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Metabolic Characterization of Four Members of the Genus *Stachys* L. (Lamiaceae)

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Abstract: Several members of *Stachys* L. (among the largest Lamiaceae genera) have been traditionally used as medicinal plants. With 54 *Stachys* taxa (species and subspecies) occurring in mainland and/or insular Greece, the present study aimed to investigate the metabolic profiling of four range-restricted local *Stachys* members: *Stachys candida* and *S. chrysantha* (protected and endangered local Greek endemics), *S. leucoglossa* subsp. *leucoglossa* (local Balkan endemic), and *S. spinulosa* (local Balkan subendemic). In this investigation, the infusions of their above-ground parts were characterized using NMR and HPLC-PDA-MS techniques. Thus, 1D- and 2D-NMR spectra were obtained to compare the chemical fingerprints of these plants. Furthermore, previously isolated compounds from *Stachys* spp. were used to identify specific constituents. NMR screening revealed the presence of: (i) phenylethanoid glycosides, mainly acteoside in *S. candida* and *S. chrysantha* (section *Candida*, *Swainsoniana* phyloclade), and (ii) flavone 7-*O*-allosylglucoside (isoscutellarein 7-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl]-(1→2)-β-D-glucopyranoside) and iridoids (monomelittoside or/and melittoside) in *S. leucoglossa* subsp. *leucoglossa* (section *Olisia*, *Swainsoniana/Olisia* phyloclade, *Swainsoniana* phyloclade) and caffeoylquinic acid (chlorogenic acid) in *S. spinulosa* (section *Campanistrum*, *Stachys* phyloclade). In total, 26 compounds were detected by HPLC-PDA-MS belonging to flavonoids, phenylethanoid glycosides, and phenolic acids. Among them, chlorogenic acid was identified in all samples as one of their main metabolites. The present study complements previous studies with first reports of constituents detected in the studied taxa, reports for the first time on the metabolic characterization of *S. spinulosa*, and discusses the chemotaxonomic significance of such findings.

Keywords: *Stachys spinulosa*; infusions; metabolic profiling; NMR; HPLC-PDA-MS; Greece

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1. Introduction

The genus *Stachys* L. (Lamiaceae family) comprises about 365 species, which are mainly distributed in the northern hemisphere [1–3] and are arranged in 19 sections [4] or different phyloclades [2]. In Greece, 54 species and subspecies of *Stachys* are distributed in parts of the mainland and/or insular country [5], and 41% of them are local endemics confined to Greece, while 28% are Balkan endemics or subendemics extending to Turkey and/or Italy.

Several *Stachys* taxa (species and subspecies) are used as infusions and decoctions in folk medicine, and their intended uses mainly concern the treatment of infections, the common cold, gastrointestinal disorders, inflammation, skin diseases, and wounds or as a remedy for asthma and anxiety implications [6]. Many of these folk uses (and related ancient or old recipes) date back to Dioscorides times in ancient Greece and Rome, traditional Chinese medicine, or Middle Eastern (Iranian and Turkish) folk phytotherapy [6]. Previous

phytochemical investigations of *Stachys* spp. have reported several constituents belonging to different chemical categories, including terpenoids, iridoids, phytosterols, polyphenols (e.g., flavonoids, phenylethanoid glycosides, and phenolic acids), polysaccharides, and others [6,7]. Many *Stachys* taxa, as well as their isolated compounds, are known to exhibit diverse pharmacological properties, such as antioxidant, anti-inflammatory, anti-diabetic, anti-microbial, anti-proliferative, and cytotoxic activities, among others [6,7].

In our continuing endeavor to explore and document in phytochemical terms the different *Stachys* taxa growing in Greece [6], we have performed a chemical investigation herein in four members of the genus *Stachys* without bracteoles (or minute bracteoles), namely *S. candida* Bory & Chaub., *S. chrysantha* Boiss. & Heldr., *S. leucoglossa* Griseb. subsp. *leucoglossa*, and *S. spinulosa* Sm. More specifically, we focused on: (i) the endangered *S. candida* and *S. chrysantha* [8], which are local Greek (Peloponnese) endemics protected by the Greek Presidential Decree 67/1981 that belong to the section *Candida* [3,5] or the phyloclade *Swainsoniana* [2], with the first being an unarmed perennial with orbicular to ovate–orbicular leaves, calyx teeth longer than wide, and white corolla with purple spots, with the other being a non–spiny perennial with elliptic–ovate to suborbicular leaves and yellow tomentose corolla; (ii) the range-restricted local Balkan endemic *S. leucoglossa* subsp. *leucoglossa* belonging to the section *Olisia* or the *Swainsoniana/Olisia* phyloclade [2], an unarmed perennial with white or pale pink corolla in remote 2–6-flowered verticillasters with an almost glabrous calyx and very small floral leaves; and (iii) the local Balkan subendemic *S. spinulosa* which belongs to the section *Campanistrum* [3,5] or the *Stachys* phyloclade [2], a hispid annual with ± scabrid stem on angles, spiny bracts and crowded verticillasters in dense spikes, and coarsely spined cauline leaves, and lower leaves cordate at the base.

S. candida and *S. chrysantha* have been previously studied regarding their phytochemical composition, as well as the anti-inflammatory activity of their methanol extracts and their flavonoids [6]. Specifically, xanthomicrol, chrysoeriol, calycopterin, chrysoeriol-7-*O*-β-D-(3″-E-p-coumaroyl)-glucopyranoside, chrysoeriol-7-*O*-β-D-glucopyranoside, and isoscutellarein-7-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl]-(1→2)-6″-*O*-acetyl-β-D-glucopyra noside were isolated from both plants, while luteolin-7-*O*-β-D-glucopyranoside was found only in *S. chrysantha* methanol extract. Additionally reported in *S. candida* were: four flavonoids (apigenin-7-*O*-β-D-glucopyranoside, isoscutellarein-7-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl-(1→2)]-β-D-glucopyranoside, 4′-methyl-isoscutellarein-7-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl-(1→2)]-β-D-glucopyranoside, and 4′-methyl-hypolaetin-7-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl-(1→2)]-β-D-glucopyranoside), one phenylethanoid glycoside (acteoside), and one phenolic acid (chlorogenic acid). However, *S. leucoglossa* (not determined by subspecies) has been chemically characterized only once regarding its content in flavonoids (namely, isoscutellarein-7-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl-(1→2)]-β-D-glucopyranoside, 4′-methyl-isoscutellarein-7-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl-(1→2)]-β-D-glucopyranoside, and 4′-*O*-methyl-hypolaetin-7-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl-(1→2)]-β-D-glucopyranoside) and iridoids (i.e., melittoside, harpagide, acetyl-harpagide, and ajugol) [9]. Nonetheless, *S. spinulosa* has not been subjected to any phytochemical study to date. Therefore, the present study aimed to investigate the metabolic profiles of the infusions of these four Greek *Stachys* taxa through NMR and HPLC-PDA-MS analyses.

2. Materials and Methods

2.1. Plant Material

The flowering above-ground parts of the studied plant species and subspecies were collected from wild-growing populations in different locations in Greece (Figure S1), and the living material was maintained ex situ at the premises of the Institute of Plant Breeding and Genetic Resources, Agricultural Organization Demeter (Table 1). Voucher specimens were identified by Dr. Nikos Krigas and deposited in the Herbarium of the Balkan Botanic Garden of Kroussia (BBGK), Institute of Plant Breeding and Genetic Resources, Agricultural Organization Demeter.

Table 1. Investigated *Stachys* taxa (species and subspecies) with abbreviations used, sample origin, date of collections, and living or voucher specimen.

Species	Abbreviation	Sample Origin	Date	Living and/or Voucher Specimen
S. candida	SCA	Mt. Taygetos	29/5/2020	GR-1-BBGK-20,164-A
S. chrysantha	SCH	Mt. Taygetos	28/2/2020	GR-1-BBGK-20,96
S. leucoglossa subsp. *leucoglossa*	SLE	Mt. Karpouzi	13/6/2020	GR-1-BBGK-20,97
S. spinulosa	SSP	Sparta	29/5/2020	GR-1-BBGK-20,164-B

2.2. Preparation of the Infusions

Precisely 4.0 g of dried comminuted aerial parts of each plant sample was added to 200 mL of boiled water for 5 min, separately. Due to the fact that *Stachys* infusions in Greece have been traditionally prepared and consumed as 'mountain tea', referring to different *Sideritis* spp., the preparation of the infusions was based on the monograph of the European Medicines Agency (EMA) concerning different species and subspecies of the genus *Sideritis* [10]. Then, the samples were filtered and concentrated to dryness using a rota evaporator under reduced pressure to yield residues of 2.3 g for *S. candida*, 2.8 g for *S. chrysantha*, 2.0 g for *S. leucoglossa* subsp. *leucoglossa*, and 1.8 g for *S. spinulosa*. Distilled water was used as a solvent for the infusions, avoiding any additive/impurity in the samples.

2.3. NMR Analysis

In the NMR experiments, a part of each sample (5.0 mg) was dissolved in 600 μL of CD_3OD. The 1D- and 2D-NMR spectra of the samples were recorded on a Bruker 400 DRX instrument at 300 K. Chemical shifts are given in ppm (δ) and are referenced to the solvent signal at 3.31/ 49.0 ppm (CD_3OD) for ^{1}H- and ^{13}C-NMR, respectively. COSY (COrrelation SpectroscopϒY) and HSQC (Heteronuclear Single Quantum Correlation) experiments were performed using standard Bruker microprograms.

2.4. HPLC-PDA-MS Analysis

HPLC-PDA-MS analysis was performed on a Thermo Finnigan system (Palo Alto, CA, USA), which consisted of an LC Pump Plus, Autosampler, and Surveyor PDA Plus Detector. The HPLC system was interfaced with an ESI MSQ Plus (Thermo Finnigan, San Jose, CA, USA) operating with Xcalibur software (version 2.1). The mass spectrometer operated in negative and positive ionization modes; the scan spectra were from m/z 100 to 1000, the gas temperature was at 350 °C, the nitrogen flow rate was at 10 L/min, and the capillary voltage was 3000 V. The cone voltage was in the range of 60–110 V. The column was an SB-Aq Zorbax (Agilent, Santa Clara, CA, USA) RP-C18 column (150 mm × 3.5 mm) with a particle size of 5 μm maintained at 30 °C. The eluents were H_2O at pH 2.8 by formic acid (0.05% v/v) (A) and acetonitrile (B) and with a flow rate of 0.4 mL/min. The gradient program was as follows: 0–7 min, 90–85%A; 7–12 min, 85–82%A; 12–25 82%A; 25–27 min, 82–75%A; 27–32 min, 75%A; 32–42 min, 75–60%A; 42–49 min, 60%A; 49–53 min, 60–90%A; 53–60 min, 90%. The injected volume of the samples was 5 μL of solution. The UV-vis spectra were recorded between 220 and 600 nm, and the chromatographic profiles were registered at 280, 330, and 350 nm.

3. Results and Discussion

The present study reports on the chemical fingerprints of the infusions of four *Stachys* taxa (*S. candida*, *S. chrysantha*, *S. leucoglossa* subsp. *leucoglossa*, and *S. spinulosa*) growing wild in Greece by means of NMR and HPLC-PDA-MS techniques. In general, LC–MS (Liquid Chromatography-Mass Spectrometry) and NMR (Nuclear Magnetic Resonance) are commonly used techniques for metabolic characterization in plants. By employing both techniques, the qualitative and quantitative strategy can be considerably improved, rendering the identification of plant extracts' constituents feasible [11].

3.1. NMR Analysis

A preliminary screening of the *Stachys* spp. infusions was first obtained by ^{1}H-NMR spectra, and the chemical categories of their constituents were identified based on peaks in specific regions. The comparative 1D-NMR fingerprints are presented in Figure 1. In addition, 2D-NMR spectra (COSY and HSQC) were acquired to provide a better overview (Figures S1–S5). In all four ^{1}H-NMR spectra, signals of polysaccharides (region: 5.40–3.10 ppm) were noticed. However, some differences in the chemical fingerprints were observed among the samples.

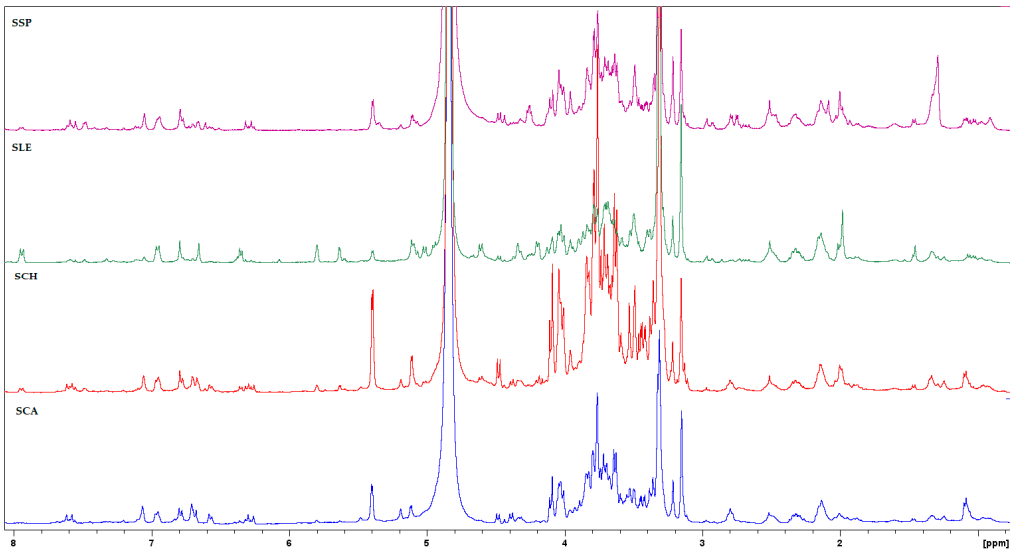

Figure 1. Overlaid ^{1}H-NMR spectra of the four investigated *Stachys* infusions in the range of δ_H 8.00–0.90: *Stachys candida* (SCA, blue color), *Stachys chrysantha* (SCH, red color), *Stachys leucoglossa* subsp. *leucoglossa* (SLE, green color), and *Stachys spinulosa* (SSP, purple color).

In the ^{1}H-NMR spectra of *S. candida* (SCA) and *S. chrysantha* (SCH) belonging to the section *Candida* or the *Swainsoniana* phyloclade, mainly signals from phenylethanoid glycosides were detected (Figure 2A,B). Specifically, at δ_H 7.60 (d, J = 16.0 Hz) and 6.28 (d, J = 16.0 Hz), we found *trans*-coupling olefinic signals ascribable to double bonds (HSQC: δc 146.8 and 113.5, respectively), at the δ_H range of 7.07–6.57, we observed signals which could belong to protons of aromatic tri-substituted rings, and at δ_H around 2.80, we spotted signals of benzylic methylene protons (HSQC: δc 35.0). In the ^{1}H-^{1}H-COSY spectra of SCA and SCH, the principal correlation peaks among protons corresponding to phenylethanoid glycosides were also detected (Figures S2a and S3a). Their HSQC spectra are presented in Figures S2b and S3b. As an effort to interpret the observed different signals in the 1D-/2D-NMR spectra of SCA and SCH infusions, we compared them with the NMR spectra of previously isolated compounds sourced from our own previous works in *Stachys* spp. Through the careful screening of the ^{1}H-NMR spectra of different compounds, we noticed that the main proton signals in the spectra of *S. candida* and *S. chrysantha* infusions could be attributed to the phenylethanoid glycoside, namely acteoside. The overlaid ^{1}H-NMR spectra of both infusions and acteoside are illustrated in Figure 2. Acteoside has been previously found in several *Stachys* taxa [6,7], including *S. candida* [6]. Furthermore, the presence of flavones has been previously reported from members of the genus *Stachys* [6,7]. In *Stachys* species of the section *Candida*, chrysoeriol and isoscutellarein derivatives have been found [6]. It should be mentioned that minor signals of isoscutellarein derivatives were also detected in the SCH sample. However, it was not feasible to identify these constituents with previously isolated compounds in the NMR spectra due to signal overlapping and their low concentration.

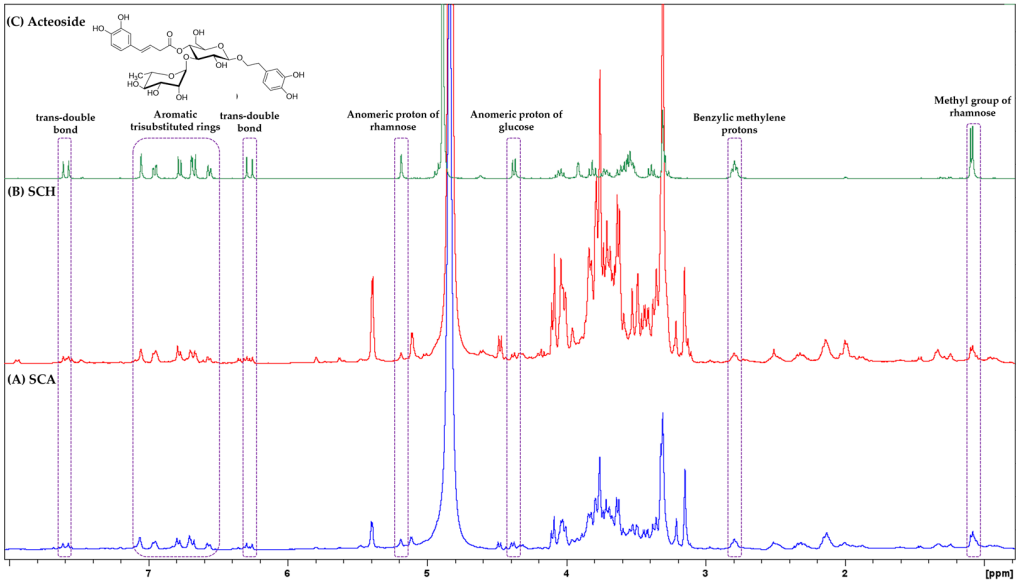

Figure 2. Overlaid ^{1}H-NMR spectra of (**A**) *Stachys candida* (SCA, blue color), (**B**) *Stachys chrysantha* (SCH, red color) infusions, and (**C**) acteoside (green color). Specific signals are indicated by purple boxes.

In the ^{1}H-NMR spectrum of *S. leucoglossa* subsp. *leucoglossa* (SLE) belonging to the section *Olisia* or the *Swainsoniana/Olisia* phyloclade, mainly signals from flavonoids and iridoids were detected (Figures 3 and 4). In the downfield region (δ_H 7.95–6.34), signals of flavones [δ_H: 7.94 (d, J = 8.7 Hz), 6.96 (d, J = 8.7 Hz), 6.79 (s), 6.66 (s)] were observed (Figure 3). In addition, a double peak (J = 6.5 Hz) appeared at δ_H 6.35. This assignment, along with the specific signals at δ_H 5.80 (s), 5.63 (d), and 5.10 (d), could belong to iridoids (Figure 4). In the ^{1}H-^{1}H-COSY spectrum, the principal correlation peaks among vicinal protons corresponding to flavones and iridoids were also detected (Figure S4a). The HSQC spectrum of SLE is presented in Figure S4b. By carefully screening the ^{1}H-NMR spectra of the SLE infusion with those of previously isolated compounds of *Stachys* spp., we noticed that the main proton signals in the spectrum of this infusion could be assigned to isoscutellarein derivatives, namely isoscutellarein-7-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl]-(1→2)-β-D-glucopyranoside, as well as to monomelittoside or/and its 5-glucoside derivative, namely melittoside (Figures 3 and 4). The presence of flavone 7-*O*-allosylglucosides has been previously reported from members of the genus *Stachys* [6,7]. In the *Stachys* species of the section *Olisia*, monoacetyl and diacetyl derivatives of isoscutellarein have been found, including reports of *S. leucoglossa* [6,9]. Furthermore, iridoids are known to be among the main metabolites in *Stachys* spp. [6,7], and previous studies have reported their presence in the members of the section *Olisia*, such as *S. recta* L. and *S. spinosa* L. [12,13], while melittoside has been found in *S. leucoglossa* [9].

In the ^{1}H-NMR spectrum of *S. spinulosa* (SSP) belonging to the section *Campanistrum* or the *Stachys* phyloclade, mainly signals from caffeoylquinic acid derivatives were detected (Figure 5). Specifically, at δ_H 7.58 (d, J = 16.2 Hz) and 6.28 (d, J = 16.2 Hz), we found *trans*-coupling olefinic signals ascribable to double bonds (HSQC: δc 147.0 and 115.7, respectively), and at the δ_H range of 7.05–6.77, the observed signals could belong to protons of aromatic tri-substituted rings. Although the signal overlapped in the middle area of the ^{1}H-NMR spectrum, signals of the oxymethine protons of the quinic acid moiety were spotted at δ_H 5.36 (HSQC: 72.3), 4.10 (HSQC: 73.0), and 3.67 (HSQC: 74.5), with its methylene protons also appearing at the δ_H range of around 2.15–1.95 (HSQC: δc 40.5). In the ^{1}H-^{1}H-COSY spectrum, the principal correlation peaks among protons of the caffeoyl and quinic acid moieties were detected (Figure S5a). The HSQC spectrum of SSP is presented in Figure S5b. By carefully screening the ^{1}H-NMR spectra of the SLE infusion with those of previously isolated compounds of *Stachys* spp., we noticed that the main proton signals in

the spectrum of this infusion could be assigned to chlorogenic acid. The overlaid ^{1}H-NMR spectra of the SSP infusion and this compound are illustrated in Figure 5. Caffeoylquinic acids, especially chlorogenic acid, have been found in many *Stachys* species [6]. Moreover, flavones have been previously reported from two *Stachys* species belonging to the section *Campanistrum* [14,15]. Even though minor signals of flavones and other derivatives were also detected in the SSP sample, we were not able to identify specific constituents with previously isolated compounds in the NMR spectra due to signal overlapping and their low concentration. It should be mentioned that this is the first study addressing the phytochemical characterization of *S. spinulosa*.

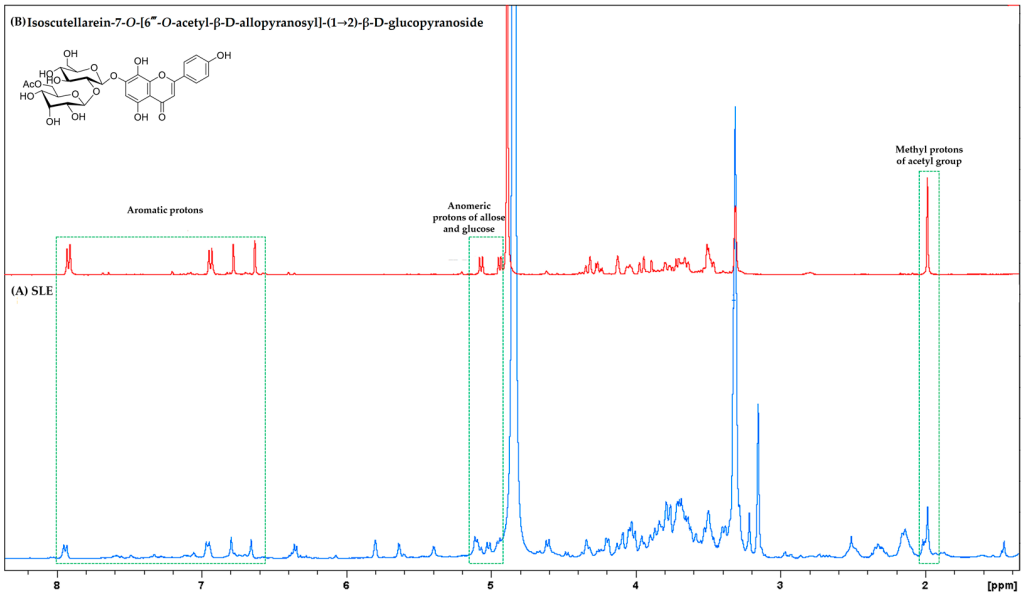

Figure 3. Overlaid ^{1}H-NMR spectra of (**A**) *Stachys leucoglossa* subsp. *leucoglossa* infusion (SLE, blue color) and (**B**) isoscutellarein-7-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl]-(1→2)-β-D-glucopyranoside (red color). Specific signals are indicated by green boxes.

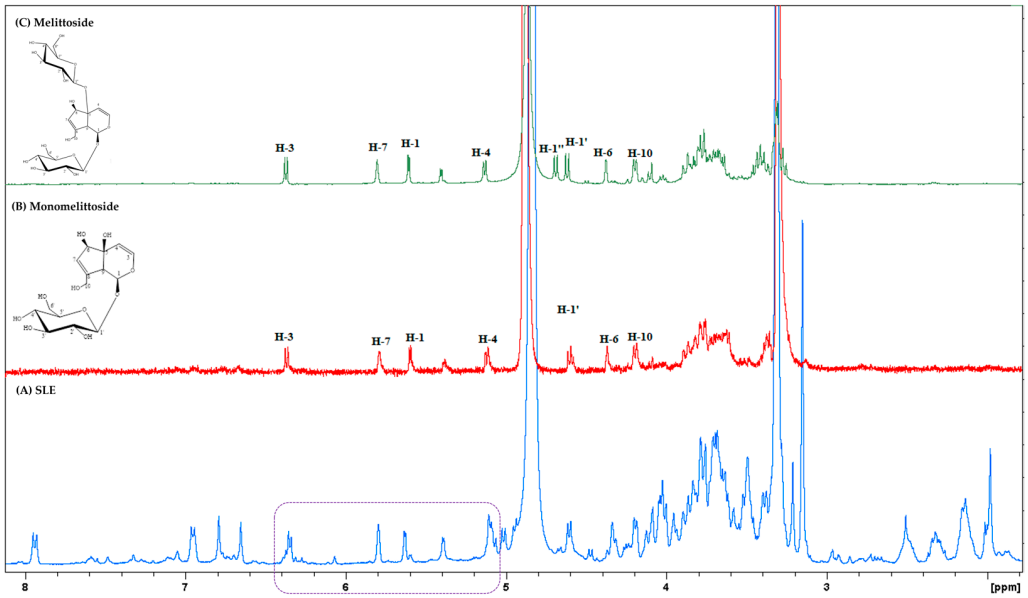

Figure 4. Overlaid ^{1}H-NMR spectra of (**A**) *Stachys leucoglossa* subsp. *leucoglossa* infusion (SLE, blue color), (**B**) monomelittoside (red color), and (**C**) melittoside (green color). Specific signals are indicated by a purple box.

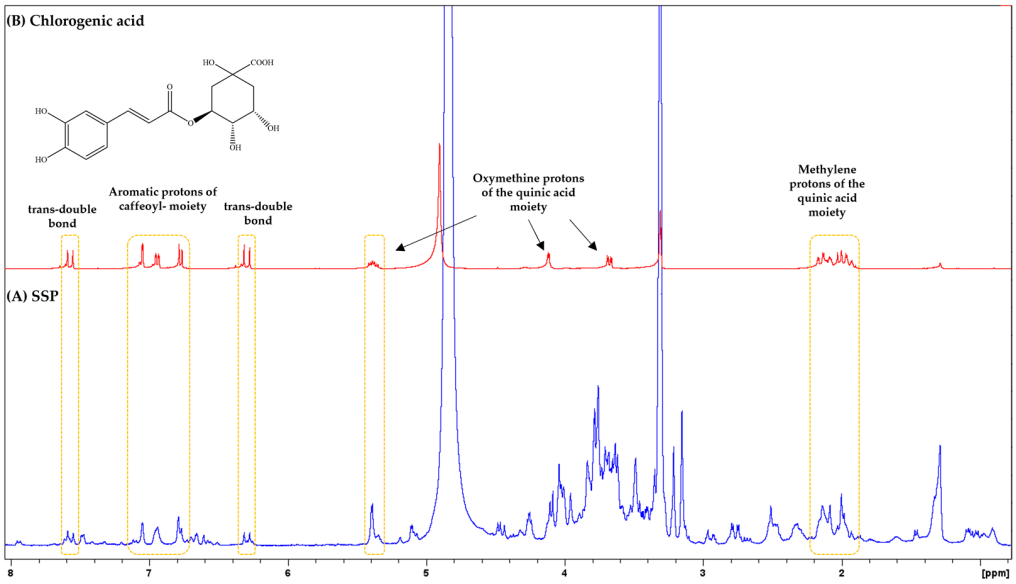

Figure 5. Overlaid ^{1}H-NMR spectra of (**A**) *Stachys spinulosa* infusion (SSP, blue color) and (**B**) chlorogenic acid (red color). Specific signals are indicated by orange boxes.

3.2. HPLC-PDA-MS Analysis

In total, 26 compounds were detected by HPLC-PDA-MS (Table 2), with them belonging to three main classes, namely flavonoids, phenylethanoid glycosides, and phenolic acids. The HPLC-PDA chromatograms of the *Stachys* infusions are illustrated in Figure S6.

Chlorogenic acid (**2**) was identified in all samples as one of the main metabolites in the *Stachys* infusions under study. The identification of the latter was based on a reference standard. An isobaric compound at an earlier retention time was assigned as an isomer, probably with a 4-substitution of the quinic acid group, as suggested by a fragment at *m*/*z* 173 [16]. Chlorogenic acid and isomers have previously been identified in the *Stachys* taxa of several sections, such as the sections *Candida* (*S. candida* and *S. horvaticii* Micevski, previously known as *S. iva* Griseb.), *Eriostomum*, and *Olisia* (*S. atherocalyx* K. Koch and *S. recta* L.), and members of the section *Stachys* [6]. However, this is the first report on the presence of chlorogenic acid and isomers in *S. chrysantha*, *S. leucoglossa* subsp. *leucoglossa*, and *S. spinulosa*.

Phenylethanoid glycosides are the main constituents of the genus *Stachys* [6,7]. In this study, nine phenylethanoid glycosides were identified in the four investigated infusions. Three detected phenylethanoid glycosides, namely lavandulifolioside (**4**), acteoside (**5**), and leucosceptoside A (**12**), were unambiguously identified by co-chromatography using compounds isolated in previous works by our group [6]. Although these compounds have previously been found in various *Stachys* taxa [6], they were detected for the first time in *S. chrysantha*, *S. leucoglossa* subsp. *leucoglossa*, and *S. spinulosa*. Acteoside has been previously isolated from *S. candida* [6], while lavandulifolioside and leucosceptoside A have not been identified in this species before. Peaks **6**–**8** were assigned tentatively as isomers of lavandulifolioside and acteoside as they had similar fragmentation patterns. Different substitution patterns of the acyl groups on the sugars might account for the differences in the retention times. Peaks **10** and **16** were tentatively assigned to stachysoside B and/or isomers, as suggested by the difference in the molecular weight (by 14 amu) when compared to lavandulifolioside. Furthermore, they were eluted at considerably longer retention times compared to lavandulifolioside. These compounds have been reported in *S. affinis* Bunge (synonym: *S. sieboldii* Miq.) [17]. Likewise, peak **19** was assigned tentatively to stachysoside C due to its pseudomolecular ion at *m*/*z* 783.0, suggesting the presence of two extra methyl groups in comparison to lavandulifolioside. The small amount of the latter in the infusion did not permit supporting further fragmentations. However, its retention time of almost

36 min agrees with this hypothesis. Such compounds are common in other *Stachys* species, such as *S. affinis* (synonym: *S. sieboldii*) [17] and *S. plumosa* Griseb. from Serbia [18].

The flavone glycosides detected in the infusions belong to the group of isoscutellarein/hypolaetin derivatives with some differentiations. Peaks **20**, **22**, and **24**–**26**, being the main metabolites, were identified based on co-chromatography with previously isolated compounds from both laboratories [6]. Accordingly, peaks **9**, **13**, **14**, **18,** and **23** were identified either as their deacetylated counterparts or isomers resulting from different acylation sites or, ultimately, as diacetylated derivatives. Regarding the section *Campanistrum*, 8-hydroxyflavone-allosylglucosides have been found in *S. arvensis* (L.) L. and *S. ocymastrum* (L.) Briq. (=*S. hirta* L.) [15]. However, there is no previous report on such compounds in *S. spinulosa*. Regarding the section *Candida*, isoscutellarein/hypolaetin derivatives have been previously reported in *S. candida*, *S. chrysantha*, and *S. horvaticii* Micevski (previously known as *S. iva*) [6]. Furthermore, monoacetyl and diacetyl derivatives of isoscutellarein have been found in *Stachys* species of the section *Olisia*, including *S. leucoglossa* [6,9]. *S. candida* and *S. spinulosa* also contained apigenin/luteolin derivatives in small amounts. In a previous study, chrysoeriol and its derivative, namely chrysoeriol 7-(3″-E-p-coumaroyl)-D-glucopyranoside, were isolated from *S. candida* [6]. Vicenin-2 was identified by the diagnostic successive losses of 120 amu [19], while peak **15** was identified as apigenin-7-O-glucoside previously isolated from *S. candida* [6]. It should be mentioned that vicenin-2 and apigenin-7-O-glucoside were detected only in *S. candida.* In addition, this study reports the presence of vicenin-2 in this species for the first time. Peaks **11** and **21** were detected only in *S. spinulosa* and were identified as acetylated luteolin and chrysoeriol dihexosides based on UV and MS fragmentations as well as on previously isolated compounds from *S. spinosa* and *S. aegyptiaca* Pers. [13,19].

Table 2. UV-VIS absorption and MS fragmentation data (positive and negative mode) of the compounds detected in the Greek *Stachys* infusions examined (SCA: *Stachys candida;* SCH: *Stachys chrysantha;* SLE: *Stachys leucoglossa* subsp. *leucoglossa;* SSP: *Stachys spinulosa*). The first reports of specific compounds for SCA, SCH, and SLE are indicated with an asterisk (*).

No.	Rt (min)	UV (nm)	Negative Mode, *m/z*	Positive Mode, *m/z*	Identification	SCA	SCH	SLE	SSP
1	10.90	298, 327	136.9, 172.9, 179.1, 191.1 [quinic acid-H]$^-$, **353.1** [M-H]$^-$, 707.1 [2M-H]$^-$	**354.9** [M+H]$^+$	Chlorogenic acid isomer	+ *	+ *	+ *	+
2	11.20	298, 324	191.1 [quinic acid-H]$^-$, **353.1** [M-H]$^-$, 707.1 [2M-H]$^-$	**354.9** [M+H]$^+$, **376.9** [M+Na]$^+$	Chlorogenic acid	+	+ *	+ *	+
3	15.36	271, 334	353.1, 473.0, **592.9** [M-H]$^-$	**595.0** [M+H]$^+$	Vicenin-2	+ *	-	-	-
4	22.28	291, 328	160.9, 593.2 [M-caffeoyl-H]$^-$, **755.1** [M-H]$^-$	479.1, **757.1** [M+H]$^+$, **779.1** [M+Na]$^+$	Lavandulifolioside (syn. Stachysoside A)	+ *	+ *	tr *	tr
5	23.73	291, 330	160.9 [caffeoyl group-H]$^-$, 461.1 [M-caffeoyl-H]$^-$, **623.1** [M-H]$^-$	**625.1** [M+H]$^+$, **647.1** [M+Na]$^+$	Acteoside	+	+ *	tr *	+
6	24.56	288, 329	160.9, 593.1, 623.2 [M-pentosyl-H]$^-$, **755.2** [M-H]$^-$	439.2, 625.1 [M-pentosyl+H]$^+$, **757.0** [M+H]$^+$, **779.1** [M+Na]$^+$	Isomer of lavandulifolioside	+ *	+ *	-	-

Table 2. *Cont.*

No.	Rt (min)	UV (nm)	Negative Mode, *m*/*z*	Positive Mode, *m*/*z*	Identification	SCA	SCH	SLE	SSP
7	27.26	289, 327	461.1 [M-caffeoyl-H]$^-$, **623.0** [M-H]$^-$	**647.1** [M+Na]$^+$	Acteoside isomer	+ *	+ *	-	-
8	28.57	289, 327	**623.1** [M-H]$^-$	**647.1** [M+Na]$^+$	Acteoside isomer	+ *	-	-	-
9	29.65	277, 307, 324	285.0 [A-H]$^-$, **608.9** [M-H]$^-$	**611.0** [M+H]$^+$	Isoscutellarein-7-*O*-allopyranosyl-(1→2)-glucopyranoside	-	-	+ *	-
10	32.03	288,329	137.0 [dihydroxytyrosol-H]$^-$, 593.1 [M-feruloyl-H]$^-$, 607.1 [M-rhamnose-H]$^-$, **769.4** [M-H]$^-$	**771.1** [M+H]$^+$, **793.2** [M+Na]$^+$	Phenylethyl glycoside, isomer I (stachysoside B, syn. Leonoside A) tentatively	+ *	-	-	-
11	32.40	255, 268, 348	285.0 [A-H]$^-$, **651.1** [M-H]$^-$	**653.1** [M+H]$^+$, **661.1** [M+Na]$^+$	Luteolin-acetyl-dihexoside	-	-	-	+
12	32.65–32.81	290, 329	136.9 [dihydroxytyrosol-H]$^-$, 446.9, **637.1** [M-H]$^-$	**639.2** [M+H]$^+$, **661.1** [M+Na]$^+$	Leucosceptoside A	+ *	+ *	-	+
13	32.70	277, 307, 327	**651** [M-H]$^-$	**653.1** [M+H]$^+$	Isoscutellarein-7-*O*-[6‴-acetyl-allopyranosyl-(1→2)]-glucopyranoside Isomer I	-	-	+	-
14	33.06–33.20	253, 287, 296, 334	301.2 [A-H]$^-$, **667.2** [M-H]$^-$	479.1, **669.1** [M+H]$^+$	Hypolaetin-acetylated derivative	+	+ *	+ *	+
15	33.44	268, 330	**430.9** [M-H]$^-$	**433.1** [M+H]$^+$	Apigenin-glucoside	+	-	-	-
16	33.66	282, 328	137.0 [dihydroxytyrosol-H]$^-$, 476.9, 637.1, **769.1** [M-H]$^-$	**771.1** [M+H]$^+$	Phenylethyl glycoside, isomer II (stachysoside B, syn. Leonoside A)	+*	+ *	-	-
17	34.05	267, 340 and 272, 287, 332			Mixture	+	-	-	-
18	34.62	277, 307, 327	**651** [M-H]$^-$	**653.1** [M+H]$^+$	Isoscutellarein-7-*O*-[6‴-acetyl-allopyranosyl-(1→2)]-glucopyranoside Isomer II	-	-	+	-
19	35.88	287, 329	**783** [M-H]$^-$	**785.1** [M+H]$^+$	Phenylethyl glycoside (stachysoside C) tentatively	+ *	-	-	-

Table 2. *Cont.*

No.	Rt (min)	UV (nm)	Negative Mode, *m*/*z*	Positive Mode, *m*/*z*	Identification	SCA	SCH	SLE	SSP
20	35.99–36.21	276, 306, 328	285.0, $[A-H]^-$, 429.1, **651** $[M-H]^-$	**653.1** $[M+H]^+$	Isoscutellarein-7-*O*-[6‴-acetyl-allopyranosyl-(1→2)]-glucopyranoside	+	+	+	+
21	36.49	269, 342	298.9 $[A-H]^-$, 623.1 $[M\text{-acetyl-}H]^-$, **665.0** $[M-H]^-$	**667.1** $[M+H]^+$	Chrysoeriol-7-*O*-acetyl-dihexoside (= stachyspinoside) tentatively	-	-	-	+
22	37.34–37.45	278, 296, 335	314.9 $[A-H]^-$, 625.2 $[M\text{-acetyl-}H]^-$, **681.1** $[M-H]^-$	**683.1** $[M+H]^+$	3′-hydroxy-4′-*O*-methylisoscutellarein-7-*O*-[6‴-acetyl-allopyranosyl-(1→2)]-glucopyranoside	+	+ *	+	+
23	41.55–41.70	278, 297 338	300.9 $[A-H]^-$, **709.0** $[M-H]^-$	**711.1** $[M+H]^+$	hypolaetin-7-*O*-di-acetyl-dihexoside	-	+ *	+ *	+
24	42.24	277, 307, 322	298.9 $[A-H]^-$, **665.0** $[M-H]^-$	**667.1** $[M+H]^+$	4′-*O*-methylisoscutellarein-7-*O*-[6‴-acetyl-allopyranosyl-(1→2)]-glucopyranoside	-	-	+	tr
25	43.66	276, 307, 324	**693.1** $[M-H]^-$	**695.1** $[M+H]^+$	Isoscutellarein-7-*O*-diacetyl-dihexoside	-	+ *	+ *	tr
26	44.16–44.26	276, 297, 338	314.9 $[A-H]^-$, 501.0 $[M\text{-hexosyl-acetyl-}H_2O\text{-}H]^-$, **723.1** $[M-H]^-$	**725.1** $[M+H]^+$	3′-hydroxy-4′-*O*-methylisoscutellarein-7-*O*-diacetyl-dihexoside	-	+ *	+ *	+

A: aglycon; tr: traces.

4. Conclusions

In the present study, the metabolic characterization of two protected and endangered local Greek endemic members of the genus *Stachys* (*S. candida* and *S. chrysantha*) and two local Balkan (sub-) endemic *Stachys* members (*S. leucoglossa* subsp. *leucoglossa*, and *S. spinulosa*) were investigated using NMR and HPLC-PDA-MS techniques. For the detection of specific constituents in the studied *Stachys* members, 1D- and 2D-NMR experiments were employed to compare their chemical fingerprints in combination with reference compounds isolated and identified in previous studies. In total, 26 compounds were detected by HPLC-PDA-MS as belonging to flavonoids (mainly isoscutellarein and hypolaetin derivatives), phenylethanoid glycosides, and phenolic acids (chlorogenic acid and its isomer). The chemical composition of *S. spinulosa* was investigated herein for the first time in detail, while knowledge of the metabolic profiling concerning the rest of the studied taxa was substantially supplemented herein with new reports of specific compounds not previously reported for one or more of the studied taxa. From a chemotaxonomical viewpoint, some compounds were found in all four *Stachys* members, evidencing a shared phytochemical relatedness. On the other hand, vicenin-2 and apigenin-glucoside were detected only in *S. candida*, while isoscutellarein-7-O-allopyranosyl-(1→2)-glucopyranoside was only detected in *S. leucoglossa* subsp. *leucoglossa*. Furthermore, *S. candida* and *S. chrysantha* had some compounds in common, evidencing (in phytochemical terms) their close affinity within the section *Candida* or the phyloclade *Swainsoniana*. Therefore, the data furnished herein

may further contribute to ongoing phytochemical and chemotaxonomical investigations regarding the distribution of different compounds and categories thereof across different members and sections of the genus *Stachys*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13102624/s1. Figure S1: Representative photographs of the studied *Stachys* taxa in their original habitats in Greece: (A) *Stachys chrysantha*, (B) *Stachys candida*, (C) *Stachys leucoglossa* subsp. *leucoglossa*, and (D) *Stachys spinulosa*. Photos: Eleftherios Dariotis (reproduced with permission); Figure S2: ^{1}H-^{1}H-COSY and HSQC spectra of *Stachys candida* (SCA); Figure S3: ^{1}H-^{1}H-COSY and HSQC spectra of *Stachys chrysantha* (SCH); Figure S4: ^{1}H-^{1}H-COSY and HSQC spectra of *Stachys leucoglossa* subsp. *leucoglossa* (SLE); Figure S5: ^{1}H-^{1}H-COSY and HSQC spectra of *Stachys spinulosa* (SSP); Figure S6. HPLC-PDA chromatograms of the *Stachys* infusions at 330 nm.

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