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Iridoids From *Stachys Byzantina* K. Koch (Lamb's Ears) And *Stachys Germanica* L. (Downy Woundwort)

Original Paper

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Abstract Iridoids are a class of secondary metabolites found in a wide variety of plants. Iridoids are typically found in plants as glycosides, most often found to glucose. The genus *Stachys* L. is one of the largest genera of the Lamiaceae family, containing iridoids.
Aim: The aim of this study was the isolation and identification of iridoids from the aerial parts of *Stachys byzantina* K. Koch and *Stachys germanica* L.
Methods: For the isolation and identification of the iridoids, different chromatographic methods (NP-TLC, CPC and RP-HPLC) were used. The structures were established by one- and two-dimensional NMR and mass spectrometry, also.
Results: Iridoids (aucubin, harpagide, ajugoside and harpagoside) were isolated and identified by combination of different chromatographic methods from *S. byzantina* and *S. germanica*.
Conclusion: Stachys species may also be used as a potential source of iridoids.

Keywords *Stachys germanica – Stachys byzantina – iridoids – harpagide – aucubin – ajugoside – harpagoside*

INTRODUCTION

The family Lamiaceae consists of approximately 200 genera of 3500 species. The woundwort (*Stachys* L.) 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, Eastern Europe, Cape Province and Chile. Ten 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. *S. officinalis* L. is found in Europe, so in Hungary as well. *S. alpina* L. likes shady places, and it is found in fresh hornbeam-beech forests. *S. germanica* L. also grows in Hungary and Slovakia, and it is quite frequent in dry grasslands and pastures. *S. byzantina* L. is found as an ornamental plant, and *S. grandiflora* Stev. ex Willd. and *S. macrantha* K. Koch are found in botanical gardens. *S. 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. *S. palustris* L. is widespread in the greater part of Europe, and it is common in Hungary, especially along marshes and bogs. *S. recta* L. is frequent on dry, stony grasses and steppe slopes. *S. 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 ploughlands and stubble fields, mainly on hard soils (Tomou et al., 2020; Tutin et al., 1972).

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 (Skaltsa et al., 1999, 2001; Tomou et al., 2020).

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Stachys species belong to the Lamioideae subfamily, and 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 C5 or C6. They typically contain a methyl or acetyl group on C8, giving C8 a quaternary character. For the most part, these iridoids cannot be detected in UV light, and 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 (El-Naggar & Beal, 1980; Kobzar, 1986; Derkach et al., 1987; Jeker et al., 1989; Calis et al., 1991; Boros & Stermitz, 1990, 1991; Isamukhamedova & Pulatova, 1992; Kartev et al., 1994; Miyase et al., 1990; Munoz & Pena, 2001).

The aim of this study was the isolation and identification of iridoids from the aerial parts of *Stachys byzantina* (lamb's ears) and *Stachys germanica* (downy woundwort).

MATERIALS AND METHODS

Stachys germanica was collected at the Medicinal Plant Garden of the Faculty of Pharmacy, Comenius University in Bratislava. *Stachys byzantina* was gathered at Hungarian biotope and the Botanical Garden, Vácrátót, Hungary. Voucher specimens were deposited in the Institute of Pharmacognosy, University of Szeged, Hungary. The aerial parts of both species were collected at the flowering time, in June. The samples were conserved at -20°C until processing.

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

Extraction and isolation of iridoids

S. byzantina and *S. germanica* were rubbed with $CaCO_3$ and extracted with methanol using 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, Germany (70–230 mesh ASTM)). Extracts were concentrated under vacuum with a Rotavapor RE (Büchi, Germany) rotary evaporation system. This was dissolved in water, and a liquid-liquid distribution was performed with chloroform. Chlorophyll of all samples was removed by using a polyamide column chromatography.

Purification and isolation of chemical compounds

Silica gel 60 G (mean particle size 15 µm) (Merck, Germany) was used for vacuum-liquid chromatography (VLC). The concentrated extract was 6 g for *S. byzantina* and 8 g for *S. germanica*. After dissolution in water, VLC fractionation was carried out by using a water pump. Eluents were chloroform:methanol:water [70:10:1 and 10:70:1] and methanol:water [40:10 and 10:40].

The composition of the fractions was checked with thin-layer chromatography (TLC) in each case, and the solvent system was $CHCl_3$:MeOH:H_2O [25:10:1], as a mixture developed in this work, while the developer was the 1% concentrated hydrochloric acid solution of 4-(dimethylamino)benzaldehyde (105°C, 5 min).

The HPLC system (Shimadzu SPD 10 A/10 AV HPLC, Shimadzu Corporation, Japan) consisted of a gradient pump, analytical sampling valve, UV detector and integrator with software. Columns: BST SI-100 10 C-18 (250 mm, Ø 4 mm; Merck, Germany) and LiChrospher RP-18 (250 mm, Ø 5 µm; Merck, Germany), solvent system: method 1: water:acetonitrile [90:10]; method 2 – eluent: water:acetonitrile [98:2]; gradient program: isocratic; flow rate: 0.5 mL/min; injection volume: 20 µL; detector UV; column temperature: 23 °C. The fractions were concentrated under vacuum used a Rotavapor RE (Büchi, Germany) rotatory evaporation system.

Structural examination

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

Identification of isolated components

Melting points were measured with a melting point apparatus MP70 (Merck, Germany).

The UV spectra were recorded in MeOH and H_2O with Shimadzu UV 2101 PC spectrophotometer (Shimadzu Corporation, Japan).

^1H, ^{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 (Bruker, Germany), at 400 MHz (^1H) and 100 MHz (^{13}C).

RESULTS AND DISCUSSION

Aerial parts of both *Stachys* species (*S. byzantina* and *S. germanica*) contained iridoids. Comparing the main iridoid component in the samples obtained from methanolic extract of drugs, harpagide was found in both *Stachys* samples. In addition to the iridoids in *S. byzantina*, ajugoside, aucubin and harpagide were identified by TLC (see Fig. 1), while those later two compounds were isolated and determined by RP-HPLC and NMR method aucubin and harpagide (see Tables 1 and 2). Harpagide and harpagoside (see Fig. 1) were detected in *S. germanica*. We could develop a method which can be used for the identification and detection of the iridoids of *Stachys* species.

In 1945, the Swedish botanist Erdtman subdivided this taxonomic family into two major subfamilies: the Lamioideae and the Nepetoideae. The Lamioideae subfamily

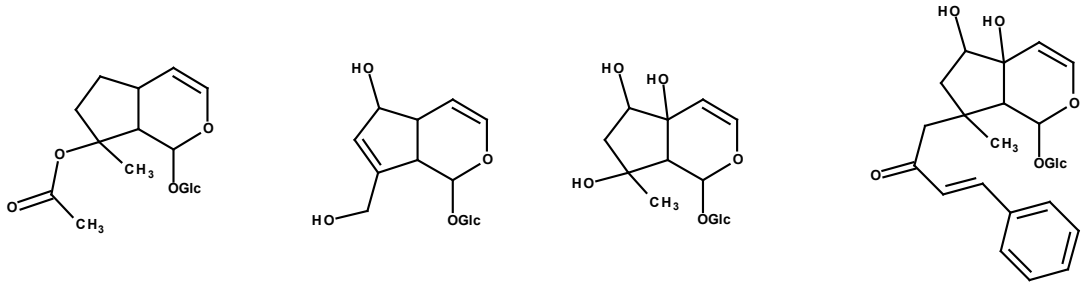

Figure 1. Isolated compounds.

Table 1. Physical and UV spectroscopic data on iridoids from S. byzantina and S. germanica.

Iridoids	M. P. [°C]	λ_{max} [nm]
Ajugoside	amorph. powder	206
Aucubin	179 – 183	210
Harpagide	amorph. powder	210
Harpagoside	amorph. powder	216

M. P., melting point.

is characterized by tricolpate and binucleate pollen, albuminous seeds, spatulate embryos, the presence of iridoid glycosides, lower content of essential oils and rosmarinic acid but higher content of phenylpropanoid glycosides, whereas the Nepetoideae have hexacolpate, trinucleate pollen, exalbuminous seeds, investing embryos, the presence of volatile terpenoids, mainly monoterpenes and high content of essential oil and rosmarinic acid. Both subfamilies contain caffeic acid and its derivatives (Erdtman, 1945; Cantino & Sanders, 1992).

The genus *Stachys* belongs to the subfamily Lamioideae. Iridoids are a class of secondary metabolites found in this genus. The first investigation of the iridoid content of in three species dates back to the beginning of the 1970s, when Adema detected iridoid glycoside in *S. palustris* and when Ukrainian researchers determined harpagide and 8-*O*-acetylharpagide in 17 *Stachys* species (Adema, 1968; Zinchenko, 1972). The main iridoid components in the *Stachys* species studied by Gritsenko et al. in 1977 were harpagide and 8-*O*-acetylharpagide. The presence of aucubin was also detected in some *Stachys* species (Gritsenko et al., 1977). Ajugol, ajugoside, harpagide and 8-*O*-acetylharpagide were detected in *S. atherocalyx*, *S. inflate* and *S. iberica* (Komissarenko et al., 1976, 1979). In 1980, the research team started an examination concerning 20 different *Stachys* species, which supplemented the previous results: beside harpagide and acetylharpagide as the main components, reptoside and diacetyl-reptoside were also found (Pakaln et al., 1980). In 1984, Lenherrr et al. performed the RP-HPLC analysis of 10 species belonging to the *S. recta* group, and melittoside, harpagide, acetylharpagide, ajugoside and ajugol were identified (Lenherrr, 1984). The data relating to the iridoids isolated until 1980 are summarized by El-Naggar & Beal (1980). El-Naggar's summary was followed by the work by Boros & Stermitz, which contains the summary of iridoids isolated between 1980 and 1990 (Boros & Stermitz, 1990, 1992). Russian researchers continued to investigate the chemical components of the *Stachys* species growing in Russia (Isamukhamedova & Pulakova, 1992; Kartev et al., 1994). Japanese researchers also conducted research into the iridoids *Stachys* species (Miyase et al., 1990). Monomelittoside, melittoside, 8-*O*-acetylharpagide, harpagide, ajugol, catalpol, 7-*O*-acetyl-8-epi-loganic acid, aucubin and 5-allosyloxy-aucubin were isolated by research made by Montenegrian, Greek, Hungarian and Italian scientists (Háznagy-Radnai et al., 2005, 2006, 2007; Munoz & Pena 2001; Kostos et al., 2001; Meremeti et al., 2004; Serrilli et al., 2005).

The isolated iridoid components of *Stachys* species found in Hungary are included in Table 3. Háznagy-Radnai et al., 2006, 2007 two iridoids, as harpagide and 8-*O*-acetylharpagide isolated in *S. byzantina and S. germanica*. Ajugoside and aucubin in both species right now.

CONCLUSIONS

The genus *Stachys* L. is one of the largest genera of the family Lamiaceae and subfamily Lamioideae containing iridoids. Iridoids (aucubin, harpagide, ajugoside and harpagoside) were isolated and identified by combination of different chromatographic methods (NP-TLC, CPC and RP-HPLC) from *S. byzantina* and *S. germanica*. The structures were established by one- and two-dimensional NMR and mass spectrometry. *Stachys* species may also be used as a potential source of iridoids.

Table 2. ^{1}H and ^{13}C NMR spectral data for isolated iridoids.

Aucubin (methanol):					
C/H	**DEPT**	**δ_C**	**δ_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 (methanol):					
C/H	**DEPT**	**δ_C**	**δ_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	81.2			H-7, H-9
9	CH	58.5	2.55 s		H-4, H-7, H-10
10	CH_2	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, 8.0)	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	3.66 dd	(11.8, 5.5)	H-5′
			3.90 d	(11.8)	

Table 3. The isolated iridoid components of Stachys species.

Species	**Iridoids**
S. alpina	harpagide, 8-*O*-acetylharpagide (Háznagy-Radnai, 2006, 2007)
S. annua	melittoside (Lenherr, 1984)
S. byzantina	harpagide, 8-*O*-acetylharpagide (Háznagy-Radnai, 2006, 2007)
S. germanica	harpagide, 8-*O*-acetylharpagide (Háznagy-Radnai, 2006, 2007)
S. grandiflora	harpagide, 8-*O*-acetylharpagide, melittoside (Háznagy-Radnai, 2006, 2007)
S. macrantha	harpagide, 8-*O*-acetylharpagide, ajugol, ajugoside, reptoside, allobetonicosid, macrathoside (Calis, 1991; Háznagy-Radnai, 2006, 2007)
S. officinalis	harpagide, 8-*O*-acetylharpagide, ajugol, ajugoside, reptoside, allobetonicosid, 6-*O*-acetylmioporoside (Kobzar, 1986; Derkach, 1987; Jeker, 1989; Háznagy-Radnai, 2006, 2007)
S. palustris	harpagide, 8-*O*-acetylharpagide, aucubin (Adema, 1968; Háznagy-Radnai, 2006, 2007)
S. recta	harpagide, 8-*O*-acetylharpagide, aucubin, ajugol, ajugoside, melittoside (Lenherr, 1984; Háznagy-Radnai, 2006, 2007)
S. sylvatica	harpagide, 8-*O*-acetylharpagide (Kukic, 2006; Háznagy-Radnai, 2006, 2007)

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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