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Determination of Iridoid Glycosides from Four Turkish *Lamium* Species by HPLC-ESI/MS

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An HPLC-ESI/MS method that enables fast detection and identification of iridoid glycosides is described. Eleven iridoid glycosides known to occur in the genus *Lamium*-lamalbide, sesamoside, 6-β-OH ipolamiide, shanzhiside methyl ester, dehydropenstemoside, barlerin (= 8-*O*-acetylshanzhiside methyl ester), 6-*O*-syringyl-8-*O*-acetylshanzhiside methyl ester, lamerioside, lamiide, eriobioside, and ipolamiide, were identified by means of their retention time and ESI/MS data. This method was successfully applied to the identification of the iridoid composition of the *n*-butanol extracts of *Lamium eriocephalum* Bentham subsp. *eriocephalum*, *L. garganicum* L. subsp. *pulchrum* R. Mill, *L. garganicum* L. subsp. *laevigatum* Arcangeli, and *L. purpureum* L. var. *purpureum* from the Turkish flora.

Key Words: *Lamium*, Lamiaceae, iridoid glycosides, HPLC, ESI/MS.

Introduction

Lamium L. (Lamiaceae) is a genus that includes almost 40 annual or perennial herbaceous plants distributed throughout Europe, Asia, and Africa.[1] *Lamium* species are known to exhibit antispasmodic, anti-inflammatory, antioxidant, free radical scavenging, and antiproliferative properties, and therefore have been extensively used in folk medicines for the treatment of trauma, fractures, putrescence, paralysis, leucorrhoea, and hypertension, as well as some gynecological conditions, such as menorrhagia, uterine hemorrhage, and vaginal or cervical inflammation.[2–7] The genus *Lamium* is represented by 30 species in the flora of Turkey.[8,9]

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The *L. album* plant is used as a folk remedy to relieve the pain of rheumatism and other arthritic ailments in western Anatolia.[10] In addition to *L. album*, *L. maculatum* and *L. purpureum* are used as tonics and for the treatment of constipation in Anatolian folk medicine.[11] *L. eriocephalum* subsp. *eriocephalum*, *L. garganicum* subsp. *laevigatum*, *L. garganicum* subsp. *pulchrum*, and *L. purpureum* var. *purpureum* from the Turkish flora have been reported to show anti-inflammatory and antinociceptive activity,[12] as well as antimicrobial and free radical scavenging properties.[13]

Lamium species are characterized mainly by iridoid glucosides.[14−23] Recently, we reported the iridoid composition of *L. garganicum* subsp. *laevigatum*[24] and *L. eriocephalum* subsp. *eriocephalum.*[25] To date, a number of papers on the application of different liquid chromatography-mass spectrometry (LC-MS) techniques for the phytochemical screening of iridoid glycosides have been published.[26−32] Recently, the efficiency of high performance liquid chromatography with mass spectrometry coupled with an electrospray ionization interface (LC-ESI/MS) has been reported for the identification of the iridoids in *Lamium* species from Bulgaria.[33]

As part of our continuing study on the diversity of iridoids in Turkish *Lamium* species, herein we report the results of LC-ESI/MS analysis of the iridoid glycosides of 2 endemic *Lamium* species, *Lamium eriocephalum* Bentham subsp. *eriocephalum* and *L. garganicum* L. subsp. *pulchrum* R. Mill, together with *L. garganicum* L. subsp. *laevigatum* Arcangeli and *L. purpureum* L. var. *purpureum* from the Turkish flora.

Experimental

Plant Material: *Lamium eriocephalum* Bentham subsp. *eriocephalum* (LEE) and *L. garganicum* L. subsp. *pulchrum* R. Mill (LGP) were collected from Aladağlar, Niğde (altitude: 2200 m) in June 2002. *L. garganicum* L. subsp. *laevigatum* Arcangeli (LGL) was collected from Uludağ, Bursa (altitude: 2300 m) in July 2005. *L. purpureum* L. var. *purpureum* (LPP) was collected from the Sıhhiye Campus of Hacettepe University, Ankara (altitude: 850 m) in March 2006. Voucher specimens were deposited in the Herbarium of Hacettepe University Faculty of Pharmacy, Ankara, Turkey (HUEF 02045, 02046, 05004, and 06005, resp.).

Extraction: The air-dried and powdered aerial plant material (5 g) of each specimen was extracted with methanol (50 mL) for 5 h at 40 °C under reflux and concentrated to dryness under reduced pressure. Concentrated methanolic extract was then suspended in water (50 mL) and partitioned with dichloromethane (2 × 25 mL) and *n*−butanol (2 × 25 mL), individually. The *n*−butanol extract was then dissolved in 50% v/v aqueous methanol and after filtration through a membrane filter (0.45 μm) a 20-μL aliquot was injected.

Iridoid standards: Lamalbide (**2**), sesamoside (**3**), 6-β-OH ipolamiide (**5**), shanzhiside methyl ester (**6**), dehydropenstemoside (**9**), barlerin (= 8-*O*-acetylshanzhiside methyl ester) (**10**), and 6-*O*-syringyl-8-*O*-acetylshanzhiside methyl ester (**11**) were previously isolated from *L. garganicum* subsp. *laevigatum.*[24] Lamerioside (**1**), lamiide (**4**), eriobioside (**7**), and ipolamiide (**8**) were isolated from *L. eriocephalum* subsp. *eriocephalum*[25]. The identity and purity of each standard compound were confirmed by HPLC-ESI-MS and NMR spectroscopy. The reference iridoids were dissolved in 50% v/v aqueous methanol (0.1 mg/mL), filtered through a membrane filter (0.45 μm), and injected (20 μL).

HPLC-ESI/MS Analysis: HPLC-ESI/MS analysis was carried out using a Waters (Milford, MA, USA) Alliance 2695 liquid chromatographic system equipped with a Waters photodiode array detector (PDA, 2996), which was connected to an orthogonal quadrupole mass spectrometer (Micromass ZQ, Manchester, UK). Both systems were controlled by MassLynx v.4.1 software (Micromass, Manchester, UK). An electrospray ionization (ESI) probe was used. The atmospheric pressure chemical ionization function was turned

off during analysis. Chromatographic separations were achieved on a Ace 5 C_{18} (Aberdeen, Scotland) column (150 × 4.0 mm i.d.; 5 μm) with a guard column (4 × 3 mm), using a methanol:water mixture for elution, with a gradient from 10:90 to 50:50 for the first 25 min and from 50:50 to 65:35 for the next 5 min, followed by elution with pure methanol for another 10 min. The flow-rate was 0.8 mL/min with the column maintained at 30 °C. ESI mass spectra were recorded, both in positive and negative ion modes. The ESI-MS parameters were as follows: Desolvation gas (N_2) flow rate: 500 L/h; cone gas flow rate: 50 L/h; drying gas temperature: 450 °C; source temperature: 120 °C; capillary voltage: 2.85 kV; cone voltage: 35.0 V; inverted electrical polarity for positive and negative ionization modes; RF lens voltage: 0 V for positive-ion mode and 0 V for negative-ion mode. The acquisition time was set to 0.4 s for both positive and negative ionization modes. The mass range was 200-1300 m/z for both positive and negative ion modes. A 0.2-s change-over time between the modes was applied.

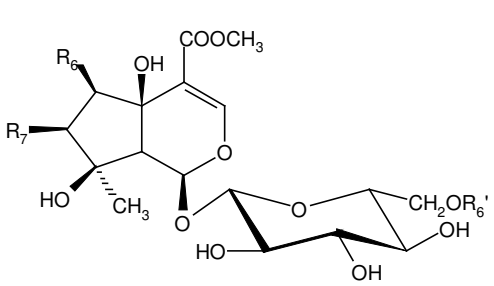
Results and Discussion

Eleven C_{10} iridoid glycosides (**1**-**11**) known to occur in various species of *Lamium* were analyzed by HPLC-ESI/MS for their occurrence in *L. eriocephalum* Bentham subsp. *eriocephalum*, *L. garganicum* L. subsp. *pulchrum* R. Mill, *L. garganicum* L. subsp. *laevigatum* Arcangeli, and *L. purpureum* L. var. *purpureum*. All iridoid references have a C-4 methoxycarbonyl substituent and differ according to the position and number of hydroxyl groups, or the presence of an epoxy substituent or a double bond in the cyclopentane ring. Except for eriobioside (**7**) all iridoids are monoglucosides. The structures of the reference iridoids are given in Figure 1. The retention time (t_R) and ESI/MS fragmentation data for each compound are listed in Table 1. Typical HPLC-MS chromatograms of the $n-$butanol extracts of the studied *Lamium* species are presented in Figure 2 (A-D). Evaluation of the retention time and MS data of the reference compounds allowed establishment of the iridoid characteristics of the *Lamium* extracts. Further information was obtained by spiking the extracts with each of the standard iridoids and repeating the analysis. In each case the identification was verified.

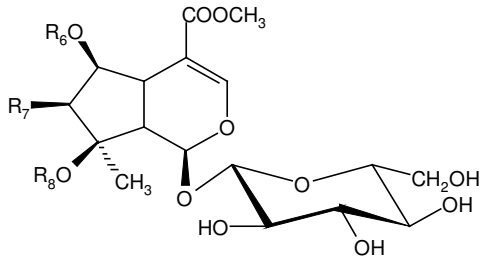
HPLC-ESI/MS characteristics of the reference iridoid glycosides

For all iridoid glycosides, a quasimolecular ion $[M+Na]^+$ in the positive ion mode was observed as the base peak. Abundant $[M+K]^+$ adducts were also observed for almost all iridoids, except 6-β-OH ipolamiide (**5**) and 6-*O*-syringyl-8-*O*-acetylshanzhiside methyl ester (**11**). Na^+ and K^+ adducts are often described for ESI mass spectra in the positive ion mode. These alkali ions are generally extracted from the glassware used for storage or during analysis. It was demonstrated that the substituents in the iridoid structure exhibited great effect on the formation of alkali adducts and the sugar moiety was also responsible for the chelation of Na^+ and K^+ [34]. Furthermore, a characteristic cluster ion at $[2M+Na]^+$ was common for all reference compounds. $[M+H]^+$ ions, generally characteristic of many glycosides in this case, however, could not be detected.

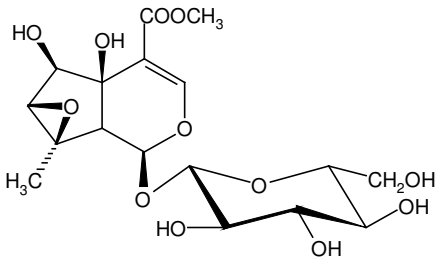
Compared with the positive ion spectra, there were many informative fragment ions in the negative ESI/MS and, therefore, further discussion will be limited to the latter. Intensive $[M-H]^-$ ions were produced under negative ion MS conditions for all reference samples, making the determination of molecular mass easier. The $[M+35]^-$ ions for which the involvement of water seemed to be necessary to produce were tentatively assigned to $[M-H+2H_2O]^-$.[33] Cluster ions corresponding to $[2M-H]^-$ were also observed in all cases. For compounds containing an acetoxy group (**10** and **11**) a unique $[M-H-HOAc]^-$ fragment ion, due to the loss of acetic acid, was observed.



	R_6	R_7	$R_{6'}$
1	H	α-OH	H
4	H	OH	H
5	OH	H	H
7	H	H	β-glucopyranose
8	H	H	H



	R_6	R_7	R_8
2	H	OH	H
6	H	H	H
10	H	H	Acetyl
11	Syringyl	H	Acetyl



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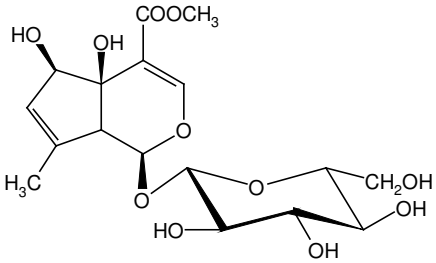

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Figure 1. Structures of the iridoid glycosides used as reference compounds.

Iridoids identified in the *n*-butanol extracts of four *Lamium* extracts

The $n-$butanol extracts from aerial parts of 4 *Lamium* species were analyzed for their iridoid content. The presence of iridoid compounds was verified by their retention times, mass spectra of both the positive and negative mode, and formation of cluster ions and fragments.

In the HPLC-ESI/MS analysis of *L. garganicum* subsp. *laevigatum* the iridoid glucosides lamalbide (**2**), sesamoside (**3**), 6-β-OH ipolamiide (**5**), shanzhiside methyl ester (**6**), dehydropenstemoside (**9**), barlerin (= 8-*O*-acetylshanzhiside methyl ester) (**10**), and 6-*O*-syringyl-8-*O*-acetylshanzhiside methyl ester (**11**) were detected. These iridoid glucosides were previously isolated and identified from this plant.[24] In addition to these compounds, another iridoid glucoside,(**4**) occuring in small amounts, showed a molecular mass of 422 and displayed the same fragmentation pattern as that of lamalbidee (**2**) and 6-β-OH ipolamiide (**5**), suggesting a closely-related structure. Lamiide (**4**) was readily identified by its retention time and MS fragmentation data. Dehydropenstemoside (**9**) was the major constituent of *L. garganicum* subsp. *laevigatum*, following compounds **10** and **3**. Penstemoside, previously reported to occur in *L. garganicum* from the Bulgarian flora,[33] however, could not be detected in the Turkish specimen. *L. garganicum* subsp. *laevigatum* could be considered the richest among the screened *Lamium* species because of its iridoid content.

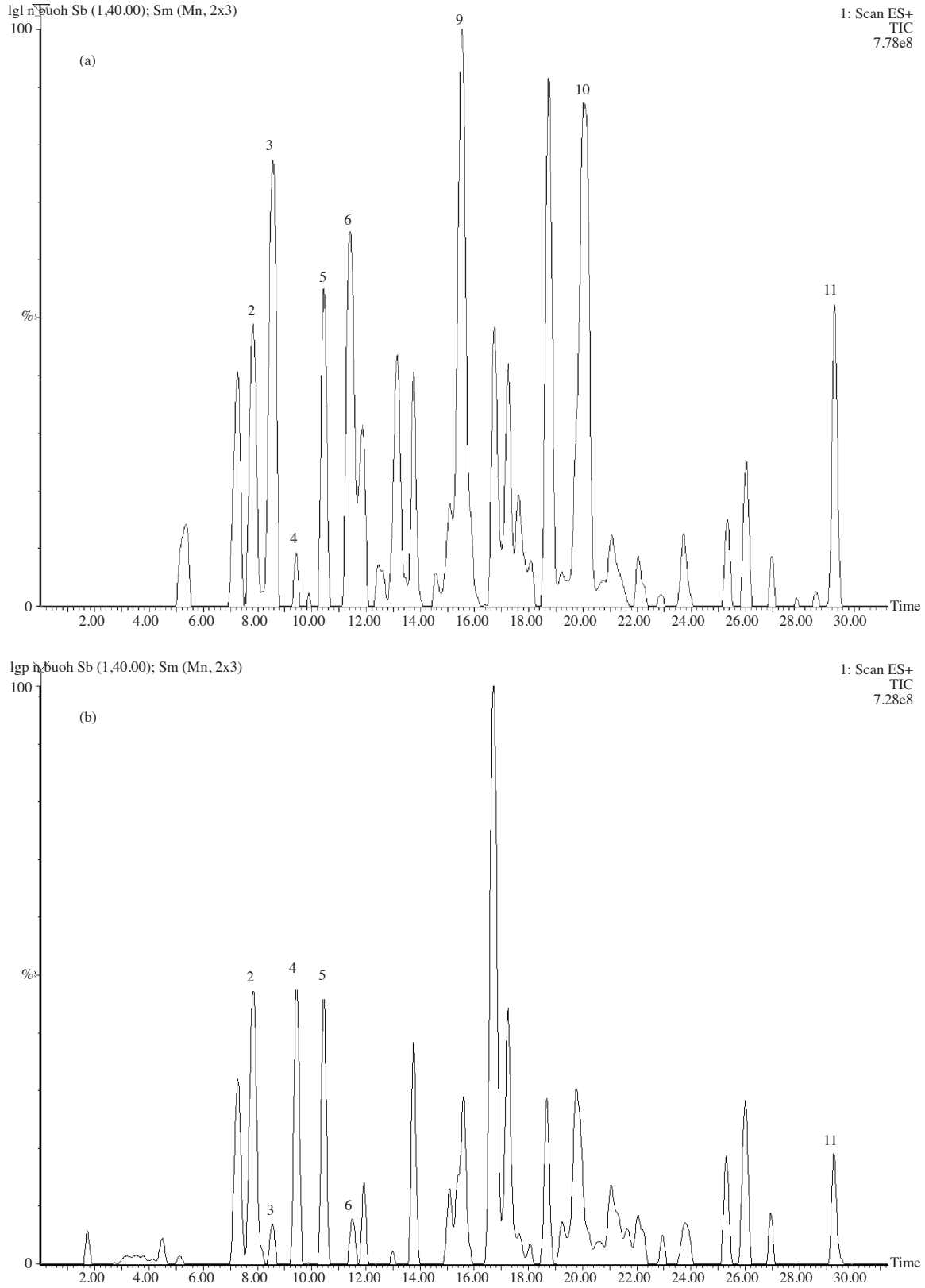

Figure 2. HPLC chromatograms of the *n*-butanol extracts of *Lamium* species. (a): *L. garganicum* subsp. *laevigatum*; (b): *L. garganicum* subsp. *pulchrum*; (c): *L. eriocephalum* subsp. *eriocephalum*; (d): *L. purpureum* var. *purpureum*.

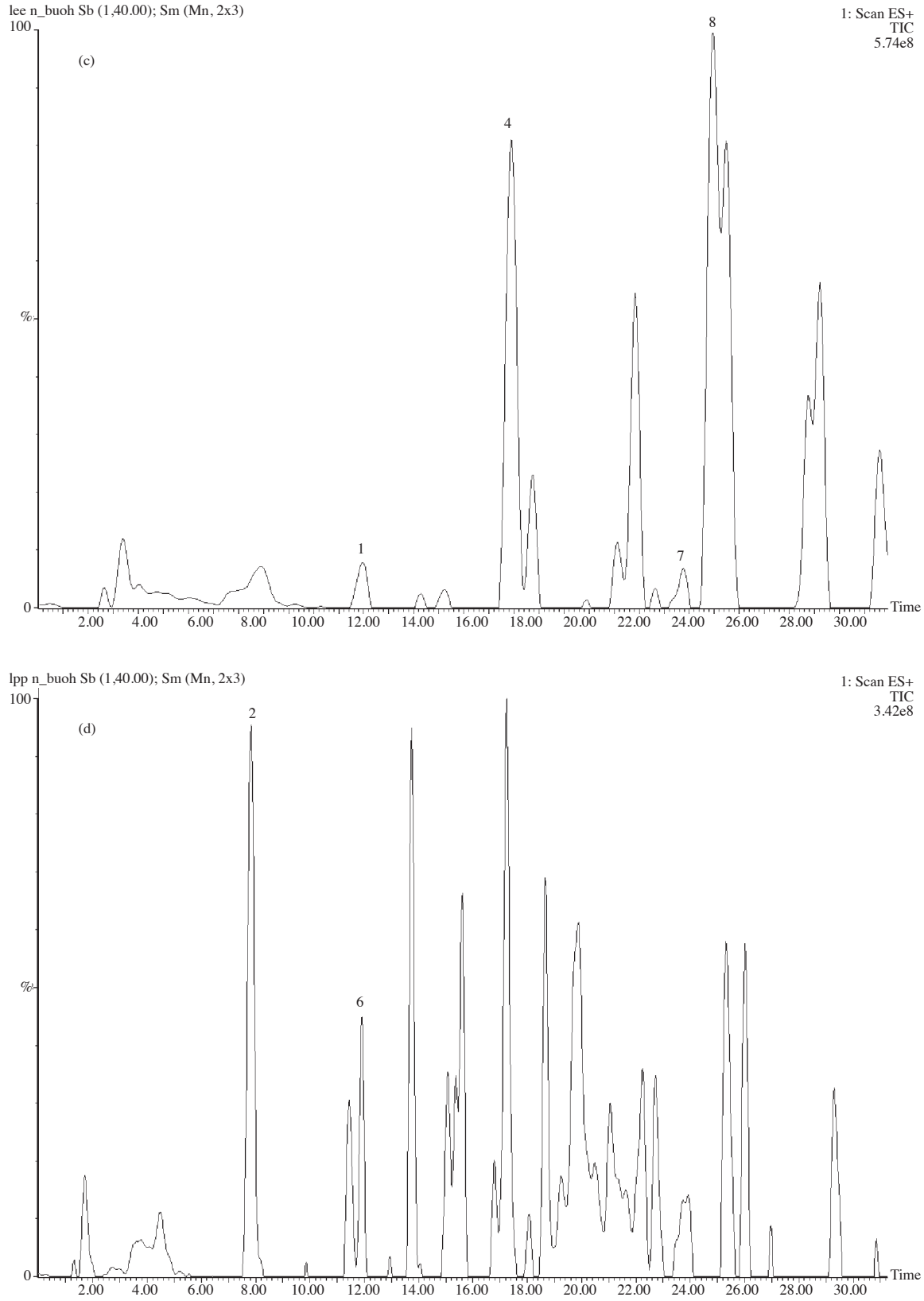

Figure 2. Continued.

Table 1. Retention time and ESI/MS fragmentation of iridoid references.

Compound No	Retention time (min)	Molecular mass	Cluster ion	Quasi molecular ions		Cluster ion	Quasi molecular ion	Fragment ions					
			$[2M+Na]^+$	$[M+K]^+$	$[M+Na]^+$	$[2M-H]^-$	$[M-H]^-$	$[M-H+2H_2O]^-$	$[M-H-HOAc]^-$	$[M-H-H_2O]^-$	$[Aglycone-H]^-$	$[Aglycone-H-HOAc]^-$	$[Aglycone-H-OCH_3]^-$
1	6.68	422	867[a]	461[a]	**445**[f]	843[c]	**421**[f]	457[c]		403[b]	259[b]		
2	8.05	422	867[a]	461[a]	**445**[f]	843[a]	**421**[f]	457[c]			259[b]		
3	8.57	420	863[b]	459[a]	**443**[f]	839[d]	**419**[f]	455[e]		401[c]	257[d]		
4	9.42	422	867[a]	461[a]	**445**[f]	843[e]	421[d]	**457**[f]		403[c]	259[a]		
5	10.36	422	867[a]	461[a]	**445**[f]	843[c]	**421**[f]	457[c]			259[b]		
6	11.40	406	835[a]	445[a]	**429**[f]	811	**405**[f]	441[c]			243[b]		212
7	12.77	568	1159[a]	607[a]	**591**[f]	1135[d]	**567**[f]	603[c]		549[d]	243[b]		
8	13.51	406	835[a]	445[a]	**429**[f]	**811**[f]	405[e]	441[e]		387[b]	243[b]		
9	15.58	404	831[a]	443[a]	**427**[f]	807[b]	**403**[f]	439[b]			241[b]		
10	20.21	448	919[a]	487[b]	**471**[f]	895[c]	**447**[f]	483[c]	387[c]		285[c]	225[c]	
11	29.46	628	1279[b]	667[a]	**651**[f]	1255[b]	**627**[f]	663[b]	567[a]		465[b]		

The relative intensities of ions are shown as: [a]1-10%; [b]11-20%; [c]21-50%; [d]51-80%; [e]81-99%; [f]100% (base peak).

In the extract of closely related *L. garganicum* subsp. *pulchrum*, 6 iridoid glucosides, lamalbide (**2**), sesamoside (**3**), lamiide (**4**), 6-β-OH ipolamiide (**5**), shanzhiside methyl ester (**6**), and 6-*O*-syringyl-8-*O*-acetylshanzhiside methyl ester (**11**), were detected. Lamiide (**4**) and 6-β-OH ipolamiide (**5**) were the major iridoid glucosides of this subspecies; however, the dehydropenstemoside (**6**) and barlerin (**10**) detected in *L. garganicum* subsp. *laevigatum* were not found in the extract. *L. garganicum* subsp. *pulchrum* is considered the second most iridoid-rich *Lamium* specimen.

In the case of *L. eriocephalum* subsp. *eriocephalum*, the previously reported iridoid glycosides lamerioside (**1**), lamiide (**4**), eriobioside (**7**), and ipolamiide (**8**) were easily detected in the extract. Lamerioside (**1**) was identified as the 7α-(OH) derivative of lamiide (**4**);[25] therefore, it displayed the same MS fragmentation pattern as **4**, though retention time data provided information allowing the distinction between these 2 isomers. Moreover, lamerioside (**1**) and lamiide (**4**) were distinguishable by their fragmentation pattern. Lamerioside (**1**) exhibited a cluster ion $[M\text{-}H]^-$ (m/z 421; 100%) as the base peak, while the $[M\text{-}H+2H_2O]^-$ (m/z 457; 100%) fragment ion was the base peak for lamiide (**4**). In addition, lamiide (**4**) exhibited a cluster ion $[2M\text{-}H]^-$ (m/z 843; 90.61%) with higher intensity than that of lamerioside (**1**) ($[2M\text{-}H]^-$ m/z 843; 32.42%) (Figure 3).

Only lamalbide (**2**) and shanzhiside methyl ester (**6**) were identified in *L. purpureum* var. *purpureum*, whilst barlerin (= 8-*O*-acetylshanzhiside methyl ester) (**10**), previously reported from the Bulgarian species of *L. purpureum*,[33] could not be found in the specimens of Turkish origin.

Iridoid glucosides detected in the *n*-butanol extracts of the studied *Lamium* species are given in Table 2. The occurrence of the iridoid glucosides in *L. garganicum* subsp. *pulchrum* and *L. purpureum* var. *purpureum* is reported for the first time. In addition, lamiide was noted for the first time in *L. garganicum* subsp. *laevigatum*. The HPLC-ESI/MS method used in this study could be considered a sensitive and rapid method that provides valuable information for iridoid screening in the genus *Lamium*.

Table 2. Iridoid glycosides detected in the studied *Lamium* species.

Iridoid Glycosides	Extracts			
	LGL	*LGP*	*LEE*	*LPP*
Lamerioside (**1**)			+	
Lamalbidee (**2**)	+	+		+
Sesamoside (**3**)	+	+		
Lamiide (**4**)	+	+	+	
6-β-OH ipolamiide (**5**)	+	+		
Shanzhiside methyl ester (**6**)	+	+		+
Eriobioside (**7**)			+	
Ipolamiide (**8**)			+	
Dehydropenstemoside (**9**)	+			
8-*O*-Acetyl shanzhiside methyl ester (**10**)	+			
6-*O*−Syringyl-8-*O*-acetyl shanzhiside methyl ester (**11**)	+	+		

LGL: *Lamium garganicum* subsp. *laevigatum*; *LGP*: *Lamium garganicum* subsp. *pulchrum*; *LEE*: *Lamium eriocephalum* subsp. *eriocephalum*; *LPP*: *Lamium purpureum* var. *purpureum.*

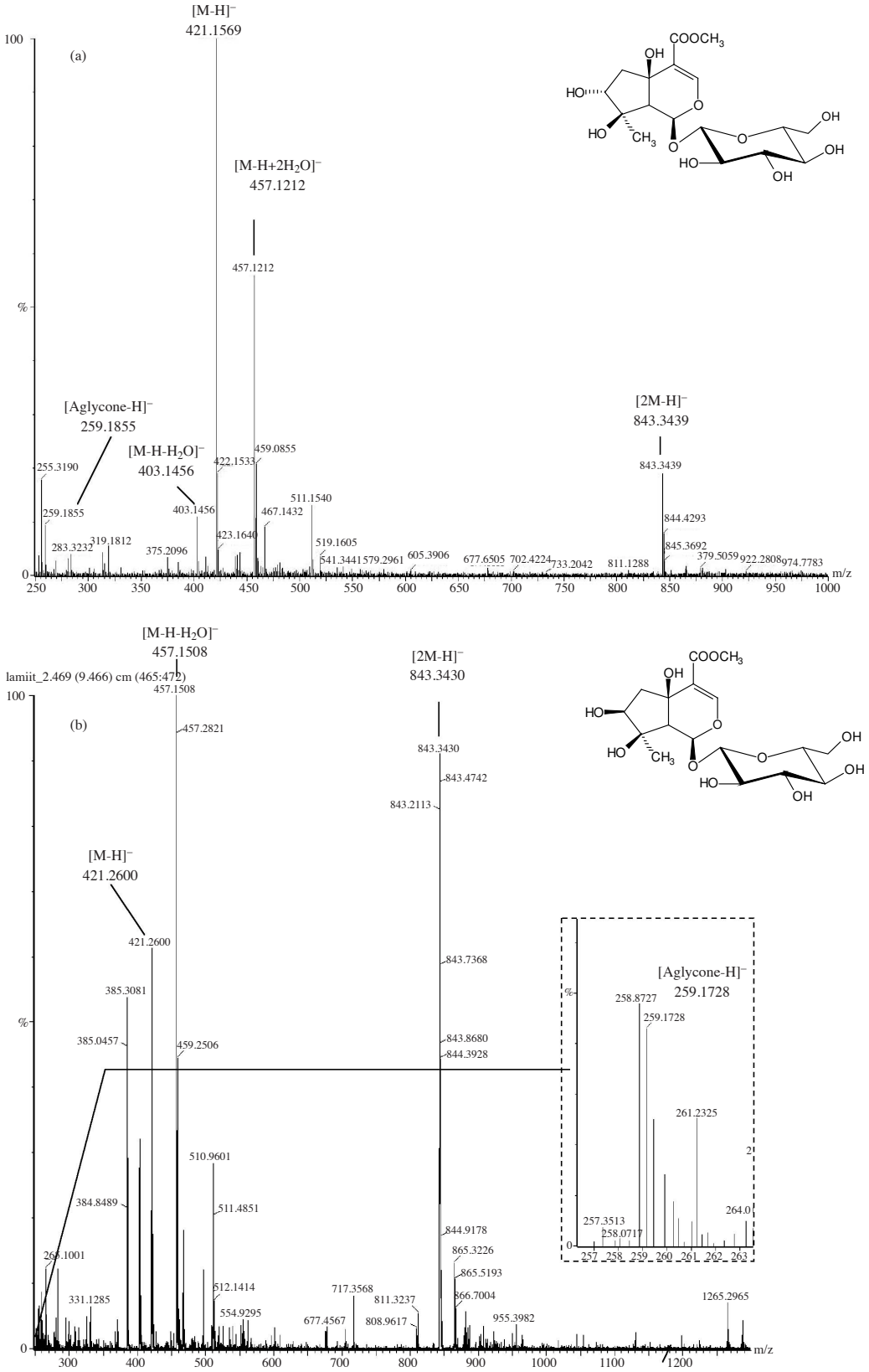

Figure 3. ESI/MS spectra of the epimeric iridoid glucosides lamerioside (**1**) and lamiide (**4**).

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