Reassessment of the polar fraction of *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande (Lamiaceae) from the Monti Sibillini National Park: A potential source of bioactive compounds

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**ABSTRACT**

**Background:** The phytochemical analysis of *Stachys alopecuros* subsp. *divulsa*, an endemic Italian species, has been recently reported and has showed the presence of 8-O-acetylharpagide (2), harpagide (3), allobetonicoside (4), and 4′-O-galactopyranosyl-teuhircoside (5). In this paper, an in depth study of its glycosidic fraction with the aim to widen the knowledge on its secondary metabolites content is reported. **Materials and Methods:** Chromatographic techniques were used for the isolation of constituents while spectroscopic and spectrometric techniques were applied for the structures elucidation. **Results:** Besides the known constituents, all of them reconfirmed, ajugoside (1), reptoside (6) and 6-O-acetyl-ajugol (7) were also identified among the iridoids while the phenolic components resulted to be chlorogenic acid (8), β-arbutin (9), verbascoside (10), and stachysoside A [11], instead. **Conclusion:** The iridoid pattern of *S. alopecuros* subsp. *divulsa* has been expanded with the identification of not previously reported compounds as well as for the phenolic fraction. Except for the reconfirmed verbascoside (10), the other phenolic compounds were recognized for the first time in the studied species. The complete NMR assignment of compound (1) by means of bidimensional techniques is reported, and both the chemotaxonomic and pharmacological relevance of the isolated compounds is largely discussed.

**KEY WORDS:** Chemotaxonomy, chlorogenic acid, iridoid, NMR, phenylethanoid glycosides, β-arbutin

**INTRODUCTION**

The genus *Stachys* comprises more than 450 species mainly distributed in warm-temperate regions of the Mediterranean basin and South-Western Asia. It represents one of the largest genera of Lamiaceae family [1], and the majority of the species belonging to this genus have been largely used in the traditional medicine. *Stachys* spp. are currently indicated as “Mountain tea” in several regions reflecting their principal use in infusions and decoctions. The genus *Stachys* is represented by annual or perennial herbs known for their interesting biological activities, i.e., anti-inflammatory [2], antispasmodic, sedative, and diuretic [3-5]. Several phytochemical components, which may be responsible of the biological activities, have been isolated from *Stachys* species, i.e., iridoids [6,7], phenylethanoid glycosides, flavonoids [8,9], acidic metabolites, polysaccharides, and terpenoids [10].

In Italy, the genus *Stachys* encompasses about 30 species and subspecies among which five species are considered endemic. *Stachys alopecuros* subsp. *divulsa* is one of these. This plant is characteristic of the mountain habitat of central Italy, being distributed only in a few regions (Umbria, Marche, Abruzzo, Lazio, and Molise) [11], on stony mountain pastures, scrubs, and screes, and preferring a calcareous, and dry soil up to 2000 m a.s.l. *S. alopecuros* subsp. *divulsa* is a perennial plant with small, hirsute, and subcylindrical ascending stem. The opposite leaves are petiolate and densely hairy on the edge and along the veins. The flowers are gathered in small verticillasters held in a dense spike, yellow-white in color and blooming from June to August-September. The fruits, which are brown colored, are constituted by 4 ovate nuculae [12]. A previous study on this subspecies revealed the presence of phenypropanoid, saponin, and iridoid glycosides [6] which...
are considered taxonomic markers for the genus and the family. In this paper, we reported a reassessment of the polar fraction constituents of this plant employing a patented method allowing the isolation of secondary metabolites of glycosidic nature.

**MATERIALS AND METHODS**

**Plant Materials**

The fresh plant materials (aerial parts) were collected at the flowering stage from a spontaneous population growing at the altitude of 1520 m a.s.l. in the territory of Pizzo Tre Vescovi, Marche, Italy (GPS coordinates: N 42°58'07", and E 13°14'10"). The botanical recognition was performed by one of us (F.M.) using available literature [12], and a representative specimen has been deposited in the Herbarium Universitatis Camericensis (CAME, included in the online edition of Index Herbariorum c/o School of Biosciences and Veterinary Medicine, University of Camerino, Italy) under the codex CAME 24825. A sample of the studied species is also stored in our laboratory (code number SAD-30062012) for any further reference.

**Instruments**

NMR spectra were recorded on a Varian (now Agilent Technologies) Mercury 300 MHz instrument and/or on a Bruker Avance III 400 MHz instrument using D$_2$O or CD$_3$OD as deuterated solvents: The chemical shifts were expressed in ppm using the HDO signal to set the 1H-spectra acquired in D$_2$O, while the internal solvent signal (m5) at 3.31 ppm was set as reference for the spectra in CD$_3$OD.

MS spectra were performed on a Q-TOF MICRO spectrometer (Micromass, now Waters, Manchester, UK) equipped with an ESI source that operated in the negative and/ or positive ion mode. The flow rate of sample infusion was 10 µL/min with 100 acquisitions per spectrum. Data were analyzed using the MassLynx software developed by Waters.

Solvents having RPE purity grade were all purchased from Sigma-Aldrich or Carlo Erba Reagenti, silica gel 60 (70-230 mesh ASTM) was purchased from Fluka.

**Bidimensional NMR Experiments**

Bidimensional spectra were performed on a Bruker Avance III 400 MHz instrument, operating at 9.4 T at 298 °K. Heteronuclear single quantum correlation (HSQC) experiments were acquired with a spectral width of 15 and 250 ppm for the proton and carbon, respectively, an average $J_{CH}$ of 145 Hz, a recycle delay of 2 s and a data matrix of 4 K × 256 points. Heteronuclear multiple bond correlation (HMBC) experiments were acquired with a spectral width of 15 and 250 ppm for the proton and carbon, respectively, a long-range coupling constant of $J_{CH}$ of 8 Hz, a recycle delay of 2 s and a data matrix of 4 K × 256 points.

**Extraction and Isolation of Polar Compounds**

A portion of 390.0 g of hair-dried plant materials was extracted with a mixture of ethanol 96% and distilled water in ratio 8:2 v/v (1.4 L). The whole was left in maceration for 5 days so that the metabolites could come into solution. The procedure was repeated three times to achieve an exhaustive extraction. The solutions, having a dark green coloration, were gathered together in a same flask and then filtered. After filtration, the organic solvent was eliminated under reduced pressure at a temperature below 45°C until a water suspension was obtained. Throughout this last part, pH was checked on normal litmus paper, and this was about 8. This step is necessary to verify that pH is not too acid, meaning not under the value of 5.5, because an extreme acidity at this step may cause secondary reactions with the formation of several artifacts coming from the hydrolysis of glycosides and esters present in the extract. The water suspension was then frozen to −20°C and later lyophilized at the same temperature to preserve temperature-sensitive compounds possibly present. The final dried crude extract obtained from this methodology weighed 86.7 g and was dark green colored.

**Adsorption Chromatography**

The crude extract (86.7 g) was subjected to an active charcoal treatment. It was adsorbed on a stationary phase consisting in a mixture of charcoal/celite/polyamide (10:1:1) (60.0 g) until no reaction to vanillin/HCl spray reagent occured according to a method reported in the patent by Ballero et al. [13]. The resulting suspension was then stratified on a Gooch funnel and eluted first with H$_2$O to eliminate the mono- and disaccharides. These usually coelute with glycosidic constituents in silica gel chromatography and may interfere during the structure identification of the metabolites of interest. The desorption chromatography was conducted in steps using EtOH/H$_2$O mixtures gradually increasing in EtOH percentages (30, 60, and 95%). In this manner, three fractions at different polarity were collected, and after freeze drying 23.9, 4.70, and 0.82 g of solids were recovered, from the 30, 60 and 95% ethanolic elutions, respectively.

**Silica Gel Column Chromatography**

After a preliminary screening on thin layer chromatographies and paper chromatography, the separation step on silica gel column was first conducted on the 60% eluate because this showed the whole iridoid components and in comparable amount among them in respect to the other fractions. The 50% fraction, containing the more polar compounds, was partitioned in a second step. The 95% fraction did not contain iridoid glucosides and was not even considered.

The first chromatographic separation was performed on an aliquot of the 60% ethanolic elution for the weight of 4.0 g using 120.0 g of silica gel (ratio 1:30). The eluting system was a mixture of n-BuOH/H$_2$O (5:2:18 v/v). From this chromatographic separation (Scheme 1) 11 compounds were isolated and identified by comparison with data reported in literature and/or by comparison with pure compounds available in our laboratory.
Verbascoside (10, 12.4 mg) [14] from the assembly of fractions 8-15; stachyoside A (11, 11.2 mg) [8,15] in mixture with verbascoside (12) in the assembly of fractions 17-24; β-arbutin (9, 3.2 mg) [16] from the assembly of fractions 30-32; reptoside (6) [17] and 6-O-acetyl-ajugol (7) [18] in mixture in ratio 1:1.5 (25.0 mg) from the assembly of fractions 45-45; ajugoside (1, 113.7 mg) [17,19] as pure compound from the assembly of fractions 53-54; 8-O-acetyl-harpagide (2) [1, 2.0 g] [20] from the assembly of fractions 70-88; chlorogenic acid (8) [21] in mixture with 8-O-acetyl-harpagide (2) in ratio 1:2 (109.4 mg) from the assembly of fractions 95-112; harpagide (3) [22] and chlorogenic acid (8) in mixture 3:1 (48.3 mg) from the assembly of fractions 118-122; allabetonic acid (4) and chlorogenic acid (8) in mixture 10:1 (29.1 mg) from the assembly of fractions 133-135; allabetonic acid (4, 100.7 mg) [23] as a pure compound from the assembly of fractions 144-163; 4′-O-galactopyranosyl-teuchicoside (5) [6] in mixture with allabetonic acid (4) in ratio 3:1 (24.7 mg) from the assembly of fractions 166-169.

A second chromatographic column was, instead, conducted on an aliquot of the 30% desorption solids (3.5 g). Similar compounds were isolated from this chromatographic step. In particular, harpagide (3), 8-O-acetyl-harpagide (2) as pure compounds, allabetonic acid (4) in mixture with 4′-O-galactopyranosyl-teuchicoside (5), and ajugoside (1) occurring in traces (relative quantities not estimated). On the other hand, phenolic compounds as well as reptoside (6) and 6-O-acetyl-ajugol (7), were not found.

### Spectral Data of the Isolated Compounds

**Ajugoside (1):** NMR data see Table 1; ESI-MS: m/z 412.80 [M+Na]+.

| Position | δ, ′H | Multiplicity, J (Hz) | δ, 13C (HSQC) | HMBC Correlations |
|----------|-------|---------------------|--------------|-------------------|
| 1        | 5.94  | d, J = 1.1          | 93.73        | C4, C5, C6         |
| 2        |       |                     |              |                   |
| 3        | 6.31  | d, J = 6.4, 2.3     | 139.72       | C3, C4, C5, C6, C7|
| 4        | 4.89  | ddd, J = 6.4, 2.1, 1.1 | 103.46   | C3, C4, C5, C6, C7|
| 5        | 2.85  | br d, J = 8.3      | 39.83        | C6, C8            |
| 6        | 4.18  | d, J = 4.0         | 75.55        | C6, C7, C8         |
| 7a       | 2.25  | d, J = 15.4        | 46.74        | C4, C5, C6, C7, C10|
| 7b       | 2.18  | dd, J = 15.4, 4.3  |              |                   |
| 8        |       |                     | 89.43        |                   |
| 9        | 2.93  | br d, J = 8.5      | 47.48        | C6, C7, C8         |
| 10       | 1.56  | s                   | 21.49        | C6, C7, C8         |

**Glucose**

| Position | δ, ′H | Multiplicity, J (Hz) | δ, 13C (HSQC) | HMBC Correlations |
|----------|-------|---------------------|--------------|-------------------|
| 1′       | 4.82  | d, J = 8.1          | 98.25        | C1, C2, C3, C5    |
| 2′       | 3.33  | dd, J = 9.3, 8.1    | 72.73        | C2, C3, C5        |
| 3′       | 3.56  | m                   | 75.65        | C1, C4, C5, C6    |
| 4′       | 3.44  | m                   | 69.65        | C4, C5, C6        |
| 5′       | 3.52  | m                   | 76.26        | C5, C6            |
| 6′a      | 3.98  | d, J = 12.5, 2.2    | 60.77        | C5, C6, C7        |
| 6′b      | 3.78  | d, J = 12.5, 6.0    | 76.77        | C5, C6, C7        |

| CH2CO    | 2.08  | s                   | 175.14       | C4, C10           |

| HSQC: Heteronuclear single quantum correlation, HMBC: Heteronuclear multiple bond correlation |
ESI-MS: m/z 413.04 [M+Na]+.

6-O-acetyl-ajugol (7): 1H NMR (300 MHz, D2O) δ: 6.34 (1H, dd, J = 4.8, 1.8 Hz, H-3), 5.50 (1H, d, J = 2.8 Hz, H-1), 4.89 (1H, dd, J = 6.4, 2.8 Hz, H-4), 4.81 (1H, obscured by the solvent signal, H-6), 4.70 (1H, m, H-2'), 2.79 (1H, br t, J = 6.5 Hz, H-5), 2.34 (1H, dd, J = 7.9, 2.7 Hz, H-9), 2.12 (1H, br dd, J = 14.0, 6.5 Hz, H-7a), 2.08 (3H, s, CH3CO), 1.93 (1H, dd, J = 13.8, 8.6 Hz, H-7b), 1.38 (3H, s, H-10).

ESI-MS: m/z 413.04 [M+Na]+.

Chlorogenic acid (8): 1H NMR (300 MHz, CD3OD) δ: 7.56 (1H, d, J = 15.9 Hz, H-β'), 7.05 (1H, d, J = 1.9 Hz, H-2'), 6.78 (1H, d, J = 8.2 Hz, H-6'), 6.26 (1H, d, J = 15.9 Hz, H-α'), 5.33 (1H, td, J = 9.1, 4.5 Hz, H-3), 4.17 (1H, dd, J = 7.9 Hz, H-7a), 2.20 (3H, s, CH3CO), 2.13-1.98 (2H, m, H-2a; H-6a), 1.09 (3H, d, J = 6.2 Hz, H-6b).

ESI-MS: m/z 377.30 [M+Na]+.

β-arbutin (9): 1H NMR (300 MHz, CD3OD) δ: 7.07 (2H, d, J = 8.6 Hz, H-3, H-5), 6.69 (2H, d, J = 8.6 Hz, H-2, H-6), 4.95 (1H, partially obscured by HDO signal, H-1'), 3.96-3.79 (m, overlapped glucose protons), 3.77-3.54 (m, overlapped glucose protons), 3.18 (1H, br t, J = 7.9 Hz, H-2').

ESI-MS: m/z 311.22 [M+K]+.

Verbascoside (10): 1H NMR (300 MHz, CD3OD) δ: 7.59 (1H, d, J = 15.9 Hz, H-β"), 7.05 (1H, d, J = 1.4 Hz, H-2'"), 6.96 (1H, dd, J = 8.2, 1.4 Hz, H-6"), 6.78 (1H, d, J = 8.2 Hz, H-5'"), 6.69 (1H, br s, H-2"), 6.68 (1H, d, J = 8.0 Hz, H-5"), 6.56 (1H, dd, J = 8.0, 1.6 Hz, H-6'"), 6.28 (1H, d, J = 15.9 Hz, H-α"), 5.19 (1H, d, J = 0.9 Hz, H-1'"), 4.38 (1H, d, J = 7.8 Hz, H-1), 2.79 (2H, br t, J = 7.3 Hz, H-β"), 1.09 (3H, d, J = 6.2 Hz, H-6'').

ESI-MS: m/z 623.19 [M-H]; m/z 647.12 [M+Na]+.

RESULTS

The reassessment of the glycosidic fraction of S. alopecuros subsp. divulsa allowed the isolation of several compounds. The majority of them were iridoids, followed by a phenolic glucoside, two phenylethanoid glycosides, and a caffeoylquinic acid. They were identified as ajugoside (1), 8-O-acetylharpagide (2), harpagide (3), allobetonicoside (4), 4’-O-galactopyranosyl-teuhircoside (5), reptoside (6), 6-O-acetyl-ajugol (7) [Figure 1] for the iridoid glycosides constituents, and as chlorogenic

Figure 1: Iridoids from Alopecurus alopecuros subsp. divulsa
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acid (8), β-arbutin (9), verbascoside (10), and stachysoside A (11) [(2-(3,4-dihydroxyphenyl)ethyl-α-L-arabinopyranosyl−(1→2)-O-6-deoxy-β-L-mannopyranosyl−(1→3)-4-O-(2E)-3-(3,4-dihydroxyphenyl)-1-oxo-2-propen-1-yl)-β-D-glucopyranoside] [Figure 2] for the phenolic compounds. The presence of previously identified iridoid glycosides (2-5) has been confirmed, together with ajugoside (1), reptoside (6), and 6-O-acetyl-ajugol (7) which resulted to be new iridoid components for this species. Among the phenolic constituents, chlorogenic acid (8), β-arbutin (9), and stachysoside A (11) were isolated for the first time from the studied species [Figure 1].

**DISCUSSION**

**Chemosystematic Implications of Isolated Compounds**

The identified iridoids have all chemosystematic relevance. In particular, 2 and 3 are considered family markers, 4 is characteristic of the section Betonica, and 5 has never been reported in other Stachys species so that it may be regarded as a chemotaxonomic marker at a subspecific level. Reptoside (6) resulted to be a rare metabolite in the Stachys genus because its occurrence has been reported only in a few species, namely Betonica officinalis (syn. of Stachys officinalis) [23] and S. macrantha [24]. 6-O-acetyl-ajugol (7), is also a rare metabolite in the Lamiaeae family since it has been found only in Leonurus persicus so far [18]. It is also worth of mention the presence of ajugoside (1) and reptoside (6) which are iridoids widely distributed in the species of the Ajugoideae subfamily. In fact, 1 was mainly reported in the Ajuga genus (e.g. Ajuga tenorei and Ajuga chamaepitys) [25,26], although its presence was also signaled in a few Lamioideae species as Melittis melissophyllum subsp. melissophyllum [19] and species of the genera Stachys and Sideritis. The occurrence of 1 in the Stachys genus is limited to a few species such as B. officinalis (syn. of S. officinalis (L.) Trevis.) [2], S. macrantha [24], S. alpina [2], Stachys germanica [2,27] and Stachys recta [2], and it has been identified also in the taxonomically related Sideritis genus like for instance in Sideritis romana [28], and Sideritis perfoliata subsp. perfoliata [29].

Considering their occurrence, compounds (1) and (6) may represent chemosystematic traits of proximity between Lamioideae and Ajugoideae subfamilies. The identification of 1 has been carried out by extensive NMR analysis on both mono-[Figures 3 and 4] and bidimensional [Figures 5 and 6] experiments since the assignment of resonances was not immediate for several signals, in particular for protons in 5, 6 and 9 positions on the iridoid skeleton as well as for protons in 3′ and 5′ positions of the glucose moiety [Figures 3 and 4].

**Figure 2:** Phenolics from Alopecurus alopecuros subsp. divulsa

**Figure 3:** 1H-NMR spectrum of ajugoside (1)
In particular, the resonance of H-6 was readily visible in the proton spectrum at 4.18 ppm, but the corresponding carbon signal was very near to a glucose carbon resonance and has been assigned by the direct HSQC correlation [Figure 5] with the signal resonating at 75.55 ppm. On the contrary, the assignment of protons and carbons in the position 5 and 9 was not immediate, and several long range correlations resulted to be crucial for discriminating the resonances of these nuclei. The signals relative to the positions 5 and 9 present resonances in the proton spectrum at 2.93 and 2.85 ppm, both as broad signals, and the study of the coupling constants gave no help for the structure elucidation. In the HSQC spectrum, a direct

**Figure 4:** $^{13}$C-NMR spectrum of ajugoside (1)

**Figure 5:** Heteronuclear single quantum correlation spectrum of ajugoside (1)
correlation between the proton signal at 2.93 ppm and the carbon resonating at 47.48 ppm was recorded as well as the correlation between the proton at 2.85 and the carbon at 39.83 ppm. This permitted to assign the respective carbons signals. In the HMBC experiments, [Figure 6] the only diagnostic correlation which permitted to differentiate the positions 5 and 9 was a long range correlation between H-3 (6.31 ppm) and the carbon resonating at 39.83 ppm, which could be assigned to C-5 of the iridoid skeleton, since almost all the proton signals correlated with both the carbons at 39.83 and 47.48 ppm. In fact, H-3 is relatively far from C-9, and this kind of signal could not be visualized with the coupling constant of 8 Hz set in the experiment. Using the HMBC correlations, it was also possible to differentiate the positions 3' and 5' on the glucopyranose unit. In particular, the resonances of the positions 1', 2', 4', and 6' (98.25, 72.73, 69.65, and 60.77, respectively) were readily assignable to the saccharidic moiety, while the signals relative to 3' and 5' might be differentiated on the basis of their correlation with near nuclei [Figures 5 and 6].

More specifically, from HMBC experiments the following long-range correlations were recognized: Between the proton signal at 3.33 ppm (H-2') and the carbon at 75.65 (C-3'); the proton at 3.34 ppm (H-4') with carbons at 76.26 (C-5') and 75.65 (C-3'); the protons at 3.98-3.78 (H-6') with carbon resonating at 76.26 (C-5'). The latter correlation is particularly crucial for the assignation of the C-5' position because H-6' cannot correlate with C-3'. The complete assignments of resonances are reported in Table 1.

Chlorogenic acid (8) has been isolated for the first time from \textit{S. alopecuros} subsp. \textit{divulsa}, although it has been already found in several \textit{Stachys} species as \textit{S. tymphaea} [8], \textit{S. lanata} [30], \textit{S. officinalis} [31], \textit{S. recta} [32], \textit{S. byzantina}, \textit{S. iberica} subsp. \textit{iberica} var. \textit{densipilosa} [33], and \textit{S. glutinosa} [34]. In most of these species, the chlorogenic acid (8) resulted to be the main polyphenolic component occurring in the polar fraction just like in this case [Figure 2].
Furthermore, β-arbutin (9) was a metabolite identified for the first time in this species. It proved to be a quite rare compound in the Stachys genus since its presence has been signaled only in S. germanica subsp. salvifolia [7]. The presence of verbascoside (10), a quite common phenylethanoid glycoside (PhGs) in Lamiaceae, has been confirmed, while stachysoside A (11), also known with the name lavandulifolioside, resulted to be as a new constituent of S. alopecurus subsp. divulsa. PhGs are compounds with a chemosystematic relevance in Asterids since their cooccurrence with iridoid glycosides, as in the studied case, has been largely confirmed [35]. The presence of 11 was noteworthy since it is not an ubiquitous compound, especially in the Stachys genus where it has been recognized only in a few species such as S. lavandulifolia [36], S. sieboldii [15], and S. tymphaea [8]. This compound resulted to be a constituent of other systematically related genera such as Leonurus [37,38], Lagochilus [39], and Sideritis [40].

Bioactivities of Isolated Compounds

The isolated compounds are responsible for several interesting activities. In particular, iridoids are known to possess anti-inflammatory properties. Harpagide (3) and its derivatives are constituents of Harpagophyllum procumbens D.C. (Pedaliaceae), a medicinal plant native to Namibia and commonly known as Devil’s Claw. This plant has been widely studied for its anti-inflammatory and analgesic effects which were demonstrated also in in vivo models, principally in rats [41-43]. The target of harpagide and its derivatives is represented by the inhibition of COX-2, an enzyme deputed to the production of the proinflammatory mediators prostaglandins and leukotrienes. Recent docking studies have demonstrated that harpagide is a selective inhibitor of COX-2, binding its active site with a strong interaction and being stabilized by 10 hydrogen bonds [44]. This confirms, on a molecular base, the observed anti-inflammatory and analgesic actions exerted by plants containing harpagide derivatives. Beside the anti-inflammatory action, harpagide (3) results a modulator of Tumor necrosis factor α (TNF-α) secretion and induces the expression of several proteins involved in cell migrations. It shows an immunomodulatory effect facilitating the leukocyte migration in the inflamed tissues [45]. The latter is a very important role because the inflammatory process is not only responsible of pain and loss of function but it is also involved in the onset and progression of degenerative diseases, including cardiovascular disorders and cancer. In a similar way, 8-O-acetyl harpagide (5) also showed a dose-related inhibition of leukocyte adhesion and transmigration in a system based on human umbilical vein endothelial cells, and human monocytic leukemia cell line THP-1 previously activated with TNF-α [46].

It was also studied, the protective effect of harpagide (3) against bone loss in ovariectomized mouse model. It resulted that this iridoid stimulates the differentiation and maturation processes of osteoblasts and this conducted to an overall improved bone properties with the recovery of mineral density. The reduced levels of biochemical markers of bone loss in the serum of treated mice (alkaline phosphatase, osteocalcin, and C-terminal telopeptide) seems to be the major mode of action of harpagide (3) [47], thus demonstrating the potential role of this iridoid in the prevention and treatment of age-dependent bone osteoporosis diseases.

From recent studies on iridoid activities, 8-O-acetyl harpagide (2) and ajugoside (1) resulted to be useful compounds in the treatment of diabetes mellitus since they were able to stimulate the cellular glucose intake and lower the glucose blood level [38]. Another target of iridoids for the antidiabetic activity is represented by the inhibitory action, verified by docking studies, on glycogen phosphorylase-α [48]. In fact, several plant species rich in iridoids have been largely used in traditional medicine for the treatment of diabetes, e.g., Scrophularia deserti Del. [49] and Leonurus sibiricus L. [58].

The phenolic fraction of S. alopecurus subsp. divulsa was mainly composed by chlorogenic acid (8), which is also endowed with interesting activities [34]. This caffeoylquinic acid is a strong antioxidant [50], a good wound healer if topically applied on excisions [51] and has also antiviral action toward hepatitis B virus [52]. Furthermore, it shows antiproliferative action [34], inhibits carcinogenesis, strongly reduces the risk of cardiovascular diseases and significantly improves the metabolism of glucose. The latter is the main reason why it has recently drawn the attention from nutritionists for its high anti-diabetic and anti-obesity properties [53]. The antioxidant and enzyme inhibitory activities in respect to acetylcholinesterase, butrylcholinesterase, and α-amylase [53] may have a protective role in neurologic diseases such as Alzheimer’s disease, and metabolic disorders as diabetes mellitus.

The other phenolic constituents also have interesting biological activities. In fact, β-arbutin (9) possesses a strong antibacterial activity and is traditionally used for urinary tract infections [54]. It is also a tyrosinase inhibitor and is employed as a skin whitener because it inhibits the melanin synthesis [55].

The phenylethanoid glycoside verbascoside (10) shows an antibacterial action especially toward S. aureus [56] and anti-inflammatory activity [57]. More recently, in an in vivo study, it resulted to be an inhibitor of xanthine oxidase which reduced uric acid concentration in rat serum. These results may suggest its potential use to treat hyperuricemia [58].

For what concerns the activity of stachysoside A (11), in literature there are mainly studies conducted on extracts obtained from species containing this compound. In this context, it is noteworthy the neuroprotective effects observed in S. sieboldii tuber extract which resulted to improve the memory dysfunction associated with vascular dementia or Alzheimer’s disease in induced-ischaemia in mice [59]. The presence of antioxidant components resulted to have a primary role in neuroprotection and PhGs are well-known antioxidants. Species belonging to Leonurus genus, all containing (11) together with iridoids, have been largely used in the traditional Mongolian medicine for the treatment of diabetes mellitus [38] and in the treatment of neurological disorders such as anxiety, depression, and nervousness [37].
CONCLUSIONS

This phytochemical study on the endemic *S. alopecuroides* subsp. *divulsa* shed light on three additional iridoid glucosides together with chlorogenic acid (8), β-arbutin (9), verbascoside (10), and stachyoside A (11) in the phenolic fraction. The presence of verbascoside has been already reported, while the other phenolics were identified for the first time in the studied species. The monoterpene glycoside pattern owned by *S. alopecuroides* subsp. *divulsa* has been disclosed with respect to literature data, showing the presence of ajugoside (1), reptoside (6), and 6-O-acetyl-ajugol (7). All these compounds have chemosystematic relevance. In particular, 1 and 6 may represent a metabolic connection between Lamioideae and Ajugoideae subfamilies while 7 is a quite uncommon compound in the Lamiaceae family since it has been found only in *L. persicus* so far. We also took advantage of this occasion to report a complete NMR assignment for compound 1. From a therapeutic perspective, the isolated compounds are known for their antioxidant, anti-inflammatory, analgesic, immunomodulatory, neuroprotective, and anti-diabetic actions. Their occurrence in the studied species provides evidence of a possible natural source of bioactive compounds for extractive purposes but also of its potential as a natural remedy of medical importance.

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