High-performance liquid chromatography with diode array detection coupled to electrospray time-of-flight and ion-trap tandem mass spectrometry to identify phenolic compounds from a lemon verbena extract

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ABSTRACT

High-performance liquid chromatography with diode array and electrospray ionization mass spectrometric detection was used to carry out the comprehensive characterization of a lemon verbena extract with demonstrated antioxidant and antiinflammatory activity. Two different MS techniques have been coupled to HPLC: on one hand, time-of-flight mass spectrometry, and on the other hand, tandem mass spectrometry on an ion-trap. The use of a small particle size C18 column (1.8 μm) provided a great resolution and made possible the separation of several isomers. The UV-visible spectrophotometry was used to delimit the class of phenolic compound and the accurate mass measurements on time-of-flight spectrometer enabled to identify the compounds present in the extract. Finally, the fragmentation pattern obtained in MS/MS experiments confirmed the proposed structures. This procedure was able to determine many well-known phenolic compounds present in lemon verbena such as verbascoside and its derivatives, diglucuronide derivatives of apigenin and luteolin, and eukovoside. Also gardoside, verbasoside, cistanoside F, theveside, campneoside I, chrysoeriol-7-diglucuronide, forsythoside A and acacetin-7-diglucuronide were found for the first time in lemon verbena.

Keywords: lemon verbena, phenolic compounds, high-performance liquid chromatography, mass spectrometry.

1. Introduction

Lippia citriodora, also called lemon verbena, is a deciduous shrub originated in South America. It was introduced into Europe at the end of the 17th century so it is nowadays cultivated in the Mediterranean area [1]. The leaves of lemon verbena contain phenolic compounds, mainly flavonoids, phenolic acids and phenylpropanoids [2-4]. Some pharmacological properties have been attributed to these compounds. In fact, lemon verbena has traditionally been used in infusions for the treatment of fever, stomach ache, indigestion and other gastrointestinal disorders, besides it can act as diuretic [5], antiinflammatory [6] and analgesic [7]. Moreover, lemon verbena products and their compounds can be considered into the food category.

As a result of these properties, a bioactive plant extract from the aerial part of *Lippia citriodora* has been developed (Monteloeder, Elche). Its main component is the phenylethanoid glycoside verbascoside, also known as acteoside, which has previously been reported to have biological activity [6-11]. Several *in vitro* and *in vivo* assays have shown that this lemon verbena extract has antioxidant and antiinflammatory effects [12]. This antiinflammatory activity might enable the use of this plant extract for the prevention and treatment of diseases related with inflammation such as osteoarthritis and rheumatoid arthritis among others.

In spite of this activity is attributed to verbascoside, other polar compounds from the leaves can be present in the extract, mainly phenolic compounds. In this way, we cannot assume that the whole biological activity of lemon verbena extract are due to just one single compound since several researches have shown interactions between phenolic compounds, mainly synergistic and antagonistic effects [13-16]. As a result, we have carried out the qualitative characterization of this commercial foodstuff.

Phenolic compounds identification in plant matrix can be a complex task as there is a wide variety of structures. They can also get bonded to five different sugar moieties or get conjugated to form dimers and trimers. Besides, a lot of polyphenol standards are not commercially available. Several separative techniques have been used to determine phenolic compounds in vegetable matrix such as gas chromatography (GC), capillary electrophoresis (CE) and mainly high-performance liquid chromatography (HPLC), all of them coupled to different detection system, mostly mass spectrometry [2,17-19]. Recently, an improvement in chromatographic performance has been achieved by the use of columns packed with small particles (sub-2 μm) [20] which provide a higher peak capacity, greater resolution, increased sensitivity and high speed of analysis [21-25].

We have developed a methodology for qualitative characterization of complex plant matrix consisting of the coupling of reversed-phase high-performance liquid chromatography (RP-HPLC) eqquiped with a small particle size column, with two different detection systems: photodiode array (DAD) and mass spectrometry with time-of-flight (TOF) and ion-trap (IT) analyzers. UV-visible spectrophotometry is a valuable tool for identifying the class of phenolic compounds, whereas MS data are useful for their structural characterization. The sensitivity together with mass accuracy and true isotopic pattern of TOF-MS analyzer provided the most probable molecular formula. On the other hand, tandem mass spectrometry carried out with IT-MS analyzer was used to determine or to corroborate structures based on fragmentation patterns. In this work we have applied the described methodology to carry out the comprehensive characterization of a lemon verbena extract.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent grade and used as received. Formic acid and acetonitrile for HPLC were purchased from Fluka, Sigma-Aldrich (Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland) respectively. Solvents were filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Instrumentation

Analysis were carried out using an Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Palo Alto, CA, USA), including a standard autosampler and a diode array detector. The HPLC column used was a Zorbax Eclipse Plus C18 (1.8 µm, 150 x 4.6 mm). The HPLC system was coupled to a microTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an ESI interface. MS/MS analysis was performed using a Bruker Daltonics Esquire 2000 IT mass spectrometer (Bruker Daltonics, Bremen, Germany) also equipped with an ESI interface.

2.3. Sample

Lemon verbena commercial extract was kindly provided by Monteloeder. Briefly, the manufacture procedure consisted of lemon verbena aerial part drying and subsequent extraction in an overheated water extractor through maceration/percolation by exhaustive recirculation of water (≤ 80 ºC) to solvent saturation. Then, aqueous

115 solution was concentrated by high vacuum at low temperature up to 20º Brix and
116 filtered through a silica-cellulose press filter (1 µm) to eliminate insoluble materials.
117 Concentration process was continued by vacuum up to 70º Brix and final syrup was
118 dried by using industrial hot plate vacuum oven system. Final ratio of extract to raw
119 material was approximately 1:7.

120 The commercial extract manufactured according to the above described procedure
121 was dissolved in water to obtain a final concentration of 1000 µg ml^{-1}. This solution was
122 filtered and injected directly into the HPLC system.

123

124 2.4. Chromatographic procedure

125

126 The separation of the compounds from lemon verbena extract was carried out at
127 room temperature with a gradient elution program at a flow rate of 0.5 ml min^{-1}. The
128 mobile phases consisted in water:acetonitrile (90:10, v/v) with 1% of formic acid (A)
129 and acetonitrile (B). The following multi-step linear gradient was applied: 0 min, 5% B;
130 25 min, 20% B; 30 min, 40% B; 35 min, 5% B. The initial conditions were held for 10
131 min. The injection volume in the HPLC system was 20 µl. The UV-Vis detection was
132 performed in the 190-450 nm range.

133

134 2.5. ESI-TOF-MS detection

135

136 As the flow rate at chromatographic conditions was set at 0.5 ml min^{-1}, to split the
137 flow was required when ESI interface was used. In order to achieve reproducible
138 results, ionization conditions need to be constant and that is not possible at regular
139 HPLC conditions since the flow is too high. In the current paper the effluent from the

HPLC column was reduced using a “T” type splitter before being introduced into the mass spectrometer (split ratio 1:3). Thus the flow which arrived to the ESI-TOF-MS detector was 125 µl min^{-1}.

The HPLC system was coupled to a TOF mass spectrometer equipped with an ESI interface operating in negative ion mode using a capillary voltage of +4 kV. The other optimum values of the ESI-TOF parameters were drying gas temperature, 190 ºC; drying gas flow, 7 l min^{-1}, and nebulizing gas pressure, 1.5 bar. The detection was carried out considering a mass range of 50-1000 m/z.

The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonics), which provided a list of possible elemental formulas by using GenerateMolecularFormula Editor. The GenerateMolecularFormula Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (Sigma Value) for increased confidence in the suggested molecular formula [26]. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm [27]. We also have to say that even with very high mass accuracy (< 1ppm) many chemically possible formulae are obtained depending on the mass regions considered. So, high mass accuracy alone is not enough to exclude enough candidates with complex elemental compositions. The use of isotopic abundance patterns as a single further constraint removes >95% of false candidates. This orthogonal filter can condense several thousand candidates down to only a small number of molecular formulas.

During the development of the HPLC method, external instrument calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois,

USA) directly connected to the interface, with a sodium formate cluster solution passing through containing 5 mM sodium hydroxide and 0.2% formic acid in water:isopropanol (1:1, v/v). The calibration solution was injected at the beginning of each run and all the spectra were calibrated prior to the compound identification. By using this method, an exact calibration curve based on numerous cluster masses each differing by 68 Da ($NaCHO_2$) was obtained. Due to the compensation of temperature drift in the microTOF, this external calibration provided accurate mass values (better than 5 ppm) for a complete run without the need for a dual sprayer setup for internal mass calibration.

2.6. ESI-IT-MS/MS detection

The mass spectrometer was run in the negative ion mode and the capillary voltage was set at 3000 V. The IT scanned at 50–1000 *m*/*z* range. The other parameters were dry temperature, 300 ºC; drying gas flow, 7 l min^{-1}; nebulizing gas pressure, 1.5 bar. The instrument was controlled by a personal computer running Esquire NTsoftware from Bruker Daltonics.

3. Results and discussion

Figure 1 shows the UV chromatogram registered at 280 nm since most of the phenolic compounds absorb at this wavelength. The figure also shows the ESI-TOF base peak chromatogram of aqueous solution of the lemon verbena extract where the peaks are identified with numbers (1-19) considering the elution order. All the compounds were identified by the interpretation of their mass spectra obtained by the

TOF-MS and the MS/MS spectra acquired with the IT-MS and also taking into account the data provided by the literature and their absorption spectra in UV-visible region. Table 1 summarizes UV-visible bands and MS data including experimental and calculated m/z for the provided molecular formulas, error, sigma value and the main fragments obtained by MS/MS, as well as the proposed compound for each peak. Figure 2 shows the structures of the proposed compounds.

Compound 1 was identified as gardoside taking into account the molecular formula provided for its accurate mass. This iridoid glycoside has been reported previously in other species from genus *Lippia*, for instance *Lippia alba* [28]. MS/MS spectrum of this compound showed fragments at m/z 211, 193, 167 and 149 corresponding to [M-glucose]$^-$ (211) and the successive losses of water (193) and CO_2 (167) respectively from the main fragment, as well as the simultaneous elimination of water and CO_2 (149) (see Fig. 3a). Another fragment was found at m/z 123, corresponding to [M-glucose-88]$^-$, which was obtained by the loss of the 3-oxopropanic acid molecule according to the fragmentation pathway shown in Figure 4. These fragments were also similar to previous data reported for other iridoid glycosides [29]. Also theveside was identified in the sample (compound 4), another iridoid glycoside. This compound presented fragments at m/z 371, 345 and 209 which were consistent with the loss of water, CO_2 and glucose moiety respectively (see Fig. 3d). The fragment at m/z 121 corresponded to the elimination of the 3-oxopropanic acid molecule from the main fragment [M-glucose]$^-$ according to the fragmentation pathway previously described for gardoside. This was also corroborated since theveside is present in other plants from genus *Lippia* [30]. Both compounds showed an UV absorbance band at 234 nm which is characteristic of these iridoid glycosides [31].

Compound 2 corresponded to verbasoside also known as decaffeoylverbascoside. Its MS/MS spectrum showed fragments at m/z 315 and 297 corresponding to the loss of rhamnose moiety followed by water elimination respectively. Other fragments were detected at m/z 161 and 135. The ion at m/z 161 was consistent with deoxyhexose group and the fragment at m/z 135 represented the hydroxytyrosol moiety after the loss of water (see Fig. 3b). This compound has been detected previously in the genus *Lippia* [32,33].

Peak 3 MS/MS spectrum gave a fragment at m/z 179 which corresponded to caffeic acid (see Fig. 3c). Cistanoside F was proposed as structure taking into account the molecular formula provided, the presence of caffeic acid in the structure and the naturally occurrence of this compound in *Lippia alba* [34]. This compound has showed previously vasorelaxant activity [35] as well as radical scavenging capacity against DPPH [36]. Therefore, it could contribute to the antioxidant activity of the extract. However, the fact that it is a minor component in the extract must be taken into account.

It was not possible to identify compounds 5 and 6 since the molecular formulas and their possible structures did not match any previous evidence in nature. Moreover, the obtained fragments did not provide any useful information to propose a tentative identification.

Several verbascoside derivatives were present in the extract such as β-hydroxyverbascoside and β-hydroxyisoverbascoside (compounds 7 and 8). They both showed the same fragmentation pattern with fragments at m/z 621 and 459 (see Fig. 3e), so it was not possible to distinguish between them. The ion found at m/z 621 was consistent with $[M\text{-}H_2O]^-$, whereas the fragment at m/z 459 corresponded to the loss of the caffeic acid moiety. The presence of these verbascoside derivatives in lemon verbena extracts has been previously reported in the literature [33,37].

MS/MS fragmentation of compounds 9, 10, 13 and 18 presented the same ion at m/z 351 which is characteristic of diglucuronide group after water elimination. As well, all these compounds showed similar UV-visible spectra typical of flavonoids although hypsochromic shifts were observed by O-glycosylation [38]. According to their molecular formulas, they were identified as luteolin-7-diglucuronide, apigenin-7-diglucuronide, chrysoeriol-7-diglucuronide and acacetin-7-diglucuronide, respectively. In the case of luteolin-7-diglucuronide, the fragment at m/z 285 corresponding to luteolin aglycone was also present (see Fig. 3f). Luteolin-7-diglucuronide and apigenin-7-diglucuronide have been previously reported in lemon verbena [33,37].

The software provided the same molecular formula for peaks 11 and 12 and they showed similar absorption spectra. They were tentatively identified as campneoside I and isomer although it was not possible to assign what peak corresponded to campneoside I. They both gave a fragment at m/z 621 which represented the loss of methoxyl group. Compound 11 also showed an ion at m/z 635 (see Fig. 3g), consistent with water elimination, and another one at m/z 459 corresponding to the loss of the caffeoyl moiety from the main fragment (m/z 621). Campneoside I has showed antibacterial activity in previous works [39].

As expected, the main peak (compound 14) was identified as verbascoside, according to MS data and by comparison with retention time and MS/MS spectrum of a standard (see Fig. 3h). Besides, two verbascoside isomers were found in the lemon verbena extract. Compound 15 corresponded to isoverbascoside. It was consistent with its fragmentation pattern, very similar to verbascoside. The fragments found at *m/z* 461 and 315 corresponded to the loss of the caffeoyl moiety and the successive loss of rhamnose, respectively. Peak 16 corresponded to another verbascoside isomer which showed the same MS/MS profile. It was tentatively identified as forsythoside A since it

is usually present in natural products [40-42] and it showed UV bands according to the data provided by the literature [43].

Compound 17 corresponded to eukovoside. Its assignment was consistent with the presence of fragments found at m/z 491, 461 and 315 which represented the loss of rhamnose moiety, feruloyl group and their successive elimination (see Fig. 3i). Martynoside was the proposed structure for compound number 19. In its MS/MS spectrum, a fragment due to the loss of rhamnose moiety at m/z 505 was observed (see Fig. 3j). Other fragments were detected at m/z 475 and 457 corresponding to the feruloyl unit elimination and the further loss of water, respectively. These phenylethanoid glycosides were also corroborated by the data reported in the literature [33,35].

4. Conclusions

A powerful analytical method has been used to carry out the comprehensive characterization of a lemon verbena extract. The combined use of HPLC separation with a small particle size column assisted by UV-Vis and mass spectrometric detections with different mass analyzers, such as TOF or IT, has proved to be an useful tool in the identification of secondary metabolites produced by plants. The utilized method simultaneously separated a wide range of iridoid glycosides, flavonoid glycosides and phenylethanoid glycosides and the successfully identification of the major compounds of this extract was done in less than 30 min.

It is also important to highlight that, to our knowledge, the compounds gardoside, verbasoside, cistanoside F, theveside, campneoside I, chrysoeriol-7-diglucuronide,

288 forsythoside A and acacetin-7-diglucuronide were described for the first time in lemon
289 verbena.
290
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300 **References**

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373

374 **Table 1.** MS and MS/MS data and UV-visible bands for each compound and their
375 proposed structures.
376
377 Note: sh, shoulder.

Peak	t_R (min)	m/z experimental	m/z calculated	Error (ppm)	Molecular formula	MS/MS Fragments	λ_{max} (nm)	Proposed compound
1	3.97	373.1147	373.1140	1.9	$C_{16}H_{21}O_{10}$	211, 193, 167, 149, 123	234	Gardoside
2	4.33	461.1670	461.1664	1.3	$C_{20}H_{29}O_{12}$	315, 297, 161, 135	238	Verbasoside
3	4.84	487.1433	487.1457	4.9	$C_{21}H_{27}O_{13}$	179	240, 328	Cistanoside F
4	6.36	389.1096	389.1089	-1.6	$C_{16}H_{21}O_{11}$	371, 345, 209, 179, 121	234	Theveside
5	9.05	387.1656	387.1661	1.1	$C_{18}H_{27}O_{9}$	369, 225, 207, 163	239	Unknown 1
6	9.25	433.2065	433.2079	3.4	$C_{20}H_{33}O_{10}$	387, 225	240	Unknown 2
7	10.71	639.1878	639.1931	8.2	$C_{29}H_{35}O_{16}$	621, 459	242, 330	β-hydroxy-verbascoside/ β-hydroxy-isoverbascoside
8	11.09	639.1936	639.1931	-0.9	$C_{29}H_{35}O_{16}$	621, 459	242, 330	
9	11.46	637.1013	637.1046	5.2	$C_{27}H_{25}O_{18}$	351, 285	253, 266 sh, 347	Luteolin-7-diglucuronide
10	15.07	621.1118	621.1097	3.3	$C_{27}H_{25}O_{17}$	351	244 sh, 266, 334	Apigenin-7-diglucuronide
11	15.66	653.2139	653.2087	7.9	$C_{30}H_{37}O_{16}$	635, 621, 459	246, 330	Campneoside I or isomer
12	15.81	653.2097	653.2087	1.6	$C_{30}H_{37}O_{16}$	621	246, 330	
13	16.94	651.1228	651.1203	-3.8	$C_{28}H_{27}O_{18}$	395, 351	252, 266 sh, 345	Chrysoeriol-7-diglucuronide
14	17.28	623.2038	623.1981	-9.1	$C_{29}H_{35}O_{15}$	461	237, 296 sh, 330	Verbascoside
15	19.45	623.1979	623.1981	0.5	$C_{29}H_{35}O_{15}$	461, 315	244, 290 sh, 326	Isoverbascoside
16	20.10	623.1969	623.1981	2.0	$C_{29}H_{35}O_{15}$	461	246, 290 sh, 330	Forsythoside A
17	22.04	637.2174	637.2138	-5.6	$C_{30}H_{37}O_{15}$	491, 475, 461, 315	245, 290 sh, 330	Eukovoside
18	25.62	635.1278	635.1254	-3.8	$C_{28}H_{27}O_{17}$	501, 351	245 sh, 267, 330	Acacetin-7-diglucuronide
19	28.28	651.2280	651.2294	2.1	$C_{31}H_{39}O_{15}$	505, 475, 457	246, 329	Martinoside

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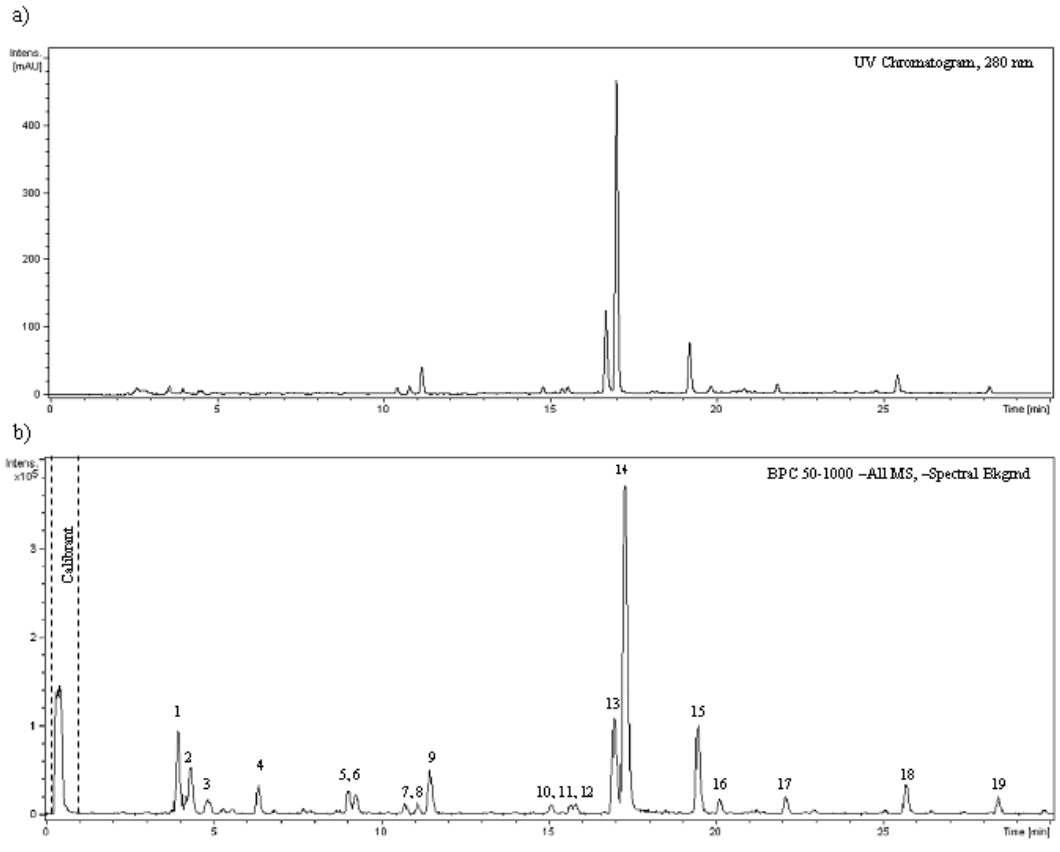


380

381 **Figure 1.** UV chromatogram obtained at 280 nm (a) and BPC (50-1000 m/z) (b) for

382 aqueous solution of lemon verbena extract.

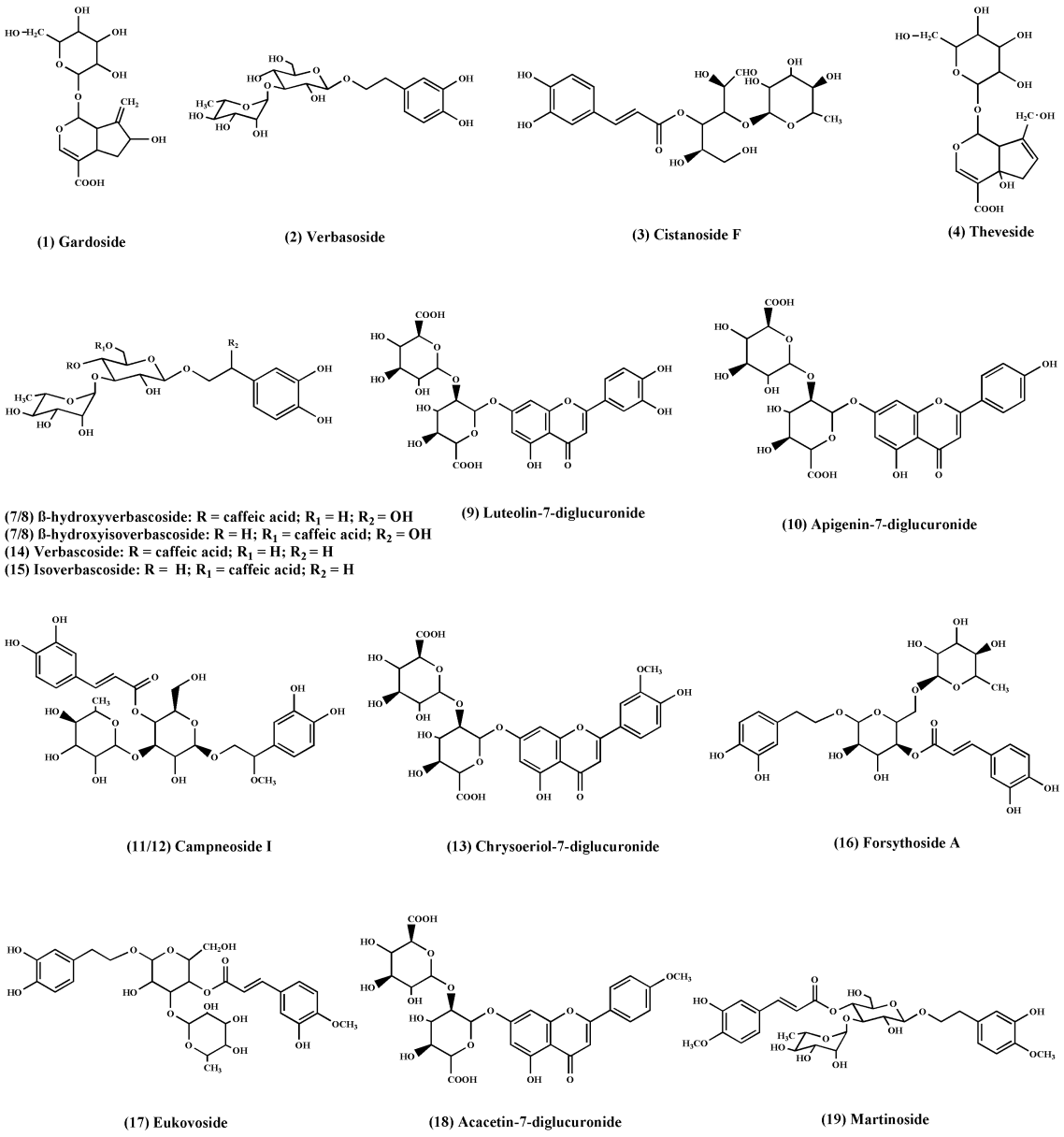
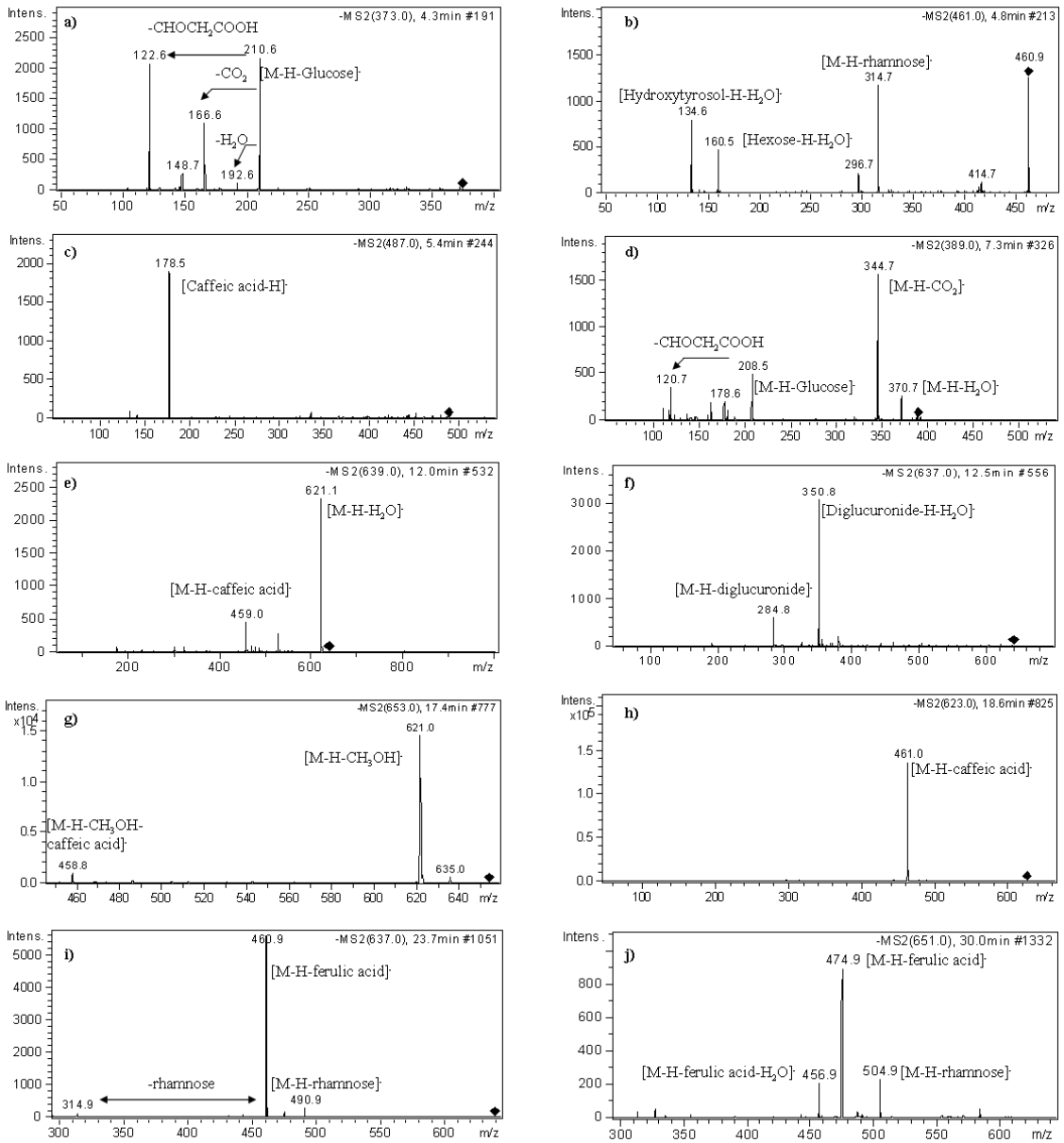
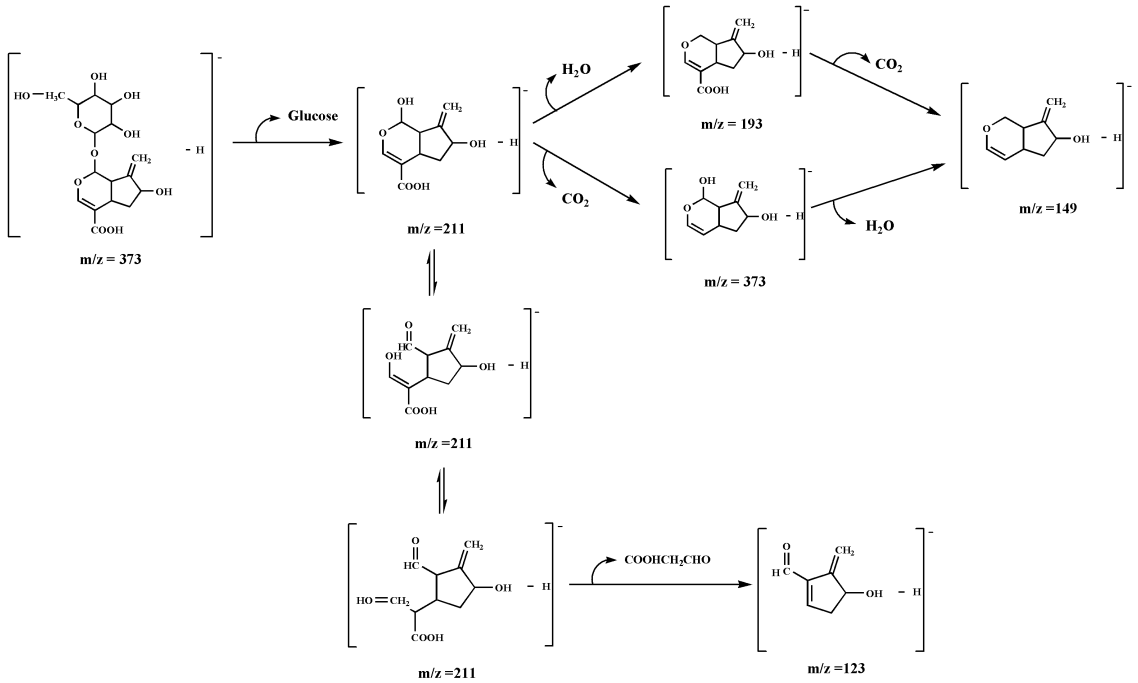


Figure 2. Chemical structures of the proposed compounds.



385

386 **Figure 3.** Most representative MS/MS spectra of compounds. The spectra correspond
387 to: a, gardoside; b, verbasoside; c, cistanoside F; d, theveside; e, β-
388 hydroxyverbascoside or β-hydroxyisoverbascoside; f, luteolin-7-diglucuronide; g,
389 campneoside I or isomer; h, verbascoside; i, eukovoside; j, martinoside.



390

391 **Figure 4.** Proposed fragmentation pathway for gardoside.