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# Glycosides of tricetin methyl ethers as chemosystematic markers in *Stachys* subgenus *Betonica*

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**Abstract**

Nine species from the genus *Stachys* L. representing subgenera *Stachys* and *Betonica* were surveyed for flavonoid glycosides by means of HPLC coupled to diode-array detection and LC-APCI-MS. Those species belonging to subgenus *Betonica* were characterised by the presence of glycosides of tricetin methyl ethers, including a new derivative, which was isolated from *S. scardica* Griseb. and identified as tricetin 3′,4′,5′-trimethyl ether 7-*O*-β-glucopyranoside by spectroscopic methods. This type of flavonoid was absent from species belonging to subgenus *Stachys* and can be considered as a chemosystematic marker for subgenus *Betonica*.


*Keywords:* *Stachys*; *Betonica*; Lamiaceae; Flavonoid glycosides; Tricetin 3′,4′,5′-trimethyl ether 7-*O*-β-glucopyranoside; Tricetin derivatives; Chemosystematics

## 1. Introduction

*Stachys* L. (Lamiaceae) is a large genus of herbs and shrubs comprising ≈300 species distributed in temperate and tropical regions of the world, with the exception of Australasia (Mabberley, 1997). The taxonomy of the genus is complicated by the wide range of variability shown by some species, and many infraspecific taxa have been described. Furthermore, taxonomic treatments of this genus have not been consistent. For example, Bentham (1848) and Koeva-Todorovska (1979) split *Stachys sensu lato* into two separate genera, *Stachys* and *Betonica* L., whereas others such as Ball (1972), in the Flora Europaea, treated *Betonica* as a section within *Stachys* at the same level as other sections of the genus. In a more recent revision, Bhattacharjee (1980) recognised *Betonica* as a separate subgenus within *Stachys* and this classification is followed here.

Characters based on the distribution of flavonoids have been used previously to resolve some taxonomic problems in *Stachys* (Lenherr et al., 1984a; Bankova et al., 1999). This approach benefits from the occurrence of several unusual types of flavonoid in the genus, as emphasised by a recent review of the chemistry of more than 25 species (Meremeti et al., 2004). For example, acetylated allosylglucosides of 8-hydroxyflavone derivatives such as isoscutellarein 7-*O*-(6‴-*O*-acetyl)-β-allopyranosyl(1‴ → 2″)-β-glucopyranoside have been found in many representatives of subgenus *Stachys* (Lenherr et al., 1984a,b; Lenherr and Mabry, 1987; Takeda et al., 1985; El-Ansari et al., 1991; Tomás-Barberán et al., 1992; Skaltsa et al., 2000; Meremeti et al., 2004). Similarly, flavonoid *p*-coumaroylglucosides occur in species of subgenus *Betonica* (Tomás-Barberán et al., 1992) and subgenus *Stachys* sections *Ambleia* (El-Ansari et al., 1991) and *Campanistrum* (Skaltsa et al., 2000). Some taxa produce highly methylated flavone and flavonol aglycones (El-Ansari et al., 1991) and the rare biflavonoid, stachysetin (diapigenin 7-glucosyl-*p*,*p*′-dihydroxytruxinate), is a constituent of *S. aegyptiaca* Person

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(El-Ansari et al., 1995). These findings prompted the present survey of the flavonoids of species of *Stachys* from the Balkan region. The hyphenated techniques of HPLC with diode array detection and liquid chromatography with atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS) were used to detect more than 20 different flavonoids, including some unusual glycosides of methyl ethers of tricetin (5,7,3′,4′,5′-pentahydroxyflavone). These included a new flavone glycoside, which was isolated from *Stachys scardica* Griseb. and identified as tricetin 3′,4′,5′-trimethyl ether 7-*O*-β-glucopyranoside. The distribution of these glycosides of tricetin methyl ethers and several other groups of flavonoid glycosides was determined for nine species belonging to the two subgenera of the genus and four different sections of the subgenera according to the classification of Bhattacharjee (1980).

## 2. Results and discussion

### 2.1. Identification of tricetin derivatives and other flavonoid glycosides

Analysis of aqueous MeOH extracts of nine species of *Stachys* using HPLC with diode array detection revealed that some contained flavonoids with UV spectra characteristic of tricetin derivatives (Table 1). LC-APCI-MS (Grayer et al., 2000) of extracts from three species belonging to subgenus *Betonica* indicated the presence of both a glucoside and a glucuronide of tricetin 3′,5′-dimethyl ether (tricin) together with glycosides of tricetin mono- and trimethyl ethers (Fig. 1). The identities of tricin 7-*O*-glucuronide (**1**) and tricin 7-*O*-glucoside (**2**) were confirmed by comparison with authentic standards. Compound **3** appeared to be a hexoside of a tricetin monomethyl ether according to MS data, and the characteristic long-wavelength band at 352 nm in the UV spectrum indicated that the methyl group was located at the 3′-OH position. Methyl ether substitution at the 4′-OH position could be excluded because this results in a hypsochromic shift of the long wavelength band from 352 to ≈330 nm (Bouaziz et al., 2001). Substitution at the 7-OH position was similarly discounted as this would require the site of glycosylation to be at either the 3′-OH or 4′-OH positions and a hypsochromic shift of the long wavelength band would be observed (Grayer and de Kok, 1998). Methylation or glycosylation at the 5-OH position was also discounted as this has a profound effect on the appearance of the UV spectrum. On this basis, compound **3** was identified as tricetin 3′-methyl ether 7-*O*-glucoside (selgin 7-*O*-glucoside). This flavonoid has been reported once before, as a constituent of the conifer *Taxodium distichum* Rich. (Geiger and de Groot-Pfleiderer, 1979), but no UV maxima were given.

Table 1
HPLC retention times, UV absorption maxima and APCI-MS data for flavonoid glycosides from *Stachys* species

| | $t_R$ (min)[a] | UV; $\lambda_{max}$ (nm) | $[M+H]^+$ (*m*/*z*)[b] | $[I+H]^+$ (*m*/*z*)[c] | $[A+H]^+$ (*m*/*z*)[d] | Identification |
|---|---|---|---|---|---|---|
| **1** | 15.0 | 248, 268sh, 352 | 507 | – | 331 | Tricin 7-*O*-glucuronide |
| **2** | 15.1 | 250, 266sh, 352 | 493 | – | 331 | Tricin 7-*O*-glucoside |
| **3** | 13.6 | 252, 265sh, 351 | 479 | – | 317 | Selgin 7-*O*-glucoside |
| **4** | 17.5 | 269, 329 | 507 | – | 345 | Tricetin 3′,4′,5′-trimethyl ether 7-*O*-glucoside |
| **5** | 12.8 | 274, 300, 343 | 465 | – | 303 | Hypolaetin 7-*O*-glucoside |
| **6** | 13.4 | 255, 267sh, 350 | 449 | – | 287 | Luteolin 7-*O*-glucoside |
| **7** | 14.4 | 275, 305, 327 | 449 | – | 287 | Isoscutellarein 7-*O*-glucoside |
| **8** | 15.0 | 266, 338 | 433 | – | 271 | Apigenin 7-*O*-glucoside |
| **9** | 15.1 | 252, 267sh, 348 | 463 | – | 301 | Chrysoeriol 7-*O*-glucoside |
| **10** | 12.7 | 274, 300, 343 | 479 | – | 303 | Hypolaetin 7-*O*-glucuronide |
| **11** | 13.2 | 255, 267sh, 350 | 463 | – | 287 | Luteolin 7-*O*-glucuronide |
| **12** | 14.6 | 274, 300, 342 | 669 | 465 | 303 | Hypolaetin 7-*O*-acetylallosylglucoside[e] |
| **13** | 15.5 | 252, 267sh, 348 | 667 | 463 | 301 | Chrysoeriol 7-*O*-acetylallosylglucoside[e] |
| **14** | 15.9 | 275, 305, 327 | 653 | 449 | 287 | Isoscutellarein 7-*O*-acetylallosylglucoside[e] |
| **15** | 16.0 | 275, 300, 340 | 683 | 479 | 317 | Hypolaetin 4′-methyl ether 7-*O*-acetylallosylglucoside[e] |
| **16** | 18.7 | 278, 306, 326 | 667 | 463 | 301 | Isoscutellarein 4′-methyl ether 7-*O*-acetylallosylglucoside[e] |
| **17** | 8.8 | 270, 335 | 595 | 577, 475, 457 | – | Apigenin 6,8-di-*C*-glucoside (vicenin-2) |
| **18** | 11.0 | 256sh, 269, 349 | 449 | 329, 299 | – | Luteolin 6-*C*-glucoside (isoorientin) |
| **19** | 12.4 | 267, 337 | 433 | 313, 283 | – | Apigenin 8-*C*-glucoside (vitexin) |
| **20** | 13.7 | 256, 355 | 611 | 465 | 303 | Quercetin 3-*O*-rutinoside |
| **21** | 15.7 | 256, 354 | 625 | 479 | 317 | Isorhamnetin 3-*O*-rutinoside |
| **22** | 19.3 | 268, 318 | 579 | – | 271 | Apigenin 7-*O*-*p*-coumaroylglucoside |

[a] For HPLC solvent system, see Section 3.1.
[b] APCI-MS (positive mode) data for the protonated molecular ion.
[c] APCI-MS (positive mode) data for other protonated fragment ions.
[d] APCI-MS (positive mode) data for the protonated aglycone ion.
[e] See Section 3.3.

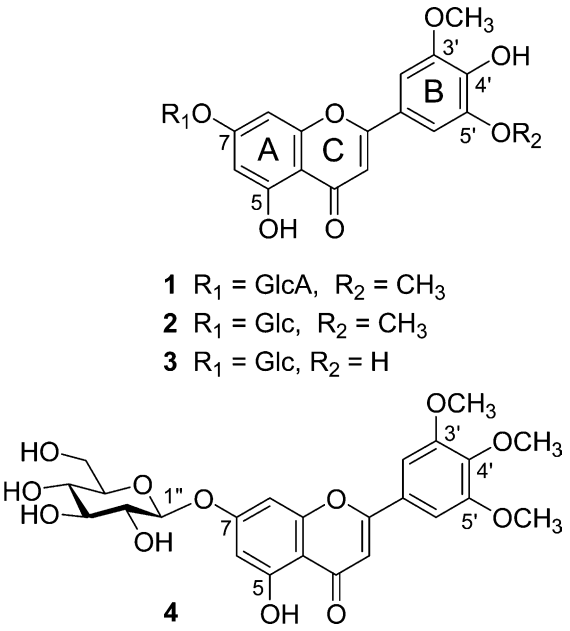


Fig. 1. Tricetin methyl ether glycosides from *Stachys* subgenus *Betonica*.

Table 2
$^1$H and $^{13}$C NMR chemical shift data for tricetin 3′,4′,5′-trimethyl ether 7-*O*-β-glucopyranoside (**4**) ($\delta$ in DMSO-$d_6$ at 37 °C)

| | Atom | Assignment | | |
|---|---|---|---|---|
| | | $\delta$ $^1$H | $\delta$ $^1$H (OH) | $\delta$ $^{13}$C |
| | 2 | | | 163.4 |
| | 3 | 7.13 *s* | | 105.2 |
| | 4 | | | 182.1 |
| | 5 | | 12.81 *br s* | 161.0 |
| | 6 | 6.49 *d* (2.1) | | 99.6 |
| | 7 | | | 163.1 |
| | 8 | 6.94 *d* (2.1) | | 95.4 |
| | 9 | | | 156.9 |
| | 10 | | | 105.4 |
| | 1′ | | | 125.7 |
| | 2′, 6′ | 7.37 *s* | | 104.4 |
| | 3′, 5′ | | | 153.2 |
| | 4′ | | | 141.0 |
| | 3′, 5′-OCH$_3$ | 3.91 *s* | | 56.3 |
| | 4′-OCH$_3$ | 3.77 *s* | | 60.1 |
| Glc | 1″ | 5.05 *d* (7.3) | | 100.2 |
| | 2″ | 3.29 *m* | 5.32 *d* (4.8) | 73.1 |
| | 3″ | 3.32 *m* | 5.04 *d* (4.6) | 76.4 |
| | 4″ | 3.17 *m* | 4.99 *d* (5.3) | 69.6 |
| | 5″ | 3.46 *m* | | 77.3 |
| | 6″ | 3.74 *m* | 4.57 *t* (5.6) | 60.6 |
| | | 3.48 *m* | | |

Analysis of the UV and APCI-MS spectra of the major flavonoid **4** suggested that it was a glycoside of a tricetin trimethyl ether with a substituted 4′-hydroxyl group (long wavelength band at 329 nm) and a single hexose residue (Table 1). The compound was isolated from *S. scardica* and its structure and molecular formula determined by NMR spectroscopy and high-resolution mass spectrometry, respectively. Complete $^1$H and $^{13}$C NMR resonance assignments for **4** were made on the basis of 1D $^1$H, 1D $^{13}$C, DQF-COSY, HSQC and HMBC experiments (Table 2). The assignment of a characteristic singlet resonance at $\delta_H$ 7.13 in the $^1$H NMR spectrum (1H, $\delta_C$ 105.2 by HSQC) to H-3 of a flavone was confirmed by the corresponding long-range connectivities to $\delta_C$ 182.1 (C-4), 163.4 (C-2), 125.7 (C-1′) and 105.4 (C-10) in the HMBC spectrum. Two *meta*-coupled protons at $\delta_H$ 6.49 (1H, $d, J = 2.1$ Hz, $\delta_C$ 99.6) and 6.94 (1H, $d, J = 2.1$ Hz, $\delta_C$ 95.4) were assigned to H-6 and H-8, respectively, of the flavone A-ring. Long-range connectivities were observed from H-6 to $\delta_C$ 163.1 (C-7), 161.0 (C-5) and 105.4 (C-10) and from H-8 to $\delta_C$ 163.1 (C-7), 156.9 (C-9) and 105.4 (C-10), as expected. A broad exchangeable singlet with a significant downfield shift to $\delta_H$ 12.81 was assigned to the 5-OH proton. The remaining aromatic resonance, a 2H singlet at $\delta_H$ 7.37 was assigned to H-2′,6′ of the flavone B-ring. Long-range connectivities from these protons to methoxyl-bearing carbons at $\delta_C$ 153.2 (C-3′,5′) and 141.0 (C-4′) and to 163.4 (C-2) and 125.7 (C-1′) were observed in the HMBC spectrum. The corresponding resonances for the methoxyl protons were found at $\delta_H$ 3.91 (6H, s, $\delta_C$ 56.3, 3′,5′-OCH$_3$) and 3.77 (3H, s, $\delta_C$ 60.1, 4′-OCH$_3$) in the 1D $^1$H NMR spectrum. These data confirmed that the aglycone was 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone (tricetin 3′,4′,5′-trimethyl ether), a conclusion consistent with the protonated aglycone $[A + H]^+$ ion at *m*/*z* 345 detected by first-order APCI-MS (positive mode) of **4** (Table 1).

The anomeric proton resonance at $\delta_H$ 5.05 (1H, $d, J = 7.3$ Hz, $\delta_C$ 100.2) in the $^1$H NMR spectrum of **4** was used as a starting point for the assignment of the remaining sugar protons by 2D NMR techniques (Table 2). This also allowed the exchangeable hydroxyl proton resonances of 2″-OH, 3″-OH, 4″-OH and 6″-OH to be assigned from the DQF-COSY spectrum. NOE connectivities observed between the anomeric proton and both H-6 and H-8 indicated that the site of glycosylation was at C-7. The $^3J_{H-1'',H-2''}$ coupling constant of 7.3 Hz and chemical shift data for the $^1$H and $^{13}$C resonances indicated that the sugar residue was an *O*-linked β-glucopyranoside. The molecular formula of **4** was determined to be $C_{24}H_{26}O_{12}$ by high-resolution MS (see Section 3.5). Thus this compound was identified as tricetin 3′,4′,5′-trimethyl ether 7-*O*-β-glucopyranoside (Fig. 1), a flavone glycoside that is new as a plant constituent but which has been recorded previously as a synthetic derivative (Lardy et al., 1983). No spectroscopic data were presented for the synthetic glycoside in the earlier report.

In addition to the tricetin derivatives **1**–**4**, 18 known flavonoid glycosides (**5**–**22**) were characterised in the

same *Stachys* extracts on the basis of their UV spectra (Upson et al., 2000), APCI-mass spectra (Grayer et al., 2000) and HPLC retention times. These data are presented in Table 1.

### 2.2. Distribution of tricetin derivatives and other flavonoids in Stachys

The distribution of flavonoid glycosides **1**–**22** in nine species of *Stachys* belonging to four different sections of the subgenera *Betonica* and *Stachys sensu* Bhattacharjee (1980), including the Balkan endemics *S. scardica, S. anisochila* Vis. et Panc. and *S. plumosa* Griseb., is given in Table 3. Here the compounds are arranged in six different groups, (i), glycosides of tricetin methyl ethers (**1**–**4**), (ii), other flavone 7-*O*-glucosides and glucuronides (**5**–**11**), (iii), flavone 7-*O*-acetylallosylglucosides (**12**–**16**), (iv), flavone *C*-glycosides (**17**–**19**), (v), flavonol 3-*O*-glycosides (**20**, **21**), and (vi), apigenin 7-*O*-*p*-coumaroylglucoside (**22**). The tricetin derivatives were present in all three species investigated from the two sections of subgenus *Betonica* (*S. officinalis* (L.) Trev., *S. scardica* and *S. alopecuros* (L.) Benth.), but absent from all species of subgenus *Stachys* sections *Eriostomum* and *Stachys*. The flavone 7-*O*-glucosides **5**–**9** were more common in species of subgenus *Betonica* than subgenus *Stachys* while flavone 7-*O*-glucuronides were restricted to subgenus *Betonica*. In contrast, flavone 7-*O*-acetylallosylglucosides occurred only in species of subgenus *Stachys*. The flavonol 3-*O*-glycosides **20** and **21** and the flavone *C*-glycoside vicenin-2 (**17**) were only detected in one species (*S. palustris* L.) of subgenus *Stachys* whereas the flavone *C*-glycosides isoorientin and vitexin were restricted to two species of subgenus *Betonica*. Apigenin 7-*O*-*p*-coumaroylglucoside (**22**) was detected in all the species surveyed with the exception of *S. alpina* L. The results of previous surveys of *Stachys* flavonoids indicate that the presence of flavone acetylallosylglucosides is a chemical character for subgenus *Stachys* (Tomás-Barberán et al., 1992; Bankova et al., 1999), a hypothesis supported by the present results. The suggestion that flavonoid 7-*O*-*p*-coumaroylglucosides are characteristic constituents of subgenus *Betonica* (Tomás-Barberán et al., 1992) was not confirmed. Glycosides of tricetin methyl ethers have not been recorded previously as constituents of *Stachys* despite the fact that the chemistry of many species (especially from subgenus *Stachys*) has been investigated extensively. The aglycone tricetin 3′,5′-dimethyl ether (tricin) has been noted as a constituent of *S. officinalis* (Kobzar and Nikonov, 1986), which belongs to subgenus *Betonica*. The latter report and the present survey indicate that glycosides and aglycones of tricetin methyl ethers are restricted to subgenus *Betonica* and could therefore be useful chemosystematic markers for this taxon.

The results of our survey and previous studies on the distribution of flavonoid glycosides in *Stachys* support the classifications given by authors who recognize *Betonica* and *Stachys* as separate genera or subgenera (Bentham, 1848; Koeva-Todorovska, 1979; Bhattacharjee, 1980) rather than classifications where these groups are treated as separate sections with an equal rank to the other sections, as proposed by others (Boissier, 1879; Briquet, 1897; Ball, 1972). According to Bhattacharjee (1980), the classification of *Betonica* at the same sectional level as other sections within the genus *Stachys* is unsatisfactory, since the morphological and anatomical gap between *Betonica* and *Stachys sensu stricto* is of a different order to those distinguishing the sections of the latter. *Betonica* is characterized by sessile flowers, whereas they are pedicellate in *Stachys*. Regarding anatomical features, Bhattacharjee (1980) noted that all representatives of the subgenus *Betonica* show the presence of adaxial phloem in the petiole and nutlets without pitted thickening in the sclerenchyma, whereas these features were not found in any species investigated from the subgenus *Stachys*. Indeed, Bankova et al. (1999) later suggested that *Betonica* should be separated from *Stachys* as a separate genus on the basis of different karyotypes and pollen and differences in chemical profiles (quantitative differences in phenylethanoid glycosides, 8-hydroxyflavone glycosides and diterpenoids). The distinction between *Betonica* and *Stachys* on the basis of the presence or absence of tricetin derivatives provides another character that supports this viewpoint.

## 3. Experimental

### 3.1. General

$^{1}$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) spectra were recorded in DMSO-$d_6$, with the solvent resonances used as internal references at $\delta$ 2.50 and 39.5, respectively. High-resolution ESIMS (positive mode) were obtained on a Bruker Apex II instrument (with an internal calibrant). Positive ion first-order APCI-MS were obtained with a quadrupole ion-trap instrument (Finnigan LCQ) using a vaporiser temperature of 550 °C, sheath and auxiliary nitrogen gas pressures of 80 and 20 psi, a needle current of 5 μA, and a heated capillary temp. of 150 °C. Samples were introduced by direct infusion, or via an HPLC fitted with a Merck (Darmstadt, Germany) LiChrospher 100RP-18 (250 × 4.0 mm i.d.; 5 μm particle size) column using a 20 min linear gradient of 25–100% MeOH in 1% aq. HOAc at 1 ml/min. The system for analytical and semi-preparative HPLC consisted of a Waters LC600 pump and a 996 photodiode array detector. For analytical HPLC, the column and gradient program were as described for LC-MS described above, but the eluting solvents were

Table 3
Distribution of tricetin derivatives and other flavonoid glycosides in *Stachys* species

| Taxon | Site of collection in Serbia (lat./long.) | Site altitude (m) | Voucher number | 7-Glucosides and glucuronides of tricetin methyl ethers | Other flavone 7-glucosides and glucuronides | Flavone 7-acetyl-allosyl-glucosides | Flavone *C*-glycosides | Flavonol 3-glycosides | Flavone *p*-coumaroyl glycosides |
|---|---|---|---|---|---|---|---|---|---|
| Subgenus Betonica | | | | | | | | | |
| Sect. Betonica | | | | | | | | | |
| *S. officinalis* (L.) Trev. | Zlatibor mountain, Partizanske vode (43°43′ N; 19°42′ E) | 979 | SOF 8736 | **1**, **2**, **4** | **11** | – | **18**, **19** | – | **22** |
| *S. alopecuros* (L.) Benth. | Mokra gora mountain, Pogled (42°49′ N; 20°22′ E) | 2047 | SAL 8740 | **1**, **2**, **3** | **5**–**7**, **9**–**11** | – | – | – | **22** |
| Sect. Macrostachya | | | | | | | | | |
| *S. scardica* Griseb. | Sveta Petka, village (42°23′ N; 21°53′ E) | 459 | SSC 8700 | **1**, **2**, **3**, **4** | **5**, **6** | – | **18**, **19** | – | **22** |
| Subgenus Stachys | | | | | | | | | |
| Sect. Eriostomum | | | | | | | | | |
| *S. germanica* L. | Nis, Sicevacka gorge (43°19′ N; 22°03′ E) | 384 | SGE 8741 | – | **8** | **13**, **15** | – | – | **22** |
| *S. alpina* L. | Kopaonik, Brzece (43°18′ N; 20°52′ E) | 1153 | SAP 8742 | – | – | **12**, **14**, **15** | – | – | – |
| Sect. Stachys | | | | | | | | | |
| *S. sylvatica* L. | Belgrade, Kosutnjak (44°46′ N; 20°26′ E) | 196 | SSY 8737 | – | – | **13** | – | – | **22** |
| *S. anisochila* Vis. et Panc. | Medvednik mountain (44°12′ N; 19°38′ E) | 822 | SAN 8701 | – | – | **14**, **16** | – | – | **22** |
| *S. plumosa* Griseb. | Lukovo village (42°29′ N; 21°59′ E) | 657 | SPL 8739 | – | **6**, **8** | **13**, **14** | – | – | **22** |
| *S. palustris* L. | Belgrade, Makis (44°46′ N; 20°24′ E) | 73 | SPA 8738 | – | – | – | **17** | **20**, **21** | **22** |

2% aq. HOAc and MeOH:HOAc:$H_2O$ (18:1:1). An identical LiChrospher column but with 10 mm i.d. was used for semi-preparative HPLC with $H_2O$ (A) and MeOH (B) as solvents. A linear gradient, starting with 50% B and changing to 60% B over 15 min, followed by isocratic elution with 100% B for 5 min was used. The column temperature was maintained at 30 °C for both analytical and semi-preparative HPLC.

### 3.2. Plant material

Plant species were collected in the Balkan peninsula from natural habitats. Details of collection sites and voucher specimen numbers are summarised in Table 3. All *Stachys* species investigated in this work were identified and authenticated by P.D.M. Voucher specimens are deposited at the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade.

### 3.3. Extraction and identification of flavonoid glycosides

Air dried leaves (0.5 g) of each *Stachys* species were crushed and boiled for ≈2 min in 10 ml 80% aq. MeOH, and then extracted at room temperature overnight. After filtration the solvent was removed by rotary evaporation. The dried extracts were redissolved in 1 ml of 80% aq. MeOH and 20 μl of each was analyzed by HPLC with diode array detection (DAD). Most species were found to contain high concentrations of caffeoylphenylethanoid glycosides such as acteoside (Nishimura et al., 1991), which obscured the flavonoid peaks in the chromatograms. For this reason each extract was separated further into flavonoid- and phenylethanoid glycoside-containing fractions by preparative PC with $H_2O$ as solvent. This procedure effectively separated flavonoid glycosides (low to medium $R_f$) from phenylethanoid glycosides (high $R_f$) and also flavonoid glucosides (low $R_f$) and glucuronides (medium $R_f$) of the same aglycone (which can be difficult to achieve by HPLC). The constituents of the flavonoid fractions (**5**–**22**) were identified from their UV and APCI-MS spectra, their HPLC retention times, and by comparison with authentic standards (**17**–**19**). Some flavonoid glycosides were identified only to type for the purposes of chemosystematic comparison, for example the site of attachment of the acyl group of apigenin 7-*O*-*p*-coumaroylglucoside (**22**) was not determined, although both 7-*O*-(3″-*O*-*p*-coumaroyl)- and 7-*O*-(6″-*O*-*p*-coumaroyl)glucosides of apigenin have been found previously in *Stachys* (El-Ansari et al., 1995). Compounds **12**–**16** were identified to type as flavone 7-*O*-acetyldihexosides from their UV and APCI-MS spectra. These are likely to be the flavone 7-*O*-acetylallosylglucosides that are well-known constituents of many species of *Stachys* (Meremeti et al., 2004).

### 3.4. Extraction and isolation of **4**

An extract of *S. scardica* was prepared by the method described in Section 3.3, but with 30 g of air-dried aerial parts of the plant and 300 ml of 80% MeOH. A preliminary purification was carried out with preparative paper chromatography (Whatman No. 3 paper) using $H_2O$ as solvent. The fraction containing compound **4** ($R_f$ value of 0.27) was further purified by semi-preparative HPLC to obtain 1 mg of sample for spectroscopic analysis.

### 3.5. Tricetin 3′,4′,5′-trimethyl ether 7-*O*-β-glucopyranoside (**4**)

Yellow solid (MeOH); UV: see Table 1; $^1H$ and $^{13}C$ NMR: see Table 2; APCI-MS (positive mode): see Table 1; HRESIMS *m/z*: 507.1493 $[M+H]^+$ (calc. for $C_{24}H_{27}O_{12}$, 507.1497).

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