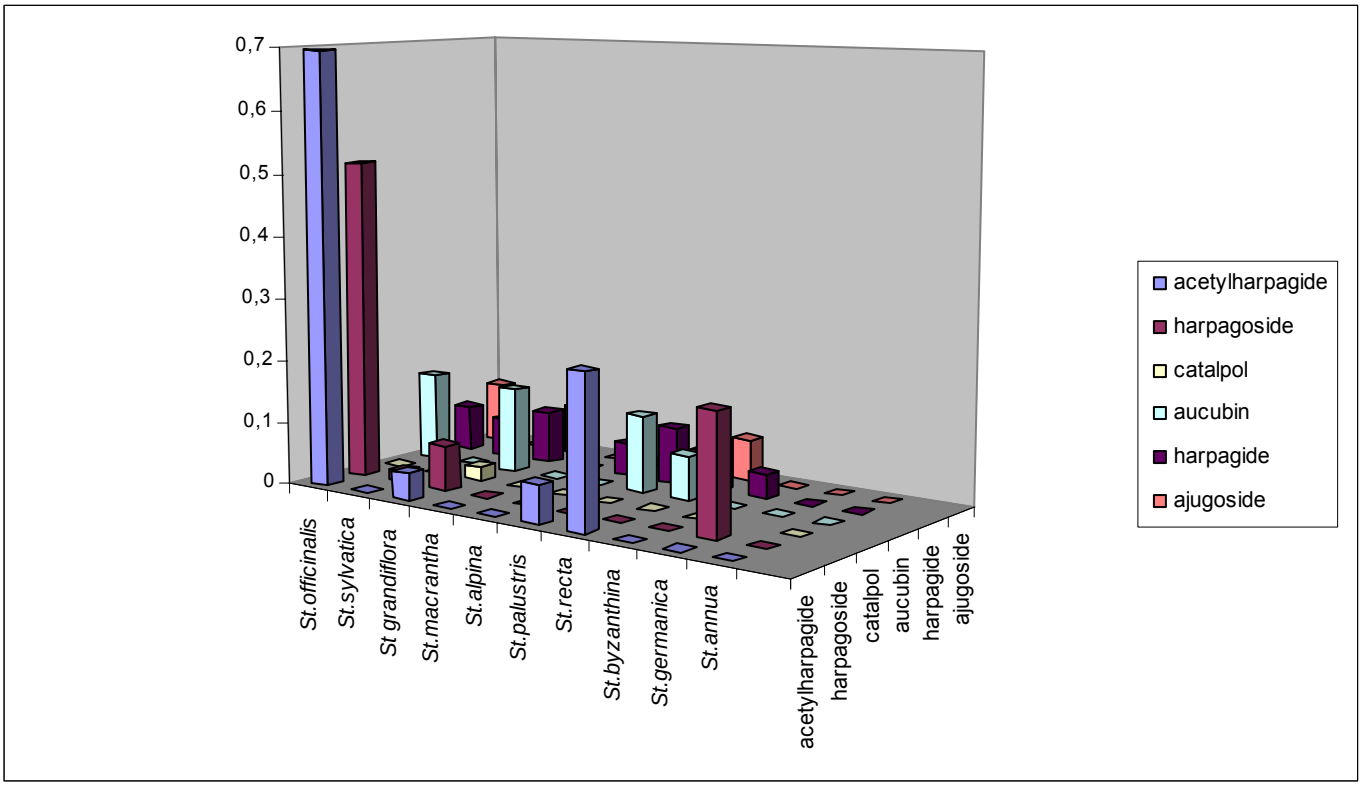



**Table 5.** TLC/densitometric measurement of isolated iridoids in the leaf of *Stachys* species

### 4.3.1. Results of TLC/densitometric examination

- The densitogram in **Figure 7.** shows the acetylharpagide, harpagoside and harpagide content in the stem, leaf and inflorescence of *St. officinalis*.
- The comparison of the data in the tables reveals that the quantity of the iridoid components contained differs according to species and organs.
- The greatest percentage of acetylharpagide was present in the inflorescence (0.78%) and leaf (0.70%) of *St. officinalis*.
- The greatest percentage of harpagoside can be detected in the leaf and inflorescence parts of *St. officinalis,* but a considerable amount is also accumulated in the leaf of *St. germanica.*
- Catalpol was only detected in *St. grandiflora* from among the species examined. No literature data were found concerning the detection of catalpol in *Stachys* species.
- The greatest percentage of aucubin occurred in the leaf of *St. officinalis* and in the inflorescence of *St. recta*
- The greatest percentage of harpagide is accumulated in the stem of *St. recta* and in the inflorescence of *St. grandiflora*.

- The greatest percentage of ajugoside is accumulated in the stem of *St. recta*.
- Our examinations are in agreement with the statements of Bentham's system concerning the *Lamioideae* subfamily insomuch as the species examined – similarly to the other taxa of this subfamily – are rich in iridoids.
- The iridoid content and iridoid composition of these *Stachys* species according to organ was detected and identified first.

## 5. Biological activity of *Stachys* species and their isolated compounds

### 5.1. Examination of the antioxidant effect of Stachys species in enzyme-independent lipid peroxidation system

The classical therapeutic use of a great number of herbs and phytotherapeutic preparations can be explained by the antioxidant effect of plant polyphenols. Antioxidants are substances which neutralize the highly reactive oxygen radicals arising in the organism. These radicals may result in various diseases through interactions.

The antioxidant effect of 6 *Stachys* species, namely *Stachys officinalis*, *St. annua*, *St. recta*, *St. macrantha, St. alpina* and *St. sylvatica* was examined in collaboration with the researchers of the Institute of Pharmacodynamics and Biopharmacy in an enzyme-independent lipid peroxidation system. [128, 129, 131, 132] The methanolic extract of each species was used for the examination. The effect was studied with the use of α-tocoferol and ascorbic acid as control materials. The extracts were prepared with the method described in Chapter 3.7. The quantitative determination of the secondary metabolic products described in Chapter 3.6 was used for the measurement of the antioxidant effect. The results of these measurements are contained in **Table 6.**

#### 5.1.1. Measurement of antioxidant activity

Enzyme-independent lipid peroxidation was tested in vitro on ox brain homogenate according to the method described in Chapter 3.7. The $IC_{50}$ values characteristic of antioxidant effect were determined with the help of the GraphPad Prism 2.01 program. Ascorbic acid and α-tocoferol were used as positive control. [128, 129]

| *Stachys* species | caffeic acid [ %] | (*O*-)-apigenin [ %] | (*O*-)quercetin [ %] | (*C*-)apigenin [ %] | pyrogallol [ %] | gallic acid [ %] | Tannin [ %] | $IC_{50}$ [mg/ml] |
|---|---|---|---|---|---|---|---|---|
| *St. recta* | 4.59 | 0.12 | 0.06 | 0.07 | 3.78 | 3.2 | 2.98 | 0.0736 |
| *St.macrantha* | 1.03 | 0.07 | 0.02 | 0.03 | 1.74 | 1.49 | 1.39 | 0.0930 |
| *St. annua* | 2.05 | 0.16 | 0.07 | 0.20 | 1.46 | 2.35 | 2.19 | 0.0888 |
| *St. officinalis* | 3.80 | 0.14 | 0.03 | 0.10 | 4.07 | 5.97 | 5.56 | 0.0806 |
| *St. sylvatica* | 3.01 | 0.13 | 0.02 | 0.17 | 8.11 | 7.43 | 2.75 | 0.0398 |
| *St. alpina* | 1.31 | 0.02 | 0.02 | 0.14 | 2.30 | 2.39 | 2.23 | 0.2194 |
| $R^2$ | 0.191 | 0.288 | 0.007 | 0.051 | **0.782** | **0.612** | 0.021 | |

**Table 6.** $R^2$ indicates the correlation between the antioxidant activity and the concentration (%) of each compound

### 5.1.2. Results of antioxidant activity

- The following components were determined with UV spectroscopy: hydroxycinnamic acid derivatives according to Arnow's method, including the determination of flavonoid content with Glasl's method, and the determination of the polyphenolic compounds according to Ph.Eu. 4. (**Table 6.**)
- Quantity of hydroxycinnamic acid derivative: 1.31 % - 4.59 %, quantity of flavonoids (*O*-apigenin 0.02 % - 0.16 %, *O*-quercetin 0.02 % - 0.07 % and *C*-apigenin 0.03 % - 0.20 %). Tannin content expressed in pyrogallol: 1.46 % - 8.11 %, polyphenol content expressed in gallic acid (1.49 % - 7.49 %) and in tannin 1.39 % - 5.56 %. **Table 6.** [133,134]
- The relationship between the antioxidant potencies and the tannins (pyrogallol) as well as the total polyphenol content (gallic acid) is depicted in the **figure 8**.
- The value of $R^2$ is the highest for the total polyphenol content expressed in pyrogallol and in gallic acid, on the basis of this it can be stated that tannins are responsible for the antioxidant effect in the *Stachys* species examined.

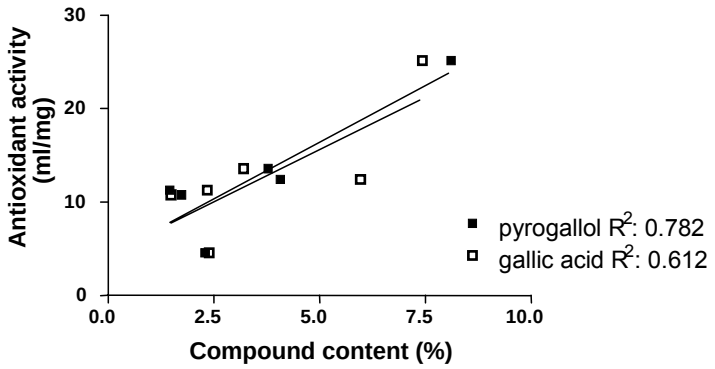



**Figure 8.** Relationship between the antioxidant potencies (defined as $1/IC_{50}$) of the *Stachys* herbs' extracts and their polyphenolic compounds expressed in pyrogallol and in gallic acid content

### 5.2. Cytotoxic activity of *Stachys* species and their iridoid components

The development of any disease, including tumour diseases, depends mainly on the eliciting factors, on the person's sensitivity, on his or her genetic makeup and on the environmental effects. Chemical carcinogens have been shown to be the most important among environmental harms. There are several protecting mechanisms against mutagens and carcinogens. Folk medicine uses medicines of plant origin against tumours already developed. Tea is made from the herb or from the leaves and is used as an antiphlogistic, spasmolytic, diuretic and for the treatment of tumour diseases.

Examinations were performed on the following species, which are native or can be made native to Hungary, and on the iridoid components isolated from them: *St. officinalis*, *St. grandiflora*, *St. byzantina*, *St. germanica*, *St. sylvatica*, *St. annua*, *St. recta*, *St. palustris*, and *St. alpina*, as well as aucubin, harpagide, harpagoside, acetylharpagide, 6-epi acetylharpagide, ajugoside. Our examinations were performed in vitro on A431 skin carcinoma, MCF7 breast adenocarcinoma, HeLa – cervix adenocarcinoma cells. After the preparation of various dilution series, the first step was to examine the species according to organs. On the basis of the data presented in **Table 7.** it was found that an effect over 25 % was shown in a dilution of 10ug/mL by the stem extract of *St. recta* on all three cell lines, by the stem of *St. palustris* on HeLa and MCF7 cell lines, by its leaf and inflorescence extract as well as by the inflorescence extract of *St.germanica* and by the stem extract of *St. byzantina*. **Table 7.** includes only those results which can be evaluated pharmacodynamically.

| | Inhibition of cell growth at 10 μg/ml (%) ± SEM | | |
|---|---|---|---|
| **Extract** | **A431** | **HeLa** | **MCF7** |
| *St. recta* stems | 41.84 ± 1.36 | 37.89 ± 0.71 | 55.40 ± 1.67 |
| *St. palustris* stems | < 25 | 27.38 ± 2.16 | 32.32 ± 6.25 |
| *St. palustris* folium | < 25 | < 25 | 28.19 ± 9.55 |
| *St. palustris* inflorescence | < 25 | < 25 | 48.36 ± 6.95 |
| *St. germanica* inflorescence | < 25 | < 25 | 34.18 ± 1.68 |
| *St. byzantina* stems | < 25 | < 25 | 39.85 ± 2.99 |

**Table 7.** Cytotoxic activity of *Stachys* species

After the evaluation of the plant extracts the isolated iridoid components (aucubin, harpagide, harpagoside, acetylharpagide and its stereoisomer and ajugoside) were examined, again with colleagues from the Institute of Pharmacodynamics and Biopharmacy on the above-mentioned three cell lines.

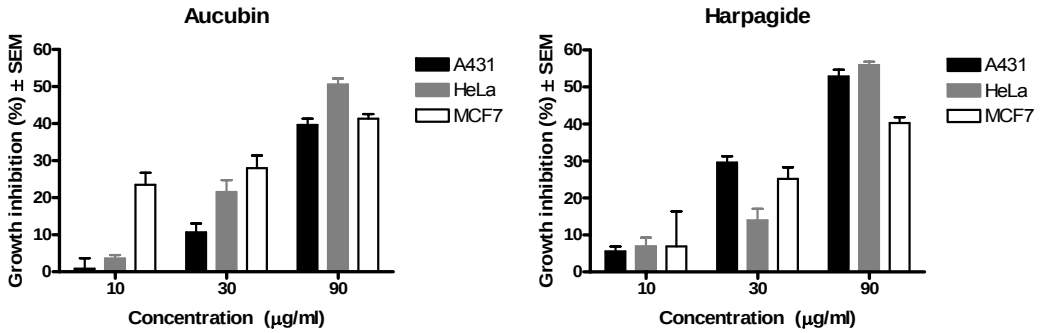



**Figure 9**. Cytotoxic activity of aucubin and harpagide

### 5.2.1. Results of cytotoxic activity

In our work the cytotoxic effect of 10 *Stachys* species (30 plant parts, 30 samples after separation according to organs) and of the iridoids isolated from two species, from *St. palustris* and from *St. recta* ( 6 samples) was examined on three human tumour cell lines with MTT test in vitro. Cisplatin and doxorubicin were used as positive control.

- From among the 33 samples examined, the stem extract of *St. recta* proved to be significantly active (inhibition % >50) in the case of breast carcinoma (on the MCF7 cell

line). 8 samples were found to be moderately active in a concentration of 10μg/mL (25-50 inhibition %). (Only the stem of *St. recta* inhibited cell growth higher than 50%.)

- On the A431 cell line only the stem extract of *St. recta* showed inhibition higher than 25 %.The % value of inhibition was 41.84 in a concentration of 10μg/mL.
- The following extracts exerted higher than 25% inhibition of proliferation: *St. recta* and *St. palustris* extract of stem on HeLa cells.
- The stem, leaf and inflorescence extract of *St. palustris*, the inflorescence extract of St. germanica and the stem extract of *St. byzantina* exerted inhibition over 25 % on the MCF7 cell line.
- The stem extract of *St. recta* proved to be effective on all the three cell lines. The results of the examination are presented in **Table 7.** Those plant extracts are indicated in this table which had inhibition higher than 25 % at least on one cell line.
- In the case of all the other samples examined, inhibition was below 25 %.
- The cytotoxic activity of the isolated components was examined in a concentration of 10-, 30-, 90μg/mL.
- Two components showed cytotoxic activity over 50 % in a concentration of 90μg/mL, aucubin on the HeLa cell line and harpagide on the A431 and HeLa cell lines. A cytotoxic effect over 40 % was exerted by aucubin on the A431 and MCF7 cell lines, and by harpagide on the MCF7 cell line in a concentration of 90μg/mL. These results are shown in **Figures 9.**.
- Our investigation revealed that aucubin and harpagide, beside the other content materials found in *Stachys* species, also contribute to the cytotoxic effect.
- These compounds were chosen a detailed concentration-response study.
- The cytotoxic effects of the Stachys extracts were found limited as the maximal inhibition of cell growths around 50% at 10 μg/mL.

## 6. Chemotaxonomic importance of the isolated compounds

With reference to Chapter 1.1, Bentham classifies the *Stachys* genus into the Stachydeae tribe. This entire tribe constitutes part of the Lamioideae subfamily formed by Erdtman in his two subfamiliar system. However, a third taxonomist, Briquet classifies the genus into an independent subfamily (subfamily Stachyoideae), only partly which agrees with the other two taxonomists' classifications. The chemical examination of the *Stachys* genus, and thus our

own examinations seem not only to confirm the classification of the genus by Erdtman and Bentham, but they are also in good agreement with the most recent classification by Cantino-Harley-Wargraf. The presence of iridoids during our examinations – with the exception of one species, *St. annua* – definitely indicates this. The low volatile oil content also agrees with this statement. If the composition of the volatile oil fraction is considered, it can be stated that irrespective of whether the plants of the Lamiaceae family have high or low volatile oil content, components described for both subfamilies are encountered. This circumstance indicates the unity of the family and also that volatile oil composition (and not the quantity of the volatile oils and their components) is less dependent on taxa.

Nowadays the previous *Stachys* and *Betonica* genera are considered together as *Stachys* genus. The name *Betonica* occurs officially on section level. In spite of this the names *Betonica officinalis* and *Stachys officinalis* are often encountered simultaneously, used for separate taxa both in international literature and index semina of botanical gardens. In order to check this, we considered it important to compare the samples under different names. Neither morphological nor chemical differences were found between *Stachys officinalis* and *Betonica officinalis*. On the basis of this we can state that neither the volatile oil nor the iridoid content or composition justifies the separate consideration of *Betonica* and *Stachys* genera.

## 7. Summary

During the examination of the volatile components of the *Stachys* species, the low volatile oil content necessitated the use of an *n*-hexane auxiliary phase. 160 components were identified with the method we used. From among these, there are 62 monoterpene components and 98 sesquiterpene components or components with a higher number of carbon atoms. Sabinene and germacrene-D is the monoterpene and sesquiterpene component occurring in the highest percentage, respectively. The greatest number of components could be identified in *St. officinalis* sample II and in *St. annua*. All the species we examined are poorer in monoterpene components. As to the *Stachys* species, no literature data were found concerning the volatile oil examination of *St. germanica, St. grandiflora* and *St macranta*, therefore it is probable that we are the first to publish data on the volatile components of these species. In the course of the examination of the *Stachys* species all the volatile components found occurred in other Lamiaceae species as well.

During the examination of iridoids our aim was to determine the main components of the species examined. In the case of two species, *St. palustris* and *St. recta,* the iridoid components of fractionated and aqueous extracts were isolated and determined parallel. Then the main iridoid components of *St. officinalis, St. alpina, St. germanica, St. byzantina, St. grandiflora, St. macrantha, St. sylvatica and St. annua* were detected with NP-TLC and RP-HPLC methods. It was found that in the species examined harpagide, acetylharpagide and aucubin are present as main components, but ajugoside, myoporoside and harpagoside can also be found. This result is in agreement with literature data in the case of harpagide and acetylharpagide, but no iridoids could be detected from *St. annua*, and we were the first to isolate aucubin from *St. palustris* and from *St*. *recta*, and harpagoside from *St. palustris* and *St. recta*. Densitometric measurements were performed for the quantitative data of the isolated components. Harpagide and acetyl-harpagide occurred in the greatest quantity in the species. This result harmonizes with the literature data only with respect to harpagide and acetyl-harpagide, as aucubin was identified in the *Stachys palustris* we examined for the first time.

As to the pharmacological effects of the species examined, antioxidant and cytotoxic effects were investigated. The antioxidant effect was examined in an enzyme-independent lipid peroxidation system. The antioxidant effect of the methanolic extracts of the species was studied with the use of α-tocoferol and ascorbic acid as control materials. Based on the examinations, tannins were found to be responsible for the effect. The study of cytotoxic effect was carried out on three cell lines, on A431-skin carcinoma, MCF7-breast and HeLa-cervix adenocarcinoma cells. From among the species examined, *St. recta* and *St. palustris* showed an effect over 50%, while out of the isolated components aucubin and harpagide had an effect over 40% in a dilution of 90ug/mL on all the three cell lines. In view of the examinations, these effects can be considered moderate.

The result of the examinations can be considered in the case of passible use as medicinal products.

**References**


1. Halmai-Novák: Farmakognózia, 1963, *Medicina könyvkiadó*, Budapest
2. Simon T.: A magyarországi edényes flóra határozója. Harasztok-virágos növények, 1992, *Tankönyvkiadó,* Budapest,
3. Tutin T. G., Heywood V. H., Burges N. A., Moore D. M., Valentine D. H., Walters S. M., Webb D. A.: 1972: *Flora Europaea*, Volume 3, University Press, Cambridge,
4. Cantino, P.D., Sanders, R.W., : *Systematic Botany* 1986, 11, 163 - 185.
5. Cantino, P.D., R.M. Harley, S.J. Wagstaff: Genera of Labiatae: Status and Classification, In R.M. Harley and T. Reynolds (eds.) *Advances in Labiatae Science* pp. 1992, 511 - 555. Royal Botanic Gardens, Kew.
6. Hegnauer, R.: *Chemotaxonomie der Pflanzen*. 1972, Vol. 1 -10. Birkhausen Verlag
7. Maly, Ernest : *Chemicke Zvesti* 1953, 7, 515-521
8. Maly, Ernest: *Journal of Chromatography A* 1985, 333(1), 288-9
9. Maly, Ernest : *Herba Hungarica* 1988, 27(2-3), 107-115
10. Haznagy-Radnai E., Sz. Czigle, I. Mathe, : *Journal of Planar Chromatography-Modern TLC* ( in press)
11. Vundac, V. B., Pfeifhofer, Hartwig W.; Brantner, A. H.; et al.: *Biochem. System. And Ecol*. 2006, 34(12), 875-881
12. Kukic, J., Petrovic, S., Pavlovic, M., et al.: *Flavour and Fragrance Journal* 2006, 21(3), 539-542
13. Radnai E, Dobos A, Veres K et al.: *Acta Horticulturae* 2003: 597 (Proceedings of the International Conference on Medicinal and Aromatic Plants Part II, 2001)-137-142
14. Mirza M; Baher, Z.F.;: *Journal of Essential Oil Research* 2003, 15(1), 46-47
15. Khanavi, M., Hadjiakhoondi, A., Shafiee, A., et al.: *Journal of Essential Oil Research* 2003, 15(2), 77-78
16. Khanavi, M., Hadjiakhoondi, A., Amin, G., et al.: *Zeitschriff fuer Naturforschung, C:Journal of Biosciences* 2004, 59(7/8), 463-467
17. Morteza-Semnani, K., Akbarzadeh, M., Changizi, S.,: *Flavour and Fragrance Journal* 2006, 21(12) 300-303
18. Skaltsa, H. D., Demetzos, C., Lazari, D., et al.: *Phytochemistry* 2003, 64(3), 743-752
19. Grujic-Jovanovic, S., Skaltsa, Helen D; Marin , P., et al: *Flavour and Fragrance Journal* 2004, 19(2), 139-144
20. Aranova,B.N; : *Okhrany Materinstva Detstva* 1964, 2, 77-82

21. Chalchat, Jean-Claude; Petrovic, S.D.; Maksimovic, Z.A. et. al.: *Journal of Essential Oil Research* 2001, 13(4), 286-287
22. Chalchat, Jean-Claude; Petrovic, S.D.; Maksimovic, Z.A. et. al.: *Journal of Essential Oil Research* 2000, 12(4), 455-458
23. Cakir, A; Duru, M.E; Harmandar, M.; et al.: *Flavour and Fragrance Journal* 1997, 12(3), 215-218
24. Tirillini, B., Pellegrino, R., Bini, L., M.,: *Flavour and Fragrance Journal* 2004, 19(4), 330-332
25. Sezik, E.; Basaran, A.; Eczacilik, F. et al.: *Journal of Faculty of Pharmacy of Istambul University* 1985, 21 98-107
26. Harmandar,M.; Duru,M.E.; Cakir, A. et al: *Flavour and Fragrance Journal* 1997, 12(3) 211-213
27. Kaya, A.; Demirci,B.; Baser, K.H.C.: *Chemistry of Natural Compounds* (Translation of Khimiya Prirodnykh Soedinenii) 2002, Volume Date 2001, 37(4), 326-328
28. Flamini,G.; Cioni,P.L.; Morelli, I. et al: *Bichemical Systematics and Ecology* 2004, Volume Date:2005, 33(1) 61-66
29. Duman,H.; Kartal,M.; Altun,L.: *Flavour and Fragrance Journal* 2005, 20(1), 48-50
30. Ozkan,G.; Gokturk, R.S. Unal,O. et al.: *Chemistry of Natural Compounds* 2006, 42(2), 172-174
31. Halim, A. F.; Mashaly, M.M.; Zaghloul, A.M. et al: *International Journal of Pharmacognosy* 1991, 29(39, 183-187
32. Mariotti, J. P., Tomi, B., Francois, C., J., et. Al.: *Rivista Italiana* 1996, 7, 536-540
33. Pelissier, Y., Marion, C., Quastana, C.et al: *Rivista Italiana* 1996, 7, 526-535
34. Mariotti, J.P.;Costa, J.;Bianchini A. et al.: *Flavour and Fragrance Journal* 1997, 12(3) 205-209
35. Skaltsa, H:D.; Lazari,D.M.; Chinou, I.B. et al.: *Planta Medica* 1999, 65(3), 255-256
36. Skaltsa, H.D.; Mavrmmati, A.; Constantinidis, T. : *Phytochemistry*, 2001, 57(2), 235-244
37. Javidna,K.; Miri,R.; Azarpira, A. et al: *Flavour and Fragrance Journal* 2003, 18(4), 299-300
38. Sefidkon, F., Nik, A. N., Bagaii, P. et al: *Flavour and Fragrance Journal* 2003, 18(4), 301-302
39. Feizbakhsh,A., Tehrani, M. S., Rustaiyan, A. et al.: *Journal of Essential Oil Research* 2003, 15(2), 72-73

40. Javidnia, K.; Mojab,F.; Mojahedi, S.A.: *Journal of Essential Oil Bearing Plants* 2003, 6(3), 174-178
41. Sefidkon,F.; Shaabani,A;: *Journal of Essential Oil Research* 2004, 16(3), 229-230
42. Norouzi-Arasi,H.; Yavari,I.; Alibabaeii, M.: *Journal of Essential Oil Research* 2004, 16(3), 231-232
43. Sajjadi, S.E.; Mehragen,I.: *Iranian Journal of Pharmaceutical Research* 2003, 2(1), 57-58
44. Sajjadi, S.E.; Somae,M.: *Chemistry of Natural Compounds* (Translation of Khimiya Prirodnykh Soedinenii 2001) 2004, Volume Date 40(4), 378-380
45. Morteza-Semnani,K.; Akbarzadeh,M.;Moshiri,K.: *Journal of Essential Oil-Bearing Plants* 2005, 8(1), 32-35
46. Sonboli,A.; Salehi,P.; Nejad Ebrahimi,S.: *Chemistry of Natural Compounds* 2005, 41(2) 171-174
47. Norouzi-Arasi,H.; Yavari,I.; Kia-Rostami,V. et al.: *Flavour and Fragrance Journal* 2006, 21(2), 262-264
48. Rezazadeh,S.; hamedani,M.P.; Dowlatabadi,R. et al.: 2006 : *Flavour and Fragrance Journal* 21(2), 290-293
49. Javidnia,K.; Rezai,H.; Miri,R. et al.: *Journal Essential Oil Research* 2006, 18(2) 146-148
50. Rustaiyan,A.; Masoudi,S.; Ameri,N. et al.: *Journal Essential Oil Research* 2006, 18(2) 218-221
51. Javidnia,K.; Miri,R.; Moein,M.R. et al: *Journal Essential Oil Research* 2006, 18(3) 275-277
52. Nik, Z.B.; Mirza,M.: *Flavour and Fragrance Journal* 2006, 21(5), 757-759
53. Petrovic,S.; Ristic,M.; Milenkovic,M. et al.: *Flavour and Fragrance Journal* 2006 , 21(2), 250-252
54. Palic, R.M.; Lazarevic,J.S.; Stojanivic, G. S: et. al.: *Journal Essential Oil Research* 2006, 18(3) 290-292
55. Adema F.: *Acta Botanica Neerlandica*, 1968, 17(5), 423-430
56. Zinchenko, T. V.; *Farmatsevtichnii Zhurnal* (Kiev) 1972, 27(1), 86-7
57. Gritsenko, E.N.; Kostyuchenko, O.I.; Fefer, I.M.; et al.: *Mater. S'ezda Farm. B. SSR*, 1977, 3.
58. Komissarenko, N. F.; Derkach, A. I.; Sheremet, I. P. et al.: *Khimiya Prirodnykh Soedinenii* 1976, 1 109-110.

59. Komissarenko, N. F.; Derkach, A. I.; Sheremet, I. P. et al.: *Khimiya Prirodnykh Soedinenii* 1979, 1 99-10.
60. Pakaln,D.A.; Komissarenko, N. F.;Sheremet, I. P. et al.: *Ikh Nar. Khoz*. 1980, 82-5
61. Lenherr, A; Meier, B.; Sticher, O.: *Planta Medica* 1984, 50(5), 403-9.
62. El-Naggar L. J., Beal J. L.: 1980: *Journal of Natural Products*, 43(6), 649-707
63. Kobzar, A. Ya.; *Khimiya Prirodnykh Soedinenii* 1986, 2, 239
64. Derkach, A. I.; Komissarenko, N. F.; Pakalns, D. et al.: *Rastitel'nye Resursy* 1987, 23(1), 92-5
65. Jeker, M., Sticher, O., Calis, I. et al.: *Helvetica Chimica Acta* 1989, 72(8), 1787-91
66. Calis I., Basaran A. A., Saracoglu I., Sticher O.: *Phytochemistry*, 1991, 31(1), 167-169
67. Boros C. A., Stermitz F. R.: *Journal of Natural Products*, 1990, 53(5), 1055-1147
68. Boros C. A., Stermitz F. R.: *Journal of Natural Products*, 1991, 54(5), 1173-1246
69. Isamukhamedova, M. P.; Pulatova, T. P.: *Khimiya Prirodnykh Soedinenii* 1992, 5, 586-587
70. Kartev, V.G.; Stepanichenko, N. N.; Auelbekov, S. A.: *Khimiya Prirodnykh Soedinenii* 1994, 6, 699-709
71. Miyase, T., Ueno, A., Kitani, T., et al.: *Yakugaku Zasshi* 1990, 110(9), 652-7
72. Munoz, O., Pena, R., C.: *Journal of Biosciences* 2001, 56(9/10)
73. Kostos, M.; Aligiannis, N.; Mitaku, S. et al.;: *Natural Product Letters* 2001, 15(6), 377-388
74. Meremeti, A.; Karioti, A.; Skaltsa, H. et al.;: *Biochemical Systematics and Ecology* 2004, 32(2) 139-151
75. Serelli, A. M. ; Ramunno, A. ,Piccioni, F. et al.: *Natural Product Research* 2005, 19(6), 561-565
76. Haznagy-Radnai, E.; Léber, P.; Tóth, E. et al.: *Journal of Planar Chromatography-Modern TLC* 2005, 18(104), 314-18
77. Serelli, A. M. ; Ramunno, A. ,Piccioni, F. et al.: *Structure and Synthesis* 2006, 20(6), 648-652
78. Haznagy-Radnai, E., Czigle, Sz., Janicsák, G. et al.: *Journal of Planar Chromatography-Modern TLC* 2006, 19(109), 187-190
79. Popa, D.P.; Orgiyan, T.M.: *Khimiya Prirodnykh Soedinenii* 1974, 3, 406
80. Tkachev,V.V., Nikonov,G.K., Atovmyan,L.O.et. al.: *Khimiya Prirodnykh Soedinenii* 1987, 6, 811-17

81. Miyase,T.; Yamamoto,R.; Ueno,A.: *Chemical & Pharmaceutical Bulletin* 1996, 44(8), 1610-1613
82. Popa, D.P., Pasechnik,G.S.: *Khimiya Prirodnykh Soedinenii* 1974, 4, 447-51
83. Adinolfi, M., Barone,G., Lanzetta, R. et al.: *Journal of Natural Products* 1984, 47(3), 541-543
84. Derkach, I.: *Rastitel'nye Resursy* 1998, 34(1), 57-61
85. Piozzi, F.; Paternostro, M.P.; Servattaz, O. et al.: *Biochemical Systematics and Ecology* 2002, 30(9), 887-889
86. Maleki N., Garjani A., Nazemiyeh H., et al.: *Journal of Ethnopharmacology*, 2001, 75, 213-218.
87. Fazio C., Passannanti S., Paternostro M. P.,et al.: *Planta Medica*, 1994, 60, 499
88. Ross S. A., Zinchenko T. V.: 1975: *Farmatsevtichnii Zhurnal* (Kiev), 30(3), 91-92.
89. Yamamoto R., Miyase T., Ueno A.: *Chemical & Pharmaceutical Bulletin*, 1994, 42(6), 1291-1296.
90. Janicsak, G., Veres, K., Kakasy, Z. et. al.: *Biochemical Systematics and Ecology* 2006, 34(5), 392-396
91. Pulatova, T.P.: *Khimiya Prirodnykh Soedinenii* 1969, 5(1), 62-63
92. Klein, G., Linser, H. : *Physiol. Chem*. 1932, 209, 75-96
93. Bankova,V.;Koeva-Todorovska,J; Stambolijska,T. et al: *Zeitschrift fuer Naturforschung, C: Journal of Biosciences* 1999, 54(11), 876-880
94. Miyase, T., Yamamoto, R., Ueno, A.: *Phytochemistry* 1996, 43(2) 475-479
95. Takeda,Y., Zhang,H., Masuda,T. et al: *Phytochemistry*, 1997, 44(7), 1335-1337
96. Ross, S. A., Zinchenko,T.V.: *Farmatsevtichni Zhurnal* 1975, 30(2), 91-92
97. Kobzar, A.Ya.,: *Khimiya Prirodnykh Soedinenii* 1986, 2, 239-240
98. Buchwald,W., Czaska, A.: *Herba Polonica* 1995, 41(4), 198-202
99. Vundac, V.B., Males, Z., Plazibat, M. et.al: *Journal of Planar Chromatography – Modern TLC* 2005, 18(104), 269-27
100. Barberan F. A. T.: *Fitoterapia*, 1986, 57(2), 67-95
101. Sheremet, I.P., Komissarenko, N.F.: *Khimiya Prirodnykh Soedinenii*, 1971, 5, 583-587
102. Sheremet, I.P., Komissarenko, N.F.: *Khimiya Prirodnykh Soedinenii* 1971, 7(3), 373-374
103. Sheremet, I.P., Komissarenko,N.F.: *Khimiya Prirodnykh Soedinenii* 1971, 7(6), 721-723

104.Bishara S. A. R., Zinchenko T. V., Nikonov G. K., et al.: *Ukrainskii Khimicheskii Zhurnal* (Russian Edition), 1976, 42(3), 284-288.

105.Ross S. A., Zinchenko T. V., Borzunov E. E.: *Ukrainskii Khimicheskii Zhurnal* (Russian Edition), 1975, 41(10), 1108-1111.

106.Ross S. A., Zinchenko T. V.: *Farmatsevtichnii Zhurnal* (Kiev), 1975, 30(5), 69-74.

107.Lennher,A.; Lahloub,M.F.; Sticher,O.: *Phytochemistry* 1984, 23(10), 2343-2345

108.Lennher,A.; Mabry,T.J.; Gretz,M.R.: *Journal of Chromatography* 1987, 388(2), 455-458

109.Tomas-Barberan, F.A.; Gil, M.I.; Ferreres, F. et. al.: *Phytochemistry*, 1992, 31(9), 3097-3102

110.Kobzar, A.Ya., Nikonov, G.K.: *Flavonoids Khimiya Prirodnykh Soedinenii* 1986, 5, 636-637

111.Pulatova, T.P.;: *Meditsinskii Zhurnal Uzbekistana* 1972, 11, 16-17

112.Varró, A. B.: Gyógynövények gyógyhatásai, 1991, *Marksped Kft*, Kaposvár

113.Grieve M.: http://botanical.com/botanical/mgmh/w/woumar39.html 2003.04.23

114.Telyat'eva R. V., Fruentov N. K.: *Voprosy Farmatsii na Dal'nem Vostoke*, 1973, 1, 136-138.

115.Danert, S., Hanelt, J.; Helm, J. és mtsi: Uránia növényvilág, Magasabb rendű növények II. ; 1981:*Gondolat kiadó*, Budapest 228, 230-231, 233

116.Rápóti, J.; Romváry ,V.: Gyógyító növények , 1997: *Medicina könyvkiadó* Rt. Budapest, 11., 83., 269, 281

117.Bruneton J.: *Pharmacognosy-Phytochemistry-Medicinal Plants*, 2nd edition, 1999: TEC&DOC Lavoisier, London, Paris, New York,

118.Wagner H., Wolff P.: *New Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutical Activity*, 1977, Springer-Verlag, Berlin, Heidelberg, New York

119Davini E., Iavarone C., Trogolo C., et al.: *Phytochemistry*, 1986, 25(10), 2420-2422.

120.Bermejo Benito P., Díaz Lanza A. M., Silván Sen A. M.,et. Al.: *Planta Medica*, 2000, 66, 324-328.

121.Skaltsa, H. D.; Bermejo, P.; Lazari D. et al: *Biological & Pharmaceutical Bulletin* 2000, 23(1), 47-53,

122.A. Háznagy, : *Gyógyszerészet* 1968, 12(12) 463-466

123.Arnow L.E. J.: *Biol Chem.* 1937, 118:531,

124.Lamaison J.L.; Petitjean-Freytet C.; Carnat A.; : *Pharm Acta Helv* 1991, 66; 185,

125.Petitjean-Freytet C.; Carnat A.; :Lamaison J.L.; *J. Pharm. Belg*. 1991, 46; 241,
126.Glasl H.; Fresenius, Z.;: *Anal. Chem* 1985, 321,325
127.*European Pharmacopoeia. 4th*. Ed. 2001: Strasbourg; Council of Europe,;p.:187,
128.Stocks, J.; Gutteridge J.M.C.; Sharp R.J. et al.: *Clin. Sci. Mol. Med*. 1974, 47, 215,
129.Zupko, I., Hohmann, J.; Rédei, D. et. al.: *Planta Med*. 2001, 67, 366
130.Mosmann T.: *J. Immunol. Methods* 1983;65: 55-63
131.Cuppett, S.L., Hall, C.A.;: *Adv. Food Nutr. Res*. 1998, 42, 245,
132.Rice-Evans C.A.; Miller N.J.; Bolwell, P.G. et. al.: *Free Radic. Res*. 1995, 22, 375,
133.Takako Y., Erbo, D.; Zhong, W.L. et.al.: *Phytoter. Res*. 1997, 11, 446
134.Dugas, A.J.; Casteneda-Acosta, J.; Boni, G.C. et al.: *J. Nat. Prod.* 2000, 63,327.
135.Duke, A.J. Handbook of Medicinal Herbs. CRC Press Boca Raton 2002, London, New York, Washigton
136.Heinrich, M.; Barnes, J., Gibbons. S.; Fundamentals of Pharmacognosy and Phytotherapy 2004, Edinburg, London, new York, Oxford, Philadelphia, St. Louis, Sydney, Toronto

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# Appendix II.

## Appendix II.

## Table 1.

**Table 1. Volatile Fraction A (KI < 1330) of various *Stachys* species /samples obtained by GC**

| KI | Sections: / Species/Samples: | Betonica | | | Eriostomum | | | | Stachys | | | | Olisia | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Sachys officinalis* I. | *Stachys officinalis* II. | *Betonica serotina* | *Stachys alpina* | *Stachys germanica* | *Stachys byzantina* | *Stachys grandiflora* | *Stachys macrantha* | *Stachys palustris* | *Stachys recta* | *Stachys sylvatica* | *Stachys annua* I. | *Stachys annua* II. |
| 792 | 1-Octen | - | tr | 0.7 | 2.3 | - | - | tr | 1.6 | 2.7 | - | - | 0.6 | - |
| 800 | Hexanal | - | - | - | - | - | - | - | - | - | - | - | - | 1.1 |
| 818 | β-*Trans*-octene | - | 2.0 | - | - | - | - | - | - | - | 8.5 | - | - | - |
| 854 | (*E*)-2-Hexanal | - | - | - | - | - | - | - | 7.9 | 2.7 | - | - | - | - |
| 909 | 2,4-(*E,E*)- Hexadienal - | - | - | - | - | - | - | - | - | - | 4.0 | - | 0.2 | 4.7 |
| 931 | $\alpha$-Thujene | tr | 0.1 | 0.9 | 0.5 | - | - | - | - | - | 0.1 | - | 0.5 | 0.4 |
| 939 | $\alpha$-Pinene | - | 3.2 | - | - | - | - | tr | - | - | 0.1 | - | - | - |
| 953 | Camphene | 0.3 | 0.1 | - | - | - | - | tr | - | - | - | - | - | - |
| 961 | Benzaldehyde | - | 0.6 | 0.1 | tr | - | - | - | 0.1 | 0.8 | 0.2 | - | 1.6 | 1.5 |
| 967 | Verbenene | tr | - | - | - | - | - | tr | - | - | - | - | - | - |
| 976 | Sabinene | 4.7 | - | 0.2 | 2.6 | 1.5 | 12.0 | 1.1 | - | - | - | 4.3 | - | 3.0 |
| 978 | 1-Octen- 3-ol | - | 3.8 | 2.4 | - | - | - | - | 4.9 | 3.2 | 5.6 | - | 3.3 | - |
| 980 | $\beta$-Pinene | tr | - | - | - | 0.1 | 0.1 | 0.6 | - | - | - | - | 0.1 | 0.2 |
| 983 | *cis* -Pinene | - | 0.1 | - | - | - | - | - | 0.6 | - | - | - | 0.2 | - |
| 986 | 3-Octanone | - | - | - | - | - | - | - | - | - | 0.1 | - | - | - |
| 986 | Menthene 3- para- | - | - | - | - | - | - | - | - | - | - | - | - | 0.1 |
| 991 | 1,8-Cineole-dehydro | - | - | - | - | - | - | - | - | - | 0.2 | - | - | - |
| 993 | 3-Octanol | - | - | 0.1 | - | - | - | - | - | - | - | - | - | 0.1 |
| 998 | Furfurol-methyl sulphide | - | 0.1 | - | - | - | - | - | 0.6 | - | - | - | - | - |
| 1018 | $\alpha$-Terpinene | - | - | - | - | - | - | tr | - | - | - | - | - | - |
| 1022 | *o*-Cymene | tr | - | - | - | tr | tr | 0.2 | - | - | - | tr | - | - |
| 1031 | Limonene | - | 0.1 | - | 0.5 | - | - | - | - | - | 1.1 | - | 0.1 | 0.1 |
| 1031 | $\beta$-Phellandrene | tr | - | 0.1 | 2.9 | 4.8 | tr | 0.3 | 0.2 | 2.6 | 1.1 | tr | - | - |
| 1033 | 1, 8-Cineole | - | 0.1 | - | - | - | - | - | - | - | - | -- | - | - |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1040 | *cis*-Ocimene | tr | 0.1 | 0.1 | 0.5 | tr | tr | 0.2 | - | 0.8 | - | tr | 0.6 | 0.9 |
| 1043 | Benzene-acetaldehyde | - | 0.1 | 0.1 | - | - | - | - | 0.8 | - | 0.5 | tr | 0.1 | 0.2 |
| 1050 | *trans*-Ocimene | tr | - | - | - | tr | tr | tr | - | - | - | - | - | 0.1 |
| 1062 | $\gamma$-Terpinene | tr | tr | - | - | tr | tr | tr | - | - | - | tr | - | - |
| 1065 | Acetofenone | - | - | - | - | - | - | - | - | - | - | - | - | 0.1 |
| 1087 | Fenchone | - | - | - | - | - | - | - | - | - | - | - | - | 0.1 |
| 1088 | Terpinolene | - | 0.1 | - | - | - | - | - | - | - | - | - | - | - |
| 1091 | Methylbenzoate | - | tr | - | - | - | - | - | - | - | - | - | - | 0.1 |
| 1098 | Linalool | 0.4 | 0.4 | 0.3 | 0.2 | tr | tr | 0.1 | 0.2 | 0.3 | 3.2 | 0.2 | 0.7 | 0.7 |
| 1102 | $\alpha$-Thujone | - | 0.1 | 0.1 | - | 0.1 | tr | - | 0.3 | 0.4 | 0.1 | - | 0.4 | 0.2 |
| 1114 | $\beta$-Thujone | - | 0.2 | tr | tr | - | - | - | 0.3 | tr | 0.3 | - | 0.2 | 0.1 |
| 1129 | *allo*-Ocimene | - | - | - | tr | - | - | - | - | - | - | - | - | 0.1 |
| 1140 | *trans*- Sabinol | - | - | - | - | - | - | - | - | - | - | - | - | 0.6 |
| 1143 | Camphor | - | 0.1 | tr | tr | - | - | - | 0.2 | 0.3 | tr | - | 0.1 | 0.4 |
| 1144 | *cis*-$\beta$ Terpineol | - | - | - | - | - | - | - | - | - | 0.2 | - | - | - |
| 1160 | *trans*-Pinocamphone | 0.3 | - | - | - | tr | 4.8 | 0.6 | - | - | 0.2 | tr | 0.2 | 0.1 |
| 1162 | Pinocarvone | - | tr | - | tr | - | - | - | - | - | - | - | - | - |
| 1163 | Benzyl acetate | - | - | - | - | - | - | - | - | - | - | - | 0.2 | - |
| 1165 | Borneol | - | - | - | - | - | - | - | - | - | 0.1 | tr | - | - |
| 1173 | *cis*-Pinocamphone | 0.6 | 0.1 | - | tr | tr | 3.8 | 1.1 | - | - | 0.1 | tr | 0.2 | - |
| 1189 | $\alpha$–Terpineol | - | tr | - | - | - | - | - | 0.2 | - | 0.3 | - | - | - |
| 1190 | Methyl salicylate | - | 0.4 | 0.1 | - | tr | - | - | - | tr | 0.3 | - | 0.1 | 0.3 |
| 1192 | Dihydro-carveol | tr | - | - | - | - | - | - | - | - | - | tr | - | - |
| 1192 | Dodecene 1 | - | tr | - | - | - | - | - | - | - | - | - | - | - |
| 1193 | Myrtenal | - | - | - | tr | - | - | - | - | - | - | - | - | 0.1 |
| 1194 | Myrtenol | - | - | - | - | - | 0.1 | tr | - | - | - | - | - | - |
| 1200 | *trans*-Dihydrocarvone | - | - | - | - | - | tr | - | - | - | - | - | 0.1 | - |
| 1204 | Verbenone | - | - | - | tr | - | - | - | 0.4 | - | tr | - | 0.1 | 0.1 |
| 1217 | *trans*- Carveol | - | - | - | - | - | - | - | - | 2.6 | - | - | - | - |
| 1220 | Fenchyl acetate (endo) | - | - | - | - | - | - | - | - | - | - | - | - | 0.1 |
| 1237 | Pulegone | - | - | - | - | - | - | - | - | - | - | - | tr | 0.1 |
| 1252 | *cis* -Myrtanol | - | - | - | - | - | - | - | - | - | 0.3 | - | - | - |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1275 | Linalool acetate dihydro | - | - | - | - | - | - | - | - | - | 0.2 | - | - | - |
| 1282 | $\alpha$–Terpine-7-al | - | - | - | - | - | - | - | - | - | 0.3 | - | - | 0.3 |
| 1283 | Anethol trans | - | - | - | - | - | - | - | - | - | - | - | - | 0.1 |
| 1298 | Carvacrol | - | - | - | - | - | - | - | - | - | - | tr | 0.1 | - |
| 1314 | (*E*,*E*)- 2,4-Decadienal | - | - | - | - | - | - | - | - | - | 0.2 | - | 0.2 | 0.1 |
| 1325 | Limonene aldehyde | - | tr | - | - | - | - | - | - | - | - | - | - | - |
| | Total: | 6.3 | 11.8 | 5.2 | 9.5 | 6.5 | 20.8 | 4.2 | 18.3 | 16.4 | 27.3 | 4.5 | 12.2 | 16.3 |

## Appendix II.

## Table 2.

**Table 2. Volatile Fraction A. (KI > 1330), obtained by GC of various *Stachys* species /samples**

| KI | Section: | Betonica | | | Eriostomum | | | | Stachys | | | | Olisia | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Species/ sample: | *Stachys officinalis* I. | *Stachys officinalis* II. | *Betonica serotina* | *Stachys alpina* | *Stachys germanica* | *Stachys byzantina* | *Stachys granflora* | *Stachys macrantha* | *Stachys palustris* | *Stachys recta* | *Stachys sylvatica* | *Stachys annua*/ Szeged | *Stachys annua*/ Vacratot |
| 1339 | $\delta$–Elemene | | | | | | | | 1.6 | | | | tr | 0.2 |
| 1351 | $\alpha$-Cubebene | tr | 0.4 | 0.1 | - | - | tr | - | - | - | - | tr | - | - |
| 1355 | Thymol acetate | - | - | - | - | - | - | - | - | - | - | - | 0.7 | - |
| 1356 | Eugenol | tr | 0.7 | 0.1 | tr | tr | tr | - | 7.9 | - | 0.1 | tr | - | 0.2 |
| 1372 | $\alpha$-Ylangene | tr | 0.4 | tr | - | | - | - | - | - | tr | tr | - | - |
| 1376 | $\alpha$-Copaene | 2.0 | 0.9 | 0.3 | 0.1 | 0.1 | tr | - | - | 1.2 | 0.1 | tr | - | tr |
| 1384 | $\beta$-Bourbonene | 2.8 | 5.6 | 2.1 | 0.3 | 0.2 | - | - | - | tr | - | 0.55 | - | 1.2 |
| 1387 | Longifolene iso- | - | - | - | - | - | - | - | - | - | - | - | 0.4 | - |
| 1388 | *cis*-Jasmone | - | - | - | - | - | - | - | 0.1 | - | - | - | - | 0.1 |
| 1390 | $\beta$-Cubebene | - | - | - | - | - | - | - | - | 0.8 | 0.2 | - | - | 0.3 |
| 1391 | $\beta$-Elemene | 10.0 | - | - | 2.0 | 0.6 | 1.3 | - | - | - | - | 0.9 | - | - |
| 1398 | Cyperene | - | tr | - | - | - | - | - | 4.9 | - | - | - | - | - |
| 1401 | Italicene | - | - | 0.6 | - | - | - | - | - | - | 0.2 | - | - | 0.1 |
| 1402 | Longifolene | - | 1.1 | 0.1 | - | - | - | - | 0.6 | - | - | - | - | - |
| 1404 | $\gamma$-Caryophyllene | - | tr | - | - | - | - | - | - | - | - | - | - | - |
| 1418 | $\beta$–Cedrene | - | - | - | - | - | - | - | - | - | - | - | - | 0.1 |
| 1418 | $\beta$-Caryophyllene | 0.9 | 13.0 | 16.5 | 4.4 | 0.2 | 0.2 | 5.1 | - | 8.7 | 0.8 | 1.3 | 0.1 | - |
| 1432 | $\beta$-Gurjunene | tr | 1.4 | 0.5 | - | 0.3 | 0.2 | - | - | - | - | 1.1 | - | 0.2 |
| 1436 | *trans*-$\alpha$-Bergamotene | - | - | 0.3 | - | - | - | - | 0.6 | - | - | - | - | - |
| 1439 | $\alpha$-Guainene | tr | 0.5 | - | - | - | - | - | - | - | - | - | - | - |
| 1439 | Aromadendrene | - | - | 3.3 | 0.5 | - | 0.1 | 10.6 | - | - | - | 0.4 | - | tr |
| 1454 | $\alpha$-Humulene | 0.2 | 3.4 | 3.1 | 0.6 | - | - | - | - | 0.4 | 0.2 | - | - | - |
| 1458 | *trans*-$\beta$-Farensene | - | - | - | - | 2.0 | - | - | 0.2 | - | - | - | - | - |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1459 | Aromadendrene dehydro- | - | - | - | - | - | - | - | - | - | - | - | - | 0.1 |
| 1461 | *allo* - Aromadendrene | - | 0.4 | - | - | - | - | - | - | - | - | - | - | - |
| 1473 | γ-Gurjunene | 0.3 | 0.3 | - | - | - | - | - | 0.8 | - | - | - | - | - |
| 1477 | γ-Muurolene | - | 3.6 | 0.2 | 23.5 | - | 0.2 | - | - | - | - | 1.1 | 0.1 | - |
| 1480 | γ-Curcumene | - | 8.2 | - | - | - | - | 16.9 | - | - | - | tr | - | - |
| 1480 | Germacrene D | 3.7 | - | 29.1 | tr | 7.3 | 21.0 | - | - | 9.9 | 0.7 | 33.1 | 3.1 | - |
| 1485 | β-Ionone | - | - | - | - | - | - | - | - | tr | 0.6 | - | 0.2 | 0.1 |
| 1485 | β-Selinene | - | - | - | 4.0 | - | - | - | - | - | - | - | - | - |
| 1491 | Valencene | - | 1.6 | 1.6 | - | - | - | - | - | 1.6 | - | - | - | - |
| 1493 | Viridiflorene | - | - | 0.3 | - | - | - | tr | 0.2 | 1.0 | 0.1 | - | - | 2.9 |
| 1493 | *epi*-Cubebol | - | 3.1 | - | - | - | 0.3 | 11.2 | 0.3 | - | - | 1.3 | - | 3.9 |
| 1494 | Bicyclo germacrene | - | - | - | - | - | - | - | 0.3 | - | - | - | 2.2 | - |
| 1495 | α-Selinene | tr | - | 0.9 | 1.6 | - | - | 0.1 | - | - | - | 1.0 | - | - |
| 1491 | Valencene | 46.1 | - | - | - | 0.1 | - | 7.9 | - | - | - | - | - | - |
| 1493 | Viridiflorene | - | 1.1 | - | 1.7 | - | - | - | 0.2 | - | - | - | - | - |
| 1499 | α-Muurolene | - | - | - | - | - | 2.0 | - | - | - | - | 2.4 | - | - |
| 1500 | *trans*-β–Guaiene | - | - | 0.4 | - | - | - | - | - | - | - | - | - | - |
| 1503 | Germacrene A | - | 0.4 | - | - | - | - | - | - | - | - | - | - | - |
| 1508 | α–Farnesene | - | - | - | - | - | - | - | - | - | - | - | 0.5 | 0.6 |
| 1513 | γ-Cadinene | 1.4 | 2.6 | 0.6 | tr | tr | - | - | - | - | - | tr | - | - |
| 1514 | Cubebol | - | - | 0.3 | - | - | - | - | - | - | - | - | - | - |
| 1524 | δ-Cadinene | 1.7 | 4.9 | 1.4 | - | 0.1 | 0.4 | 1.3 | 0.2 | 1.0 | 0.3 | 2.4 | 1.0 | 0.5 |
| 1532 | Cadina-1,4-diene | - | 0.2 | - | - | - | - | - | - | - | - | - | - | - |
| 1538 | α–Cadinene | - | 0.3 | 1.0 | - | - | - | - | - | - | - | - | - | - |
| 1542 | α-Calacorene | tr | 0.4 | - | - | - | - | - | - | - | - | tr | - | - |
| 1556 | Germacrene B | - | - | 0.2 | - | - | - | - | - | - | - | tr | - | - |
| 1563 | β-Calacorene | tr | 0.2 | - | - | - | - | - | - | - | - | tr | - | - |
| 1570 | (*Z*)- 3-Hexenyl benzoate | - | - | - | - | - | - | - | - | - | 0.2 | - | - | - |
| 1574 | Germacrene D - 4 | - | - | - | tr | - | - | - | 0.4 | - | - | - | - | - |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | - ol | | | | | | | | | | | | | |
| 1576 | Spathulenol | 0.5 | 0.3 | 6.3 | - | 0.5 | 0.2 | - | - | - | 0.2 | 1.4 | 0.8 | 1.9 |
| 1581 | Caryophyllene-oxide | 0.5 | 1.8 | - | 1.1 | 0.6 | - | - | - | 0.7 | - | 1.5 | - | - |
| 1584 | $\beta$-Copaen-4-$\alpha$-ol | tr | 0.6 | - | - | - | - | - | - | - | - | 5.8 | - | - |
| 1590 | Viridiflorol | tr | - | - | - | - | - | - | - | - | - | 1.4 | - | 0.1 |
| 1606 | $\beta$–Oplopenone | - | 0.2 | 0.4 | - | - | - | - | - | - | - | - | - | - |
| 1606 | Humulene-epoxide | tr | 0.7 | - | - | - | - | 0.7 | - | - | | 0.2 | - | - |
| 1618 | Cedranone (5-) | - | - | - | - | - | - | - | - | - | - | - | - | 0.1 |
| 1627 | Epi-cubenol | - | 0.7 | 0.3 | - | - | - | - | - | - | - | - | - | - |
| 1630 | $\alpha$-Acorenol | - | 0.6 | 0.4 | -- | - | - | - | - | - | - | tr | - | - |
| 1630 | Eudesmol $\gamma$ | - | 0.3 | 0.4 | - | - | - | - | - | - | - | - | - | - |
| 1640 | Cadinol $\tau$ | - | 1.3 | 1.0 | tr | - | - | - | 18.3 | tr | - | - | - | - |
| 1642 | Cubenol | - | 0.6 | - | - | - | - | - | | tr | - | tr | - | - |
| 1645 | $\alpha$-Muurolol | 1.5 | 0.7 | 0.4 | - | tr | tr | - | | - | 0.2 | 0.2 | - | 0.3 |
| 1653 | $\alpha$-Cadinol | 1.5 | 2.0 | 1.2 | 0.3 | tr | 0.7 | 0.4 | | 0.4 | - | 4.0 | 0.6 | 0.7 |
| 1671 | $\beta$ - Bisabolol | - | - | 1.1 | - | - | - | - | | - | - | - | 0.3 | - |
| 1672 | Valerianone | - | - | - | 0.7 | - | 1.5 | - | | - | - | - | - | - |
| 1683 | $\alpha$-Bisabolol | - | - | 1.1 | - | - | 0.2 | - | | - | - | - | - | - |
| 1686 | epi-$\alpha$–Bisabolol | - | - | - | 1.2 | - | - | - | | - | - | - | - | 1.2 |
| 1688 | Cedren 13-ol 8- | - | 1.2 | - | - | - | - | - | | - | - | - | - | - |
| 1700 | Heptadecane | - | - | - | - | - | - | - | | - | 0.1 | - | - | - |
| 1700 | Caryophyllene acetate | - | - | - | - | - | - | - | | - | - | - | 0.8 | - |
| 1704 | Cedroxide | - | - | - | 0.4 | - | - | - | | - | - | - | - | - |
| 1756 | Aristolene | - | - | - | - | - | - | - | | 1.1 | - | - | - | - |
| 1757 | Acoradienol | - | - | - | tr | - | - | - | | - | - | - | - | - |
| 1762 | Benzyl benzoate | - | - | - | - | - | - | - | | - | 0.7 | - | 0.9 | 1.0 |
| 1778 | $\gamma$–Eudesmol acetate | - | - | - | - | - | - | - | | - | tr | - | - | - |
| 1799 | δ-cadinene 14-OH | - | 0.7 | - | - | - | - | - | | - | - | - | - | - |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1800 | Octadecane | - | - | - | - | - | - | - | - | - | - | 0.6 | - | |
| 1860 | lanceol acetate | - | - | - | - | tr | - | - | - | - | - | - | - | |
| 1872 | Cubetene | - | - | - | 0.4 | - | - | - | - | - | - | - | - | |
| 1891 | epi-Laurenene | - | - | - | 2.6 | 0.2 | - | - | - | - | - | - | - | |
| 1900 | Nonadecane | - | - | - | - | - | - | - | - | - | - | 2.2 | - | |
| 1924 | Beyerene | - | - | 0.1 | - | - | - | - | - | - | - | - | - | |
| 1927 | Methyl-hexadecanoate | - | 0.6 | 3.0 | 2.7 | - | 0.7 | - | 12.7 | 2.4 | - | 15.4 | 1.7 | |
| 1961 | 13-*epi*-Manool | - | - | - | - | - | - | - | - | - | - | - | - | |
| 1989 | Manoyloxide | - | - | - | - | - | - | - | - | 0.2 | tr | 0.5 | - | |
| 1993 | Ethyl-hexadecanoate | 3.1 | - | - | - | 32.7 | 17.2 | 14.2 | - | - | 4.4 | - | 19.9 | |
| 2000 | Eicosan | - | 2.4 | - | - | - | - | - | - | - | - | tr | - | |
| 2010 | 13-*epi*-Manoyloxide | - | tr | - | - | - | - | - | - | - | tr | 0.1 | 0.1 | |
| 2034 | Kaurene | - | 1.2 | - | - | - | - | - | - | 0.2 | tr | - | 0.1 | |
| 2054 | Abietatriene | - | - | - | - | - | - | - | - | - | - | 1.3 | - | |
| 2056 | Manool | - | - | - | - | - | - | - | - | - | tr | - | 1.9 | |
| 2080 | Abietadiene | - | - | - | 0.4 | - | - | - | - | - | - | - | - | |
| 2082 | Octadecanol | - | - | - | - | - | - | - | - | - | - | - | - | |
| 2092 | Methyl-linoleate | - | 6.2 | - | 5.5 | - | - | - | 1.4 | 3.1 | - | 2.3 | 2.0 | |
| 2107 - | Laurenan-3-one | - | - | 3.1 | - | - | - | - | 14.4 | 12.0 | - | 19.5 | 9.5 | |
| 2126 | Nezukol | - | - | - | 6.2 | - | - | - | - | 3.7 | - | 1.1 | 1.2 | |
| 2200 | Docosane | - | - | - | - | - | - | - | - | 4.9 | - | - | - | |
| 2300 | TricosaneN | - | 0.2 | - | - | - | - | - | 0.4 | 0.5 | - | 1.3 | 1.4 | |
| 2302 | Abietal | - | - | - | - | - | - | - | - | - | tr | - | - | |
| 2400 | Tetracosane | - | - | - | - | - | - | - | - | 0.1 | - | 0.2 | - | |
| 2500 | Pentacosane | - | 0.4 | - | - | - | - | - | 1.8 | 0.4 | - | 1.2 | - | |
| | Total: | 76.2 | 77.4 | 81.8 | 60.2 | 44.9 | 46.1 | 68.4 | 57.5 | 32.2 | 64.4 | 57.4 | 53.4 | |

## Appendix II.

## Table 3.

**Table 3**. Number of separated compounds and their proportions in the total distillates

| Species | Number of components | Fraction A. (Monoterpenes) | Fraction B. (Sesquiterpenes) | Total proportion of the distillets (in %) |
|---|---|---|---|---|
| *St. officinalis* (sample I) | 40 | 14 | 26 | 82.5 |
| *St. officinalis* (sample II) | 73 | 26 | 47 | 89.2 |
| *Betonica serotina* | 50 | 14 | 36 | 87.0 |
| *St. alpina* | 42 | 15 | 27 | 69.7 |
| *St. germanica* | 30 | 12 | 18 | 51.4 |
| *St. macrantha* | 28 | 14 | 14 | 63.1 |
| *St. byzantina* | 32 | 13 | 19 | 66.9 |
| *St. grandiflora* | 27 | 16 | 11 | 72.6 |
| *St. palustris* | 32 | 12 | 20 | 73.9 |
| *St. recta* | 53 | 26 | 27 | 59.5 |
| *St. sylvatica* | 47 | 12 | 35 | 68.9 |
| *St. annua* (Szeged) | 51 | 24 | 27 | 67.3 |
| *St. annua* (Vácrátót) | 62 | 31 | 31 | 69.7 |

## Appendix II.

## Table 4.

**Table 4. Ratios of various types of compound present in *Stachys* and *Betonica* species**

| Section | Betonica | | | Eriostomum | | | Stachys | | | | Olisia | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species/samples/ Compounds | *Stachys officinalis* I | *Stachys officinalis* II | *Betonica serotina* | *Stachys alpina* | *Stachys germanica* | *Stachys byzantina* | *Stachys grandiflora* | *Stachys macrantha* | *Stachys palustris* | *Stachys recta* | *Stachys sylvatica* | *Stachys annua/ I.* | *Stachys annua/ II.* |
| *Aliphatics* | | | | | | | | | | | | | |
| Alkanes ,alkenes | - | 5.0 | 0.7 | 2.3 | - | - | tr | 1.6 | 4.9 | 14.6 | - | 6.2 | 1.5 |
| Alcohols | - | 3.8 | 2.5 | - | - | - | - | 6.1 | 3.2 | 5.6 | - | 3.3 | 0.1 |
| Aldehydes | - | - | - | - | - | - | - | 7.9 | 2.7 | 4.2 | - | 0.3 | 5.9 |
| Ketones | - | - | - | - | - | - | - | - | - | 0.1 | - | - | - |
| Fatty acids and aliphatic esters | 3.1 | 6.8 | 3.0 | 8.2 | 32.7 | 18.0 | 14.2 | 9.5 | 14.1 | 5.5 | 4.4 | 17.8 | 23.5 |
| *Terpenoids* | | | | | | | | | | | | | |
| Monoterpene hydrocarbons | 5.0 | 3.8 | 1.3 | 7.0 | 6.4 | 12.1 | 2.4 | 1.0 | 3.4 | 2.5 | 4.3 | 1.5 | 4.8 |
| Oxygenated monoterpenes | 1.41 | 1.2 | 0.4 | 0.2 | 0.1 | 8.7 | 1.8 | 2.3 | 3.6 | 5.5 | 0.2 | 2.8 | 3.2 |
| Sesquiterpene hydrocarbons | 69.1 | 51.0 | 62.9 | 41.9 | 11.1 | 25.3 | 42.0 | 3.1 | 24.6 | 2.6 | 44.4 | 7.5 | 6.2 |
| Oxygenated sesquiterpenes | 4.0 | 14.7 | 12.8 | 3.7 | 1.0 | 3.0 | 12.2 | 1.9 | 2.2 | 1.0 | 15.9 | 2.7 | 8.5 |
| Diterpenoids | - | 1.2 | 3.2 | 6.6 | - | - | - | 28.9 | 14.4 | 16.0 | tr | 22.5 | 12.8 |
| *Aromatics* | tr | 1.7 | 0.4 | tr | tr | tr | - | 1.2 | 0.8 | 2.0 | tr | 2.0 | 3.3 |
| Unknown | - | 2.9 | 5.5 | 30.1 | - | - | - | 23.0 | 13.4 | 2.9 | - | 19.7 | 14.3 |

# Appendix II.

## Table 5.

**Table 5**
$^{1}H$ and $^{13}C$ NMR spectral data for compounds iridoids

aucubin MeOH

| C/H | DEPT | $\delta_C$ | $\delta_H$ | *J (Hz)* | HMBC (C→H) |
|---|---|---|---|---|---|
| 1 | CH | 96,1 | 4,94 d | (7,1) | H-1', H-3, H-9 |
| 3 | CH | 140,1 | 6,3 dd | (6,3; 1,8) | H-1, H-4 |
| 4 | CH | 104,1 | 5,08 dd | (6,3; 4,0) | H-3, H-5 |
| 5 | CH | 44,8 | 2,65 m | | H-3, H-4, H-7, H-9 |
| 6 | CH | 81,3 | 4,43 m | | H-4, H-5, H-7, H-9 |
| 7 | CH | 128,9 | 5,76 s | | H-9, H-10 |
| 8 | C | 81,2 | | | H-7, H-9 |
| 9 | CH | 46,5 | 2,89 t | (7,3) | H-1, H-7 |
| 10 | $CH_2$ | 60,0 | 4,16 d | (15,4) | H-7 |
| | | | 4,34 d | (15,4) | |
| 1' | CH | 98,5 | 4,67 d | (7,8) | H-1, H-2' |
| 2' | CH | 73,5 | 3,22* | | H-1' |
| 3' | CH | 76,6 | 3,37* | | H-1', H-2', H-4' |
| 4' | CH | 70,2 | 3,28* | | H-3', H-5', H-6' |
| 5' | CH | 76,9 | 3,26* | | H-1', H-4', H-6' |
| 6' | $CH_2$ | 61,2 | 3,64 dd | (12,2; 5,2) | H-5' |
| | | | 3,85 d | (12,2) | |

harpagide MeOH

| C/H | DEPT | $\delta_C$ | $\delta_H$ | *J (Hz)* | HMBC (C→H) |
|---|---|---|---|---|---|
| 1 | CH | 92,0 | 5,74 s | | H-1', H-3, H-9 |
| 3 | CH | 141,3 | 6,31 d | (6,5) | H-1, H-4 |
| 4 | CH | 107,2 | 4,95 dd | (6,5; 1,5) | H-3, H-6, H-9 |
| 5 | C | 70,4 | | | H-1, H-3, H-7, H-9 |
| 6 | CH | 77,0 | 3,70* | | H-4, H-7, H-9 |
| 7 | $CH_2$ | 45,8 | 1,80 dd | (13,8; 3,8) | H-10 |
| | | | 1,91 dd | (13,8; 4,8) | |
| 8 | C | | | | |
| 9 | CH | 58,5 | 2,55 s | | H-4, H-7, H-10 |
| 10 | $CH_3$ | 23,6 | 1,25 s | | H-7, H-9 |
| 1' | CH | 98,1 | 4,58 d | (8,0) | H-1, H-2' |
| 2' | CH | 73,1 | 3,21 dd | (9,1; 8) | H-1' |
| 3' | CH | 76,4 | 3,38* | | H-1', H-2' |
| 4' | CH | 70,4 | 3,29* | | H-3' |
| 5' | CH | 77,0 | 3,30* | | H-4', H-6' |
| 6' | $CH_2$ | 61,4 | ,366 dd | (11,8; 5,5) | H-5' |
| | | | 3,90 d | (11,8) | |

acetylharpagide DMSO

| C/H | DEPT | $\delta_C$ | $\delta_H$ | *J (Hz)* | HMBC (C→H) |
|---|---|---|---|---|---|
| 1 | CH | 92,1 | 5,87 s | | H-1', H-3, H-9 |
| 3 | CH | 140,8 | 6,36 d | (6,3) | H-1, H-4 |
| 4 | CH | 107,0 | 4,88 d | (6,3) | H-3, H-9 |
| 5 | C | 70,8 | | | H-1, H-3, H-7, H-9 |
| 6 | CH | 75,4 | 3,57 s | | H-7 |
| 7 | $CH_2$ | 43,9 | 1,79 dd | (14,7; 4,5) | H-13 |
| | | | 2,07 d | (14,7) | |
| 8 | C | 86,3 | | | H-7, H-9, H-13 |
| 9 | CH | 53,9 | 2,65 s | | H-4, H-13 |
| 11 | C | 170,0 | | | H-12 |
| 12 | $CH_3$ | 21,5 | 1,93 s | | |
| 13 | $CH_3$ | 21,5 | 1,37 s | | H-7 |
| 1' | CH | 96,8 | 4,39 d | (7,8) | H-1 |
| 2' | CH | 72,6 | 2,99 dt | (8,3; 4,0) | H-1', H-3' |
| 3' | CH | 76,3 | 3,14* | | H-1', H-2', H-4' |
| 4' | CH | 69,7 | 3,08* | | H-5', H-6' |
| 5' | CH | 76,6 | 3,14* | | H-4' |
| 6' | $CH_2$ | 60,5 | 3,47 m* | | H-5' |
| | | | 3,69 dd | (11,6; 3,6) | |

ajugoside MeOH

| C/H | DEPT | $\delta_C$ | $\delta_H$ | *J (Hz)* | HMBC (C→H) |
|---|---|---|---|---|---|
| 1 | CH | 94,7 | 5,85 d | (1,6) | H-1', H-3, H-9 |
| 3 | CH | 141,6 | 6,21 dd | (6,3; 2,3) | H-1, H-4 |
| 4 | CH | 104,3 | 4,71 d | (6,3) | H-3, H-5, H-6, H-9 |
| 5 | CH | 41,8 | 2,81 d | (8,6) | H-1, H-3, H-7, H-9 |
| 6 | CH | 77,0 | 4,01 d | (4,5) | H-4, H-5, H-7 |
| 7 | $CH_2$ | 48,7 | 2,09 dd | (15,1; 4,5) | H-9, H-10 |
| | | | 2,20 d | (15,1) | |
| 8 | C | 90,2 | | | H-1, H-6, H-7, H-9, H-13 |
| 9 | CH | 49,6 | 2,88 d | (8,6) | H-4, H-6, H-7, H-13 |
| 11 | C | 173,3 | | (15,4) | H-12 |
| 12 | $CH_3$ | 22,3 | 2,00 s | | |
| 13 | $CH_3$ | 22,9 | 1,53 s | | H-9 |
| 1' | CH | 100,0 | 4,65 d | (8,1) | H-1, H-2' |
| 2' | CH | 74,9 | 3,20* | | H-1', H-3' |
| 3' | CH | 78,1 | 3,39* | | H-1', H-2', H-4' |
| 4' | CH | 71,8 | 3,28* | | H-3', H-5', H-6' |
| 5' | CH | 78,2 | 3,31* | | H-1', H-4', H-6' |
| 6' | $CH_2$ | 63,0 | 3,67 dd | (12,1; 5,5) | H-4', H-5' |
| | | | 3,88 dd | (12,1; 1,8) | |

harpagoside MeOH

| C/H | DEPT | $\delta_C$ | $\delta_H$ | *J (Hz)* | HMBC (C→H) |
|---|---|---|---|---|---|
| 1 | CH | 94,7 | 6,18 s | | H-1', H-3, H-9 |
| 3 | CH | 144,0 | 6,42 d | (6,5) | H-1, H-4 |
| 4 | CH | 107,0 | 4,94 dd | (6,5; 1,5) | H-3, H-6, H-9 |
| 5 | C | 73,5 | | | H-1, H-3, H-7, H-9 |
| 6 | CH | 77,8 | 3,77 d | (4,0) | H-4, H-7 |
| 7 | $CH_2$ | 46,3 | 2,03 dd | (15,1; 4,5) | H-10 |
| | | | 2,28 d | (15,1) | |
| 8 | C | 88,8 | | | H-1, H-6, H-7, H-9, H-10 |
| 9 | CH | 55,7 | 2,94 s | | H-2, H-4, H-6, H-7, H-10 |
| 10 | $CH_3$ | 22,7 | 1,54 s | | H-7, H-9 |
| 12 | C | 168,8 | | | H-13, H-14 |
| 13 | CH | 120,2 | 6,51 d | (16,1) | H-14 |
| 14 | CH | 146,2 | 7,67 d | (16,1) | |
| 15 | ArH | 135,8 | | | |
| 16 | ArH | 129,3 | 7,59* | | H-13, H-14, H-17, H-19 |
| 17 | ArH | 130,6 | 7,40 * | | H-16, H-20 |
| 18 | ArH | 130,1 | 7,40* | | H-16, H-20 |
| 19 | ArH | 130,6 | 7,40* | | H-16, H-20 |
| 20 | ArH | 129,3 | 7,59* | | H-13, H-14, H-17, H-19 |
| 1' | CH | 100,1 | 4,63 d | (8,1) | H-1, H-2' |
| 2' | CH | 74,6 | 3,23* | | H-1', H-3' |
| 3' | CH | 77,7 | 3,40* | | H-1', H-2', H-4' |
| 4' | CH | 71,8 | 3,31* | | H-3', H-5', H-6' |
| 5' | CH | 78,2 | 3,34* | | H-1', H-4', H-6' |
| 6' | $CH_2$ | 63,1 | 3,73 dd | (12,1; 5,5) | H-4', H-5' |
| | | | 3,94 dd | (12,1; 1,5) | |