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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„The Lord has brought medicines into existence from the earth,
and the sensible man will not despise them."
Ecclesiasticus 38,4

1. Introduction

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 labiate 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.

Some species grow in Hungary, too. *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. *St. germanica* L. also grows 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.

Some members of the *Stachys* genus (extracts or their content material) have significant antibacterial, antifungal and antiphlogistic effects and they can also be useful in anoxia, hepatitis and nephritis. It is proved by literature data that *Stachys* species have long been used in folk medicine for the treatment of genital tumours and cancerous ulcers.

Stachys species belong to the Lamioideae subfamily, thus they contain volatile oils in traces, but they have a great number of other secondary metabolic products, e.g. iridoids. As for their structure, their iridoids usually have 9 C atoms, with an OH group on C_5 or C_6. They typically contain a methyl or acetyl group on C_8, giving C_8 a quaternary character. For the

most part these iridoids cannot be detected in UV light, therefore a developing reagent is needed to make them visible. Their structure is relatively simple, at the same time they are very sensitive to acids and enzymes, the presence of which leads to the decomposition of the compound.

2. Aims

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 active agent pattern 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 made native to Hungary. In view of this, my aims were the following:

- to examine the volatile components of *Stachys* species
- to isolate iridoids from *Stachys* species
- to work out a simple routine method for the examination of the iridoid components
- to identify the iridoid content, composition and the percentage of each component in Hungarian *Stachys* species
- 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 plant parts used 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 following *Stachys* species were examined: *St. officinalis* L., *St. officinalis* subsp. betonica L., *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. The plants were classified

and the sample materials were categorized 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 in the Department of Pharmacognosy of the Faculty of Pharmacy of the University of Szeged.

3.2. Reagents and test materials

Solvents of analytical purity were supplied by Reanal (Budapest, Hungary), those of HPLC purity by Merck (Darmstadt, Germany).

3.3. Methods used during isolation

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 and their components were identified with NP-TLC chromatography and with the GC/FID, GC/MS gas chromatographic methods.

Extraction and isolation of iridoids:

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) and were extracted with methanol with the help of an ultrasonic shaker and Gerhardt shaker. A wide range of separation technique procedures was used during isolation: column chromatography (on aluminium oxide and polyamide stational phase), vacuum column chromatography with silica gel as the stational phase and reversed-phase HPLC as the final step of purification. The steps of purification were followed on silica gel layer.

Structure examination:

The isolated iridoids were identified on the basis of their physical and spectroscopic properties. The basic information concerning the structure of the compounds was provided by their NMR spectra.

4. Results and evaluation

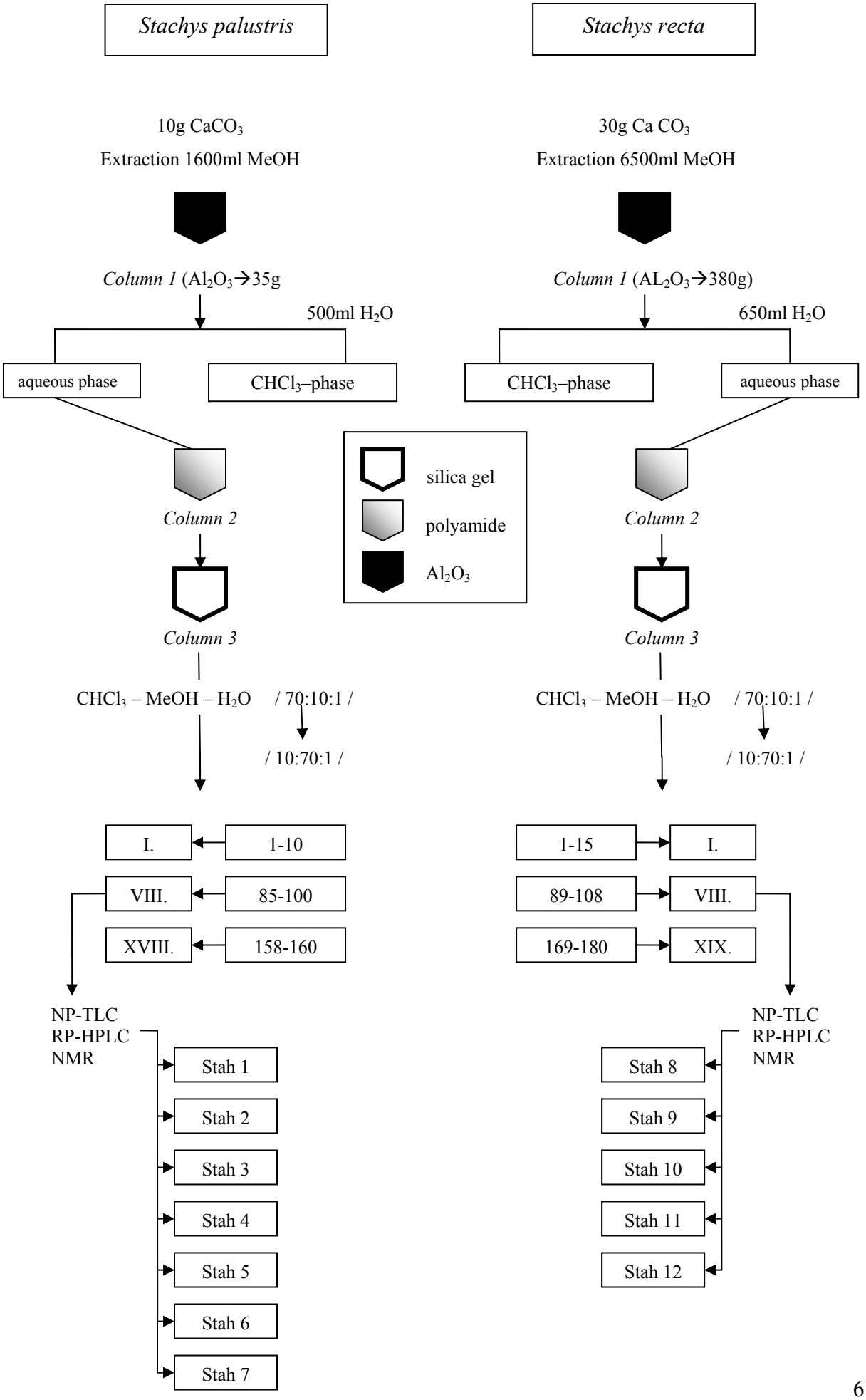
4.1. Results of gas chromatography

- All the species we examined contain a very small quantity of volatile oil.
- An n-hexane 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 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 components 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 monoterpene components occur in the greatest %: sabinene 12% (*St. byzantina*), 1-octen-3-ol 5.6% (*St. recta*), *β*-phelladrene 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. betonica), 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).
- The greatest number of components could be identified in *St. officinalis* Sample II and in *St. Recta.* The components identified in the species examined amount to 51-89 % of the total volatile oil content.
- With respect to the *Stachys* genus sections examined, the *Eriostomum* section is the one which contains the smallest number of monoterpenes. The greatest number of monoterpenes was observed in the *Stachys* and *Olisia* sections. The *Betonica* section is the richest in sesquiterpenes from among the section species examined, but the *Olisia* and *Stachys* sections are also worth mentioning both regarding the number and the quantity 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.
– 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.
– The above statements apply only to the species examined.

4.2. Results of iridoid isolation

– 12 iridoid components were isolated and identified during our work.
– 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* acetylharpagide, stah-6 is myoporoside and Stah-7 is harpagoside. The 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.
– We were the first to isolate aucubin, ajugoside and myoporoside from *St. palustris*. Moreover, harpagide, acetylharpagide, 6-*epi* acetylharpagide and ajugoside 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 the course of the identification of the iridoid components of further *Stachys* species, the iridiod components we isolated and identified were also used.
– In addition to harpagide, acetylharpagide and aucubin, ajugoside and harpagoside can also be identified from *St. officinali*s.
– From *St. sylvatica* only ajugoside, harpagoside and harpagide could be identified.
– Aucubin, harpagide, acetylharpagide and ajugoside were identified from *St. grandiflora*. This was the only Stachys species we examined in which the presence of a small amount of catalpol could be detected.
– From *St. macranta* harpagide could be identified
– Aucubin and harpagide can be seen on the NP-TLC and RP-HPLC chromatogram of *St. alpina.*
– *St. byzantina* contains aucubin, harpagide and ajugoside.
– Harpagide and harpagoside can be identified in *St germanica.*
– The presence of iridoids could not be detected in *St. annua.*


Stachys palustris
Stachys recta
10g CaCO3
Extraction 1600ml MeOH
30g Ca CO3
Extraction 6500ml MeOH
Column 1 (Al2O3→35g
500ml H2O
Column 1 (AL2O3→380g)
650ml H2O
aqueous phase
CHCl3–phase
CHCl3–phase
aqueous phase
silica gel
polyamide
Al2O3
Column 2
Column 2
Column 3
Column 3
CHCl3 – MeOH – H2O / 70:10:1 /
/ 10:70:1 /
CHCl3 – MeOH – H2O / 70:10:1 /
/ 10:70:1 /
I. 1-10
VIII. 85-100
XVIII. 158-160
1-15 I.
89-108 VIII.
169-180 XIX.
NP-TLC
RP-HPLC
NMR
NP-TLC
RP-HPLC
NMR
Stah 1
Stah 2
Stah 3
Stah 4
Stah 5
Stah 6
Stah 7
Stah 8
Stah 9
Stah 10
Stah 11
Stah 12

4.3 Working out a simple routine method for the examination of the iridoid components

An aqueous extract was prepared from 5g of fresh plant (*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 evaporated (0.15g) and dissolved in 2 mL of methanol : water/8:2, then NP-TLC and RP-HPLC examinations were carried out. Then aqueous extraction was performed for the TLC, TLC/densitometric and HPLC chromatographic methods used for the detection of the iridoid components of further *Stachys* species examined.

4.4 Results of TLC/densitometry

The percentage proportion of iridoids in the stem, leaf and inflorescence of the species examined is revealed by TLC/densitometry.

- The greatest percentage of acetylharpagide is present in the inflorescence (0.78%) and stem (0.70%) of St. officinalis.
- The greatest percentage of harpagoside can be detected in the leaf (0.51%) and inflorescence parts (0.43%) of *St. officinalis.*
- Catalpol was 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* (0.43%) and in the inflorescence of *St. recta* (0.42%).
- The greatest percentage of harpagide is accumulated in the stem of *St. recta* (0.58%) and in the inflorescence of *St. grandiflora* (0.84%)
- The greatest percentage of ajugoside is accumulated in the stem and inflorescence of the *Stachys* species we examined.
- Iridoids could not be identified in *St. annua* with densitometry. 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 iridoid components.

5. Biological effect study of Stachys species and their content material

5.1 Examination of the antioxidant effect of Stachys species

The classical therapeutic use of a great number of herbs and phytotherapeutic preparations can be explained by the antioxidant effect of plant polyphenols. The antioxidant effect of 6 *Stachys* species, namely *Stachys officinalis*, *St. annua*, *St. recta*, *St. macrantha, St. alpina* and *St. sylvatica* was examined in an enzyme-independent lipid peroxidation system. Enzyme-independent lipid peroxidation was tested *in vitro* on cattle brain homogenate. 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. The value of R^2 is the highest for the total polyphenol content expressed in gallic acid and pyrogallol, on the basis of this it can be stated that tannins are responsible for the antioxidant effect in the *Stachys* species examined.

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

From among the *Stachys* species we examined, *St. recta and St. officinalis* have been used in folk medicine for the treatment of genital tumours. 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 and ajugoside. Our examinations were performed in vitro on A431 skin carcinoma, MCF7 breast carcinoma, Hela – cervix carcinoma cells. The first step was to examine the species according to organs. 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* on the MCF7 cell line. Cytotoxic activity over 50 % was shown in a concentration of 90μg/mL by aucubin on the HeLa cell line and by harpagide on the A431 and HeLa cell lines. Cytotoxic activity over 40 % was obtained for aucubin on the A431 and MCF7 cell lines and for harpagide on the MCF7 cell line in a concentration of 90μg/mL.

6. Chemotaxonomic importance of the isolated compounds

Bentham classifies the *Stachys* genus into the Stachydeae tribe. This entire tribe constitutes part of the Lamioideae subfamily formed by Erdtman. However, a third taxonomist, Briquet classifies the genus into an independent subfamily (subfamily Stachyoideae), which agrees with the other two taxonomists' classifications only partly. 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 Wargraf and Cantino

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