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NMR Fingerprint Comparison of Cultivated *Sideritis* spp. from Cyprus

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Abstract: Medicinal and Aromatic Plants (MAPs) play an essential role in global health systems, since more than 80% of population use natural products in primary healthcare. Given that the global use of herbal medicines is exponentially increasing, as well as many MAPs products are introduced into the market, many cultivation practices are applied to produce high quality and standardized plant raw materials. Thus, the present study focuses on the chemical fingerprints of two cultivated *Sideritis* species. In Cyprus, *Sideritis cypria* Post and *S. perfoliata* L. subsp. *perfoliata* are widely used in traditional medicine. To date, there is no research work presenting the comparative chemical profiling between the aforesaid species using NMR methods. In this study, 1D and 2D NMR experiments were used to compare the chemical fingerprints of these species originated from conventional cultivation practices. Iridoids (ajugol, monomelittoside, and melittoside) and one flavone (4′-methyl-isoscutellarein 7-O-[6‴-O-acetyl]-β-D-allosyl(1→2)glucoside) were present in the infusion of *S. perfoliata* subsp. *perfoliata*. The phenylethanoid glycoside, acteoside, was detected in both samples. The phytochemical profiles of these cultivated species were similar to those of *Sideritis* species, indicating the positive impact of cultivation practices in MAPs.

Keywords: *Sideritis*; NMR fingerprints; comparison; cultivation; Cyprus

Citation: Tomou, E.-M.; Lytra, K.; Chrysargyris, A.; Tzortzakis, N.; Skaltsa, H. NMR Fingerprint Comparison of Cultivated *Sideritis* spp. from Cyprus. *Agronomy* **2021**, *11*, 1503. https://doi.org/10.3390/agronomy11081503

Academic Editor: Heike Knicker

Received: 30 June 2021
Accepted: 26 July 2021
Published: 28 July 2021

1. Introduction

Medicinal and Aromatic Plants (MAPs) are sources of specialized products with therapeutic potential which could lead to the discovery of new drugs [1]. In last decade, the cultivation of MAPs is considered as a great aspect for developing high quality and standardized natural raw materials in adequate quantity for industrial scale production, as well as for avoiding their potential extinction.

As the cultivation of MAPs is continuously increasing, rapid and precise analytical methods for identifying their chemical fingerprint are essential need. Nuclear Magnetic Resonance (NMR) fingerprinting has enabled and accelerated analytical procedures and authentication process of plant extracts, since NMR method is a fast, simple, reproducible, and non-destructive technique [2].

In recent years, mountain tea (*Sideritis* spp., Lamiaceae), one of the most popular herbal preparations worldwide, is being sold as a high added value natural product in global markets. In Cyprus, the infusions of the aerial parts of *S. cypria* Post and *S. perfoliata* L. subsp. *perfoliata* are used in traditional medicine as agents for various ailments such as stomach discomforts, respiratory disorders, influenza, and common cold [3]. Previous studies on extracts and infusions of these species revealed their rich nutrition and phytochemical profiles, as well as their great in vitro biological effects, including antioxidant, anti-inflammatory, antimicrobial, and cytotoxic activities [4–9].

At present, there is no report on comparative metabolite profiling of *S. cypria* and *S. perfoliata* subsp. *perfoliata*, using NMR methods. Therefore, in this study, an analytical method based on 1D and 2D NMR spectra is proposed to compare the chemical fingerprints of these species, originated from conventional cultivation practices.

2. Materials and Methods

2.1. Plant Materials

Harvesting was performed from one-year-old plants cultivated at Athalassa farm (Nicosia, Cyprus) by the Department of Agriculture of Ministry of Agriculture, Rural Development and Environment of Cyprus (*Sideritis cypria*) in 2019 and at Akrotiri (Limassol, Cyprus) (*Sideritis perfoliata* subsp. *perfoliata*) in 2018. Plants of both species were produced from genetic material originated from the mother plantations of the National Department of Agriculture at Athalassa. Taxonomic identification of the species was verified by authors based on visual observations on mother plantations following the keys available to the online Flora of Cyprus—a dynamic check list [10]. All plants were at the flowering stage (June), while conventional cultivation practices were applied.

2.2. Preparation of the Infusions and NMR Spectroscopic Analysis

Precisely, 2 g of aerial parts of each plant were added in 100 mL boiled water for 5 min, separately [11]. Then, 2 mL of each sample were transferred in pre-weighed vials and were concentrated in a vacuum rotary evaporator at room temperature, yielding 0.5 g (*S. cypria*) and 0.4 g (*S. perfoliata* subsp. *perfoliata*). Distilled water was used as solvent for the infusions, being sure that any additive/impurity will not be included in the samples.

For NMR measurements, each sample was dissolved in 600 μL of CD_3OD. 1D and 2D NMR spectra were recorded on Bruker 400 DRX instrument at 300 K. Chemical shifts are given in ppm (δ) and are referenced to the solvent signal at 3.31 ppm (CD_3OD). COSY (COrrelation SpectroscopϒY) experiment was performed using standard Bruker microprograms. Parameters used to obtain (i) ^{1}H-NMR spectra: ns = 16, rg = 101 and acquisition time = 1 min and 30 s and (ii) COSY spectra: ns = 4, rg = 101 and acquisition time = 19 min and 30 s.

3. Results and Discussion

The two infusions of *S. cypria* and *S. perfoliata* subsp. *perfoliata* were prepared separately, following the monograph of EMA for *Sideritis* plants [11]. To obtain the first overview of the chemical classes of their constituents, the two samples were subjected to Thin Layer Chromatography (TLC) on silica gel with visualization under UV light (254 and 366 nm). In UV light at 366 nm, dark and blue spots appeared, possibly corresponding to flavonoids and phenolic derivatives. It is noteworthy to mention that terpenoids could not be easily detected in UV light, but after spraying with vanillin-sulfuric acid reagent and heating, they could be observed as purple spots. The TLC fingerprints of both samples after use of the aforesaid solution and heating revealed three major chemical classes: (i) terpenoids, (ii) flavonoids, and (iii) phenolic derivatives. Specifically, the infusion of *S. cypria* showed rich content in phenolic derivatives, whereas the infusion of *S. perfoliata* subsp. *perfoliata* seemed to contain additionally terpenoids.

As a next step, the ^{1}H-NMR spectra of the samples were obtained, unveiling the presence of different phytochemical classes of compounds. The comparative 1D-NMR fingerprints are presented in Figure 1. Based on the hydrogen peaks, the ^{1}H-NMR spectra of both infusions were divided into four main specific regions (Figure 1).

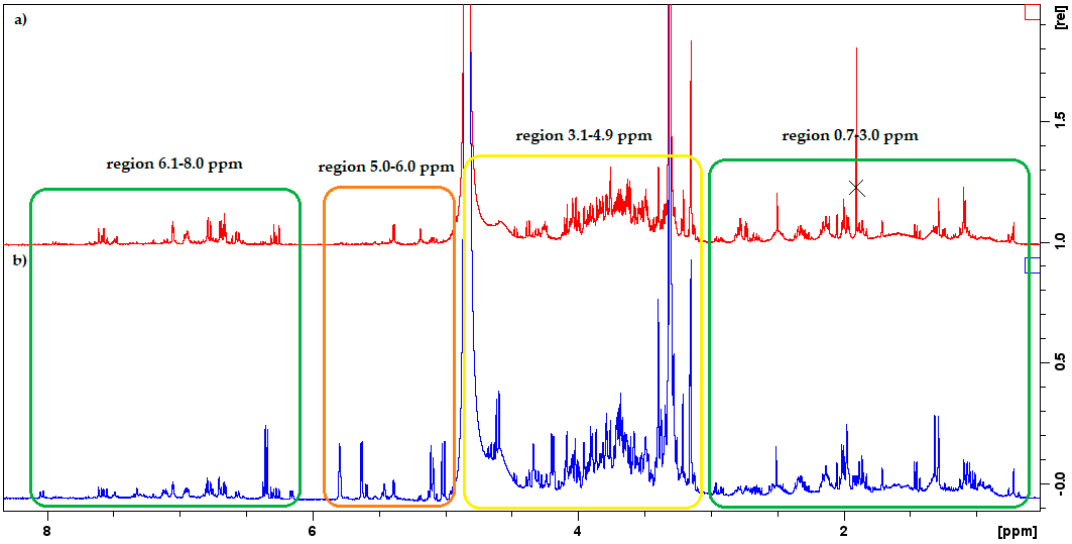
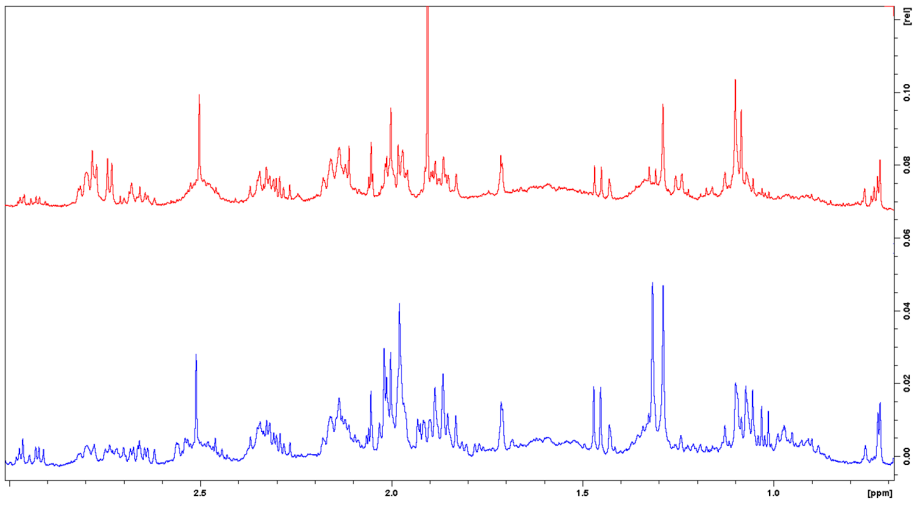


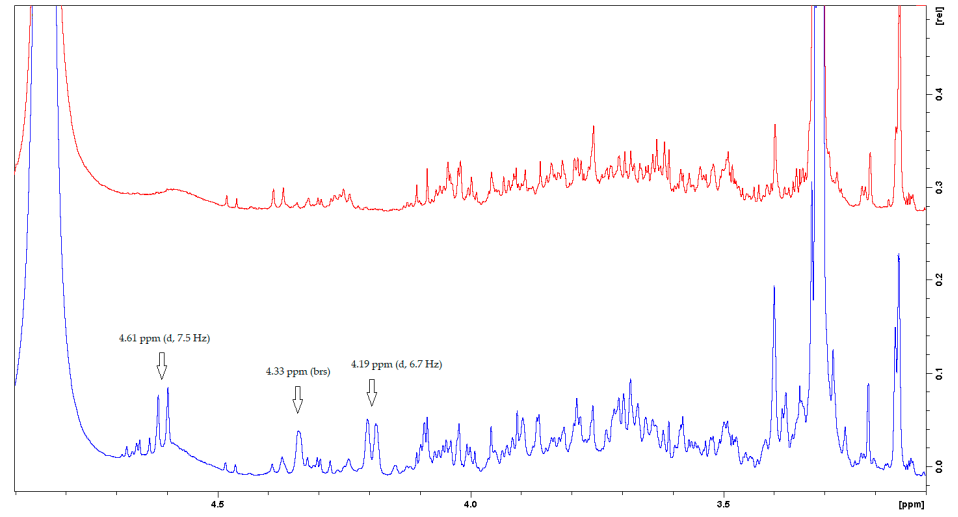
Figure 1. Overlaid ^{1}H-NMR spectra of both samples: (**a**) infusion of *S. cypria*; (**b**) infusion of *S. perfoliata* subsp. *perfoliata*.

In the upfield region (δ 0.7–3.0 ppm) there appeared mainly signals of terpenoids. The second region (δ 3.1–4.9 ppm) included signals especially of sugars, whereas in the third region (δ 5.0–6.0 ppm) peaks of anomeric hydrogens of sugars and hydrogens of iridoids were illustrated. In the downfield region (δ 6.1–8.0 ppm) signals of flavonoids, phenylethanoid glycosides and phenolic acids were observed. Comparing the ^{1}H-NMR fingerprints, both spectra were similar in the first region (Figure 2a). In the second region, three different peaks were spotted in the spectrum of the infusion of *S. perfoliata* subsp. *perfoliata* at δ 4.61 ppm (d, 7.5 Hz), 4.33 ppm (brs), and 4.19 ppm (d, 6.7 Hz) (Figure 2b). Furthermore, some differences were detected in the last two regions between the samples (Figure 2c,d). Particularly, at the region among δ 5.0 to 6.0 ppm in the ^{1}H-NMR spectrum of the infusion of *S. perfoliata* subsp. *perfoliata* were observed hydrogen signals which could belong to iridoids and anomeric hydrogens of sugars, while in the spectrum of the infusion of *S. cypria* less peaks were shown at this region. Although the fingerprints of both samples were approximately similar at δ 6.1–8.0 ppm, some hydrogen signals at δ 6.15 ppm (dd, 1.9/6.1 Hz), 6.34 ppm (d, 6.6 Hz), 7.11 ppm (m), 7.32 ppm (d, 2.3 Hz), and 8.03 ppm (d, 8.3 Hz) were found only in the spectrum of the infusion of *S. perfoliata* subsp. *perfoliata*.

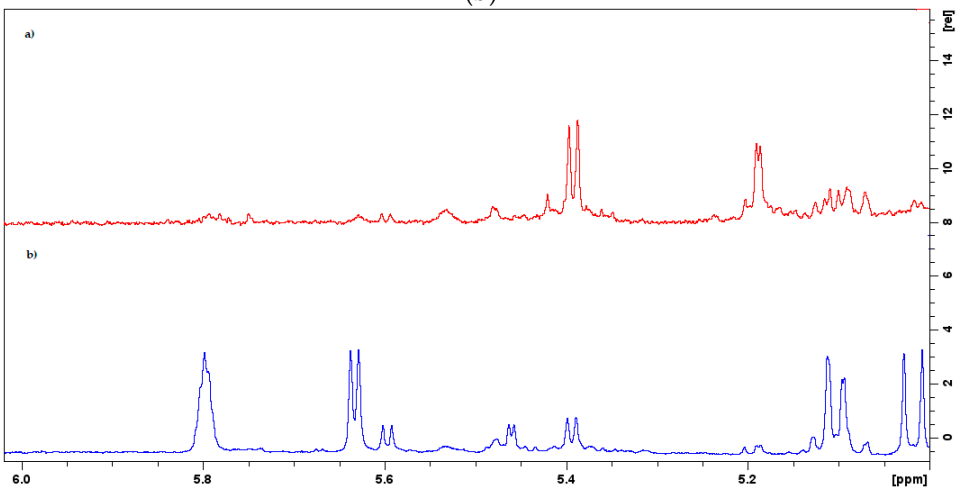


(**a**)

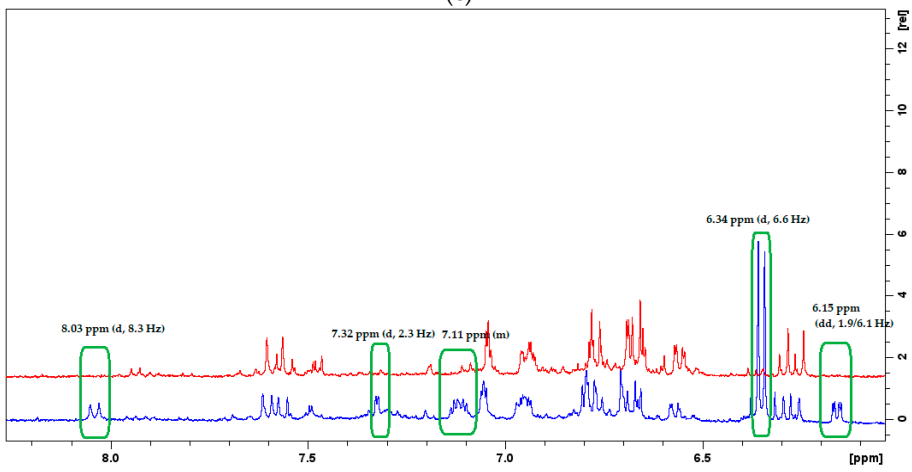
Figure 2. *Cont.*



(**b**)



(**c**)



(**d**)

Figure 2. Overlaid ^{1}H-NMR spectra of *S. cypria* (red colour) and *S. perfoliata* subsp. *perfoliata* (blue colour) infusions. (**a**) Zoom-in of the region between 0.7 and 3.0 ppm; (**b**) Zoom-in of the region between 3.1 and 4.9 ppm; (**c**) Zoom-in of the region between 5.0 and 6.0 ppm; (**d**) Zoom-in of the region between 6.1 and 8.0 ppm. The different peaks were signed in green boxes.

To get a better understanding about the observed differences of both samples, 2D ^{1}H–^{1}H–COSY spectra were carefully studied. The comparative spectra were presented in Figure 3, where the different correlation peaks were signed with green colour boxes. In the ^{1}H–^{1}H–COSY spectrum of the infusion of *S. perfoliata* subsp. *perfoliata*, we observed the major correlation peaks between hydrogens corresponding to iridoids, flavones and phenylethanoid glycosides. To be mentioned that the ^{1}H–^{1}H–COSY spectrum of the infusion of *S. cypria* revealed principally correlation peaks among hydrogens which belong to flavones and phenylethanoid glycosides.

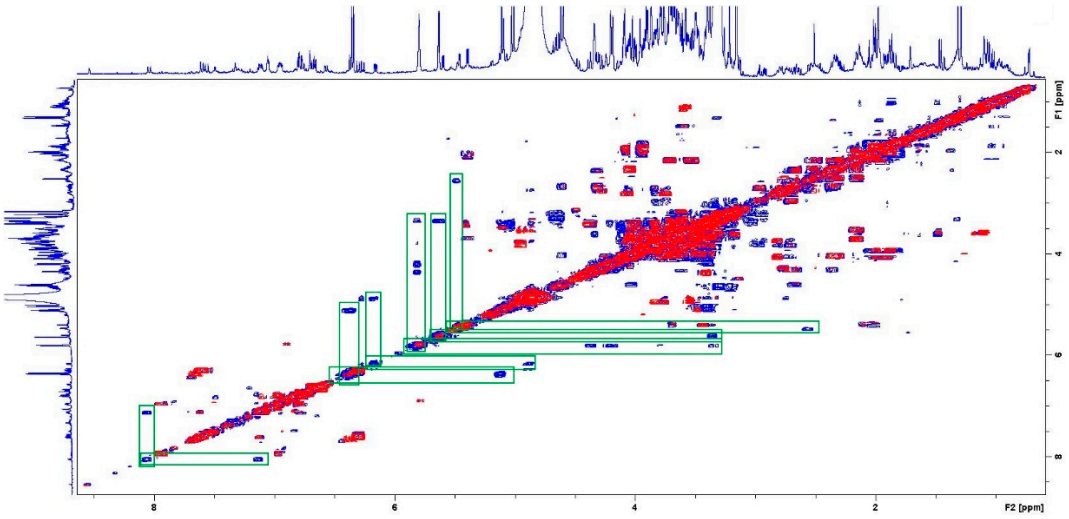


Figure 3. Overlaid ^{1}H–^{1}H–COSY spectra of both samples: infusion of *S. cypria* (red colour) and infusion of *S. perfoliata* subsp. *perfoliata* (blue colour). The different correlation peaks were signed with green colour boxes.

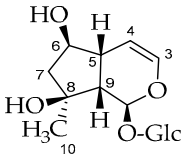
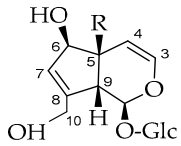
As an effort to interpretate the observed different signals in the 1D-/2D- NMR spectra of infusion of *S. perfoliata* subsp. *perfoliata* with those of *S. cypria*, we compared them with NMR spectra of previously isolated compounds from our works in *Sideritis* spp. [5,8,9,12,13]. Through the screening of the ^{1}H-NMR spectra of different compounds, we carefully noticed that the main different hydrogen signals in the spectrum of infusion of *S. perfoliata* subsp. *perfoliata* could be attributed to iridoids, namely ajugol [14] and monomelittoside and its 5-glucoside (melittoside) [15,16]. This assumption was also confirmed by the 2D ^{1}H–^{1}H–COSY spectra. The overlaid ^{1}H-NMR spectra of the infusion of *S. perfoliata* subsp. *perfoliata* and the three aforesaid iridoids are illustrated in Figure 4. In addition, we reported, herein, the hydrogen signals of these compounds and their corresponding signals in ^{1}H-NMR spectrum of the infusion (Table 1). Iridoids were not spotted in the ^{1}H-NMR spectrum of the infusion of *S. cypria*. Thus, we could assume that a principal difference among these infusions might be the absence of hydrogen signals belonging to iridoids in the infusion of *S. cypria* based on their NMR fingerprints. In *Sideritis* species, acetylated or not acetylated derivatives of hypolaetin, isoscutellarein, and their methylated derivatives are major constituents [11,17,18]. Isoscutellarein 7-O-allosyl(1→2)glucoside derivatives were also unveiled through this NMR process in both infusions. In the ^{1}H-NMR spectrum of the infusion of *S. perfoliata* subsp. *perfoliata*, a deshielded assignment at δ 8.03 ppm, d (8.3 Hz) was also observed which could be attributed to flavone structures with AB-type aromatic system in B ring. In addition, the hydrogen peaks at δ range 6.69–6.80 ppm could correspond to flavones. The 2D ^{1}H–^{1}H–COSY spectrum of this infusion demonstrated correlation peaks of this hydrogen with a hydrogen in the aromatic area at δ 7.12 ppm, d (8.3 Hz) (Figure 3), giving rise to the assumption that these hydrogens belong to flavones. Remarkably, the large deshielded shifts of these aromatic hydrogens indicated that this compound might be a 4′-methyl-isoscutellarein 7-O-allosyl(1→2)glucoside derivative. Given that some singlet hydrogen peaks were depicted at δ approximately 2.0 ppm, we could assume that the aforesaid compound could be acetylated. The overlaid ^{1}H-NMR spectra of

the infusion of *S. perfoliata* subsp. *perfoliata* and the previously isolated compound from this species [5], 4′-methyl-isoscutellarein 7-O-[6‴-O-acetyl]-β-D-allosyl(1→2)glucoside [19], are presented in Figure 5. Regarding the phenylethanoid glycosides, in the ^{1}H-NMR spectra of both samples we could observe the presence of characteristic hydrogen assignments of these compounds. However, it is not feasible to accurately identify the specific derivatives, as we cannot clearly detect the number and type of sugar moieties due to overlapped assignments in the δ region 3.1–5.5 ppm. Based on previous studies in genus *Sideritis*, the most common representative of this chemical class is acteoside which was identified in the ^{1}H-NMR spectra of both samples (Figure 6) [11,17].

Consequently, the major differences between the infusions of *S. cypria* and *S. perfoliata* subsp. *perfoliata* were: (i) iridoids, which were detected only in the 1D-/2D- NMR spectra of *S. perfoliata* subsp. *perfoliata* and (ii) the acetylated flavone, 4′-methyl-isoscutellarein 7-O-[6‴-O-acetyl]-β-D-allosyl(1→2)glucoside, which was found only in the latter species. However, both infusions presented assignments of flavones and phenylethanoid glycosides in the NMR spectra. These results are of great importance since both samples seemed to include bioactive constituents with various biological properties [17,18,20].

The presented NMR fingerprint method might be proposed as one of many potential tools for differentiation between the species.

Table 1. The main hydrogen chemical shifts of the ^{1}H-NMR spectra of ajugol and derivatives of melittoside and their corresponding peaks in ^{1}H-NMR spectrum of the infusion of *S. perfoliata* subsp. *perfoliata* (CD_3OD, 400 MHz).

Chemical Compound	δ_H [1] (ppm)	δ_H [2] (ppm)
Ajugol  Melittoside derivatives  *Monomelittoside* R=OH *Melittoside* R=O-Glc	Aglycon: 6.16 (dd, H-3), 5.46 (d, H-1), 4.87 (*, H-4), 3.93 (m, H-6), 2.73 (dd, H-5), 2.54 (dd, H-9), 2.04 (dd, H-7a), 1.79 (dd, H-7b), 1.31 (s, CH_3-10) Glucose: 4.64 (d, H-1′), 3.20–3.90 (H-2′ to H-6′) *Monomelittoside* Aglycon: 6.35 (dd, H-3), 5.79 (s, H-7), 5.64 (d, H-1), 5.10 (d, H-4), 4.34 (brs, H-6), 4.22 (d, H_2-10), 3.35 (*, H-9) Glucose: 4.60 (d, H-1′), 3.28–3.88 (H-2′ to H-6′) *Melittoside* Aglycon: 6.37 (dd, H-3), 5.80 (s, H-7), 5.60 (d, H-1), 5.12 (d, H-4), 4.38 (brs, H-6), 4.20 (d, H_2-10), 3.31 (*, H-9) Glucose: 4.61 (d, H-1′), 3.26–3.90 (H-2′ to H-6′) Glucose: 4.67 (d, H-1″), 3.25–3.90 (H-2″ to H-6″)	Aglycon: 6.16 (dd, H-3), 5.46 (d, H-1), *(H-4), *(H-6), 2.73 (*, H-5), 2.55 (dd, H-9), 2.05 (dd, H-7a), 1.79 (dd, H-7b), 1.32 (s, CH_3-10) Glucose: 4.64 (d, H-1′), *(H-2′ to H-6′) *Monomelittoside* Aglycon: 6.35 (dd, H-3), 5.80 (s, H-7), 5.63 (d, H-1), 5.10 (d, H-4), 4.33 (brs, H-6), 4.19 (d, H_2-10), * (H-9) Glucose: 4.61 (d, H-1′), * (H-2′ to H-6′) *Melittoside* Aglycon: 6.35 (dd, H-3), 5.80 (s, H-7), 5.63 (d, H-1), 5.10 (d, H-4), 4.37 (brs, H-6), 4.19 (d, H_2-10), *(H-9) Glucose: 4.61 (d, H-1′), * (H-2′ to H-6′) Glucose: 4.67 (d, H-1″), * (H-2″ to H-6″)

[1] hydrogen chemical shifts of the pure isolated compounds; [2] hydrogen chemical shifts of the infusion; * overlapped hydrogen signals; Glc: glucose

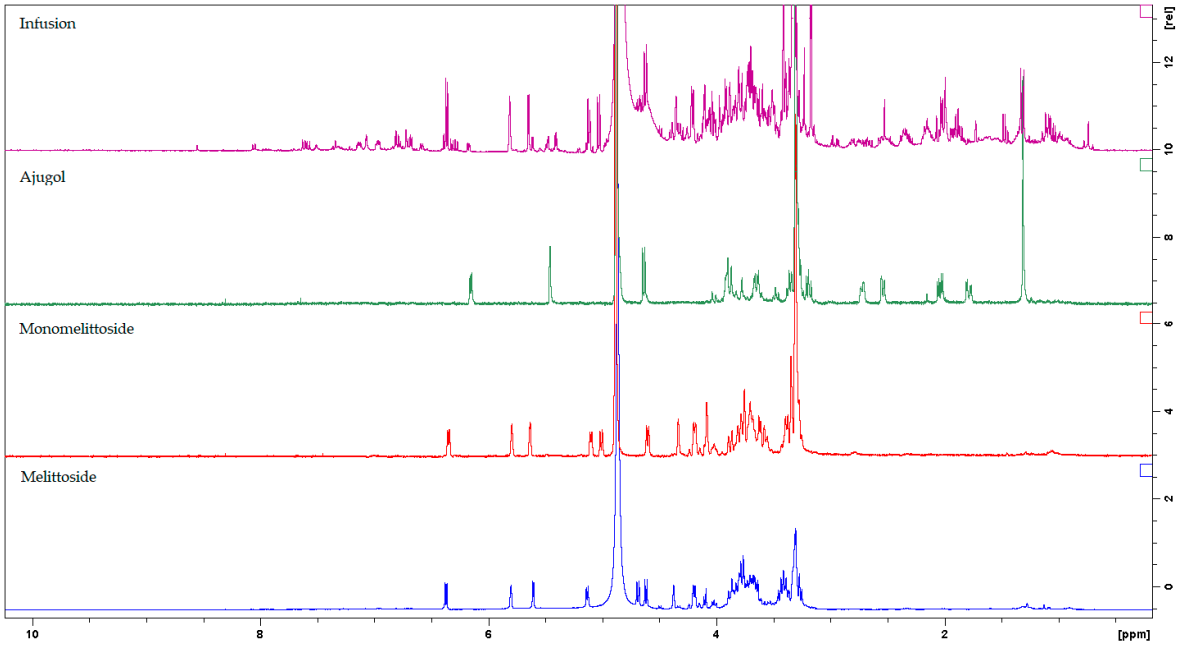


Figure 4. The overlaid ^{1}H-NMR spectra of the infusion of *S. perfoliata* subsp. *perfoliata* and of the three iridoids.

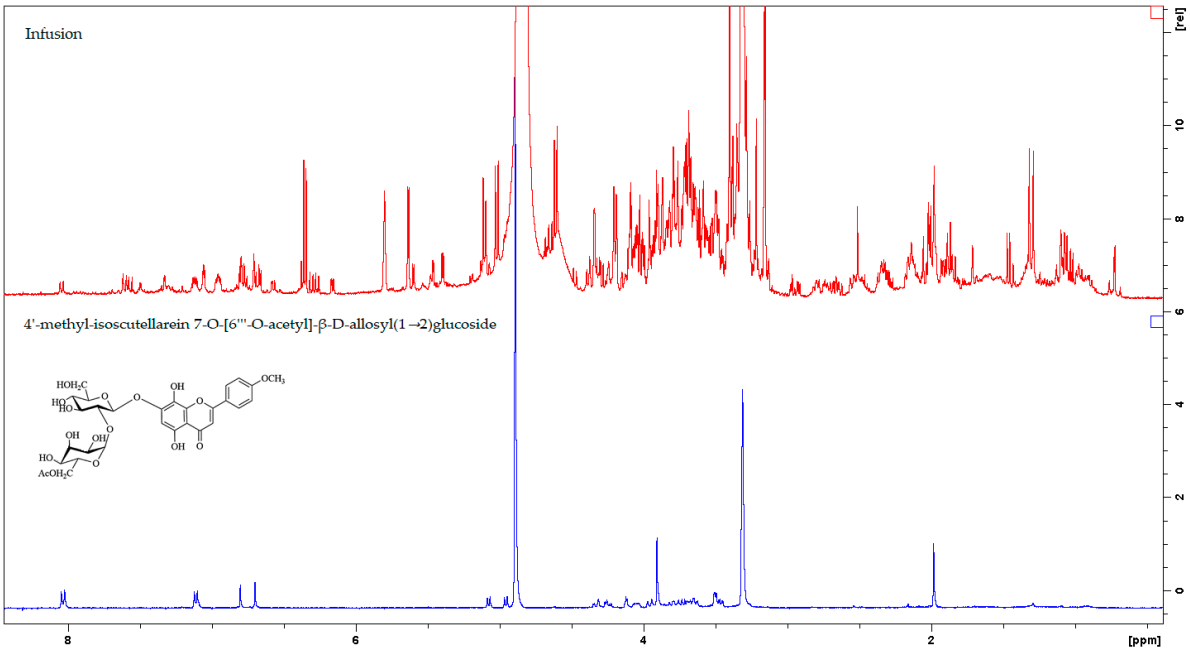


Figure 5. The overlaid ^{1}H-NMR spectra of the infusion of *S. perfoliata* subsp. *perfoliata* and of 4′-methyl-isoscutellarein 7-O-[6‴-O-acetyl]-β-D-allosyl(1→2)glucoside.

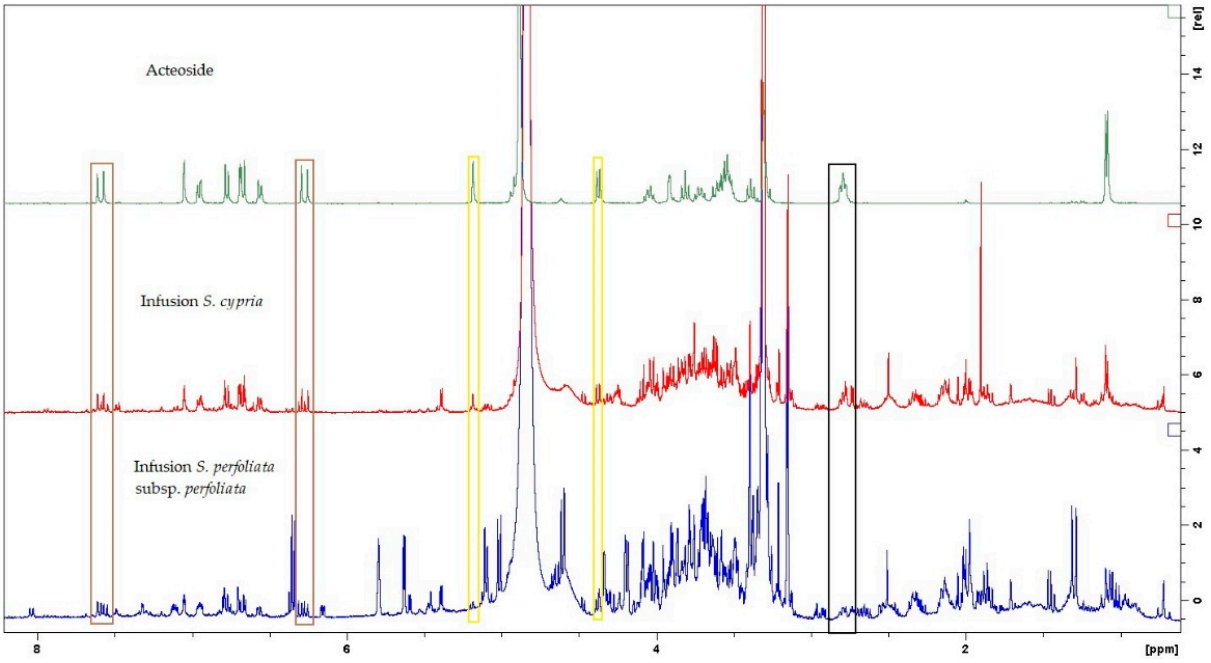


Figure 6. The overlaid ^{1}H-NMR spectra of the infusions of *S. cypria* and *S. perfoliata* subsp. *perfoliata* and of acteoside. The signal in the black box refers to the methylene hydrogens of the phenylethyl group. The signals in the brown boxes refer to the hydrogens of the trans bond (d, 16.0 Hz) of the caffeoyl group, whereas the signals in the yellow boxes show the anomeric hydrogens of acteoside.

4. Conclusions

The present study focused on the comparison of two cultivated *Sideritis* species from Cyprus, using an NMR process, revealing their similarities and differences. 1D-/2D-NMR spectra were applied for studying the chemical fingerprints of each infusion from *S. cypria* and *S. perfoliata* subsp. *perfoliata*. Both samples were rich in bioactive specialized products. However, the major differences between the samples were the presence of iridoids and one flavone in the infusion of *S. perfoliata* subsp. *perfoliata*. This work showed that the phytochemical profiles of these cultivated species were close to the typical profiles of *Sideritis* species, contributing to achieve a better insight to the potential impact of cultivation practices in MAPs.

Author Contributions: Conceptualization, E.-M.T. and H.S.; NMR investigation, E.-M.T. and K.L.; cultivation practices, A.C.; writing—original draft preparation, E.-M.T., A.C. and K.L.; review and editing, H.S. and N.T.; supervision, H.S. and N.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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