Polyphenols in *Stachys* and *Betonica* Species (Lamiaceae)

Vassya Bankova[a,*], Jordanka Koeva-Todorovska[b], Tatyana Stambolijska[b], Maria-Desislava Ignatova-Groceva[b], Daniela Todorova[a] and Simeon Popov[a]

[a] Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria. Fax: 003592–700–225. E-mail: iochnp@bgcict.acad.bg
[b] Faculty of Biology, Sofia University, Sofia 1000, Bulgaria

* Author for correspondence and reprint requests

Z. Naturforsch. **54c,** 876–880 (1999); received March 15/June 6, 1999

Lamiaceae, *Stachys, Betonica*, Phenylethanoids, Flavonoids, Chemotaxonomy

Above-ground parts and roots from four *Stachys* species (*S. germanica* L., *S. sylvatica* L. and the Balkan endemics *S. thracica* Dav. and *S. plumosa* Griseb.) *as well as of* three *Betonica* species (*B. officinalis* L. and the Balkan endemics *B. bulgarica* Deg. et Neic. and *B. scardica* Griseb.) were screened for phenols (phenylethanoid glycosides, flavonoid glycosides and the phenolic diterpene betolide). Three phenylethanoid glycosides, a flavonoid glycoside and the phenolic diterpene betolide were isolated and identified, most of them for the first time in the investigated species. The results obtained support the view that *Stachys* and *Betonica* are well separated genera.

Introduction

Betonica and *Stachys* species are widely used in folk medicine and recently in the official medicine (Ovcharov *et al.,* 1992). Most of the biologically active substances in these plants are polyphenols, compounds often used in taxonomic studies. *Betonica* and *Stachys* are represented in Bulgaria with 4 and 18 species, respectively (Koeva-Todorovska, 1979). The separation of the Linnei's genus *Betonica* from *Stachys* is based on well defined differences in the general morphological characteristics and especially in the morphology of caryotypes and pollen of both genera (Koeva-Todorovska, 1979; 1988).

It was of interest to investigate the polyphenol composition of some *Stachys* and *Betonica* species distributed in Bulgaria, especially the endemic ones, and to use the phytochemical evidence to clarify this classification problem.

Materials and Methods

Plant material

Above-ground parts and roots from *S. germanica* (near the city of Sofia, Knyazhevo, 5.08.1992; (990663), *S. sylvatica* (near the city of Sofia, Knyazhevo, 4.08.1992; (99065), *S. plumosa* (Znepole region, village Gorno Uyno, 12.08.1993; (99061); Osogovo Mt., village Bogoslov, 1.08.1994; (99066), *S. thracica* (Strandja Mt., village Bogoslov, 23.05.1994), *B. bulgarica* (Middle Balkan Mt., Korudere, 20.07.1993 and 25.07.1994; (85963), *B. scardica* (Rudina Mt., Borovski dol, 11.08.1993; (99060), *B. officinalis* (near the city of Sofia, Knyazhevo, 2.08.1992; (99064). The numbers in brackets correspond to the voucher specimens of the plants, determined by Dr. J. Koeva-Todorovska and deposited in the herbarium of the Faculty of Biology, Sofia University (SO).

Isolation

Dried ground roots from *S. germanica* (620 g) were extracted twice with 3 l ethanol. The ethanol extract was concentrated *in vacuo*, diluted with water and extracted successively with petrol ether (3x) and ethyl acetate (3x). The ethyl acetate extract was subjected to column chromatography on silica gel with mobile phase dichloroethane – methanol with increasing percentage of methanol. After repeated column chromatography on silica gel with mobile phases dichloroethane-methanol and dichloroethane-methanol-water, **1** (20 mg), **2** (14 mg) and **4** (17 mg) were isolated. From the aerial parts (800 g) by the same way **1**, **2** and **4** were isolated.

Dried ground roots from *B. bulgarica* (45 g) were extracted twice with 0.25 l ethanol. The ethanol extract was concentrated *in vacuo*, diluted with

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water and extracted successively with petrol ether (3x) and ethyl acetate (3x). From the petrol ether extract, using silica gel column chromatography with mobile phase hexane-ethyl acetate with increasing polarity, 36 mg of **5** were isolated. The same way as described above from the ethyl acetate extract, **1** (110 mg), **2** (38 mg) and **3** (77 mg) were isolated.

The extraction procedure was applied for *S. sylvatica* (82 g aerial parts, 25 g roots), *S. plumosa* (20 g aerial parts, 15 g roots), *S. thracica* (60 g aerial parts, 10 g roots), *B. bulgarica* (165 g aerial parts, 45 g roots), *B. scardica* (75 g aerial parts, 25 g roots) and the following substances were isolated: from *S. sylvatica*: aerial parts – **1**, **2** and **4**, roots – **1** and **2**; from *S. plumosa*: aerial parts – **1**, **2** and **4**, roots – **1**, **2** and **4**; *S. thracica*: aerial parts – **1**, **2** and **4**, roots – **1**, **2** and ; *B. bulgarica*: aerial parts – **1**, **2** and **3**, roots – **1**, **2**, **3** and **5**; *B. scardica*: aerial parts – **1**, **2** and **3**, roots – **1**, **2**, **3** and **5**.

Identification

The identification of the isolated compounds was carried out by measuring their UV, ^{1}H and ^{13}C NMR spectra and comparing the results with published data, as follows. For compound **1**: UV, ^{1}H and ^{13}C NMR spectra identical with data published by Ikeda *et al.,* 1994 for acteoside; for compound **2**: UV, ^{1}H and ^{13}C NMR spectra identical with data published by Ikeda *et al.,* (1994) for martinoside; for compound **3**: UV, ^{1}H and ^{13}C NMR spectra identical with data published by Miyase *et al.* (1990) for forsythoside B; for compound **4**: UV (incl. those with shift reagents), ^{1}H and ^{13}C NMR spectra identical with data published by El-Ansari *et al.* (1991) for 4'-O-methylisoscutellarein 7-O-(2''-O-6'''-O-acetyl-β-D-allopyranosyl-β-D-glucopyranoside); for compound **5**: UV, ^{1}H,^{13}C NMR and mass spectra identical with data published by Tkachev *et al.* (1987) for betolide.

The Rf values on silica gel plates for the isolated compounds are as folllows:

In mobile phase chloroform-methanol-water 80:20:1 v/v, **1** – 0.15; **2** – 0.25; **3** – 0.07; **4** – 0.32. Chloroform-methanol-water 30:20:4 v/v, **1** – 0.73; **2** – 0.80; **3** – 0.65; **4** – 0.90. chloroform-acetic acid-methanol 18:1:3 v/v, **1** – 0.60; **2** – 0.68; **3** – 0.51; **4** – 0.75. Compound **5** in mobile phase petrol ether-ethyl acetate 4:1 Rf = 0.60, and chloroform-acetone 8:1 Rf = 0.75.

Analysis of phenolics

Sample preparation

Of each extract (petrol ether and ethyl acetate) a part corresponding to 1 g plant material was weightened and dissolved in 1 ml of chloroform for the petrol ether extracts and in 2 ml of methanol/chloroform 1:1 v/v for the ethyl acetate extracts.

Preparation of the standard solutions

The standard solutions of the isolated pure compounds (purity >95%, 1H-NMR) **1**, **2**, **3, 4** and **5** were prepared by dissolving 2 mg of pure compound in 2 ml of solvent. The solvent was methanol/chloroform 1:1 v/v for **1**, **2**, **3** and **4**, and chloroform for **5**.

TLC semiquantitative analysis of petrol ether extracts

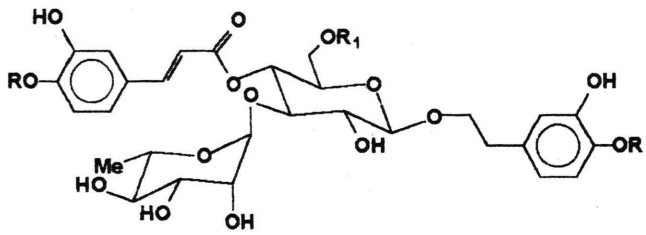
An aliquot (30 μl) of the sample solutions together with 12 μl of the standard solution of **5** were applied to silica gel plates, developed with petrol ether-ethyl acetate 4:1 and chloroform-acetone 8:1. The evaluation of the spots was made after viewing the plates in UV light (254 nm) and after charring.

TLC semiquantitative analysis of ethyl acetate extracts

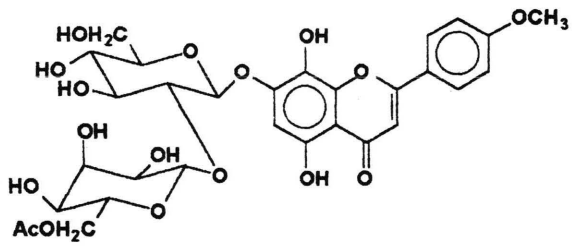
An aliquot (20 μl) of the sample solutions together with 12 μl of the standard solutions of **1**, **2**, **3** and **4** were applied to silica gel plates and developed with three mobile phases: chloroform-methanol-water 80:20:1, 30:20:4 v/v and chloroform-acetic acid-methanol 18:1:3 v/v. The evaluation of the spots was made after viewing the plates in UV light (254 and 366 nm) and after charring with sulfuric acid.

Results and Discussion

Seven species were investigated: *Betonica officinalis* L., the Bulgarian endemic *B. bulgarica* Deg. et Neic., the Balkan endemic *B. scardica* Griesb., *Stachys germanica* L., *S. thracica* Dav. (Balkan en-



1. R = R_1 = H;
2. R = Me, R_1 = H.
3. R = H; R_1 = Apiose



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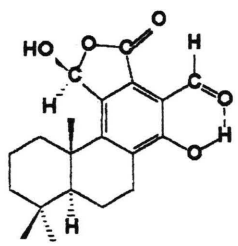

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Fig. 1

Fig. 1. Phenolic components of *Stachys* and *Betonic* species.: **1** acteoside (Ikeda *et al.*, 1994); **2** martinoside (Ikeda *et al.*, 1994); **3** forsythoside (Miyase *et al.*, 1990); **4** 7-O-(2"-O-6"'-O-acetyl-β-D-allopyra-nosyl-β-D-glucopyranoside) (El-Ansari *et al.*, 1991); **5** betolide (Tkachev *et al.*, 1987).

demic), *S. sylvatica* L. and *S. plumosa* Griesb. (Balkan endemic). Roots and above-ground parts were investigated separately. Part of the species (see Table I) were investigated in two consecutive years.

The MeOH extracts of the investigated species showed considerable differences in the polyphenol composition. The concentrated methanolic extracts were extracted subsequently with petroleum ether and ethyl acetate. The ethyl acetate extracts were subjected to column chromatography on silica gel to afford pure phenylethanoid glycosides, identified by comparing their ^{1}H and ^{13}C NMR: acteoside **1** (Ikeda *et al.*, 1994), martinoside **2** (Ikeda *et al.*, 1994) and forsythoside B **3** (Miyase *et al.*, 1990) (from *Stachys* and *Betonica* species, see Table I.) and the flavonoid glycoside **4** (El-Ansari *et al.*, 1991) (from *Stachys* species only). The petroleum ether extract was separated on silica gel

Table I. Chemical composition of *Betonica* and *Stachys* species.

Sample	1	2	3	4	5
B.officinalis	++++	–	+	–	+++
	x	x	**x**	–	–
B.bugarica 1993	+++	+	+++	–	++++
	xxx	x	x	–	–
B.bulgarica 1994	+++	+	+++	–	++++
	xxx	x	x	–	–
B.scardica	++	+	+++	–	++
	xx	xx	xx	–	–
S.germanica	+++	++	–	++++	–
	xxx	xx	–	xx	–
S.sylvatica	++	+	**+**	**+**	–
	xxx	xx	**x**	x	–
S.plumosa 1993	+	+	**+**	++	–
	xx	x	**x**	x	–
S.plumosa 1994	++	+	**+**	+++	**+**
	xx	x	**x**	x	–
S. thracica	+++	++	**+**	++	–
	xxxx	xx	**x**	xx	–

+ – roots.
x – above-ground parts.

to isolate the unpolar phenolic diterpene betolide **5** (Tkachev *et al.*, 1987), from *Betonica* species only. Semiquantitative determinations were performed by TLC, using known quantities of pure compounds as standards, chromatographed parallel with the extracts. The data obtained are summarised in Table I.

The polyphenol composition of three *Betonica* species was studied. No differences in the polyphenol composition in two different years were observed but differences were noted between the polyphenol composition of the roots and the above-ground parts. In the roots, two groups of polyphenols were found, the phenylethanoid glycosides **1**, **2** and **3** and the phenolic diterpene betolide **5**. Acteoside **1** and martinoside **2** are known constituents of *Stachys* species (Nishimura *et al.*, 1991; Cometa *et al.*, 1993; Ikeda *et al.*, 1994), and of *Betonica* species (Calis *et al.*, 1992; Miyase *et al.*, 1990). Till now forsythoside B **3** was found in *Forsythia* (Endo *et al.*, 1982) and recently in *B. officinalis* (*S. officinalis*) (Miyase *et al.*, 1990). This compound appeared to be the main phenolic glycoside in the roots of *B. bulgarica* and *B. scardica*. Acteoside **1**, martinoside **2** and forsythoside B **3** were found for the first time in *B. scardica* and *B. bulgarica*. Martinoside appeared in relatively low concentrations and was not detected in *B. officinalis* roots. Betolide **5**, identified earlier only in *B. officinalis* roots (Tkachev *et al.*, 1987) was found in relatively high concentrations in all *Betonica* root samples, the highest in *B. bulgarica*.

In the above ground parts of the *Betonica* samples no betolide (which corresponds to earlier investigations, Tkachev *et al.*, 1987), and lower concentrations of forsythoside B were found. The flavonoid glycosides were in low concentrations and we did not succeed to identify any of them.

The polyphenol composition of *Stachys* differed from that of the investigated *Betonica* species. No betolide **5** in *Stachys* samples was found, with the exception of traces in the roots of *S. plumosa*. Besides acteoside **1** and martinoside **2**, the flavonoid glycoside **4** was identified (found earlier in *Stachys* species, El-Ansari *et al.*, 1991). Only traces of forsythoside B **3** were present. Like in *Betonica* species, almost no changes in the polyphenol composition in two consecutive years were observed. There were only some qualitative differences in the polyphenol composition between the roots and above-ground parts. In the roots of *S. plumosa* and *S. germanica* higher concentrations of **4** were found. Traces of forsythoside B in most of the *Stachys* samples and relatively high concentrations of martinoside **2** and especially acteoside **1** were shown. Their concentrations in the roots and the above-ground parts showed no significant differences.

The significant amount of betolide **5** in the roots of *Betonica*, accompanied with high concentrations of forsythoside B **3** and at the same time the absence of the characteristic for *Stachys* flavonoid glycoside **4**, could be used as taxonomic features for *Betonica*. Contrary to *Betonica*, in *Stachys* no betolide **5**, and only traces of forsythoside B **3** were found, but the flavonoid **4** was present.

Evidently the established chemical composition in the studied samples *Stachys* and *Betonica* differed considerably, which is an indication that they belong to two well separated genera. This is in agreement with the hypothesis that *Betonica* could be separated from *Stacys* as a different genus (Koeva-Todorovska, 1988; Calis *et al.*, 1992).

Acknowledgements

The authors are grateful for the partial financial support by the National Foundation for Scientific Research of Bulgaria (Contract X-513).

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