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HPLC/ESI-MS and NMR analysis of flavonoids and tannins in bioactive extract from leaves of *Maytenus ilicifolia*

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Abstract

Maytenus ilicifolia is an important plant in Brazilian folk medicine, used in many gastric disorder treatments. Low molecular weight components present in the leaves have been characterized as afzelechin, epiafzelechin, catechin, epicatechin, gallocatechin and epigallocatechin, as detected by HPLC and ESI-MS. Condensed tannins have also been found, consisting of di-, tri-, tetra-, and penta-, hexa, and heptamers. ESI-MS analyses were performed in positive and negative ionization modes, and in contrast with other investigations, negative ionization improved sensitivity for obtaining molecular ions. Moreover, the tandem-MS profile with negative detection provided characteristic fragments, such as those found at *m/z* 543 [(epi)afzelechin–(epi)afzelechin], *m/z* 561 [(epi)afzelechin–(epi)catechin], and *m/z* 577 [(epi)catechin–(epi)catechin] or [(epi)afzelechin–(epi)gallocatechin]. The analysis of the fragments also indicated the presence of additional ether linkage between C2 and C7, present in A-Type proanthocyanidins, and were identified at *m/z* 559 [(epi)afzelechin–(epi)catechin], *m/z* 575 [(epi)catechin–(epi)catechin] or [(epi)afzelechin–(epi)gallocatechin] and at *m/z* 591, [(epi)catechin–(epi)gallocatechin]. CID-fragmentation was used for tannins sequencing, as well as 3D NMR HMQC–TOCSY and COSY, which provided fingerprint assignments for identification of cathechin at δ 4.55/82.1 (H-2/C-2), 3.96/68.1 (H-3/C-3) and 2.82-2.50/27.7 (H-4^a-H-4^b/C-4), and epicathechin δ 4.78/79.1 (H-2/C-2), 4.15/66.7 (H-3/C-3) and 2.82-2.73/28.5 (H-4^a-H-4^b/C-4). Since HMQC–TOCSY gives a high resolution heteronuclear connectivity, it is useful for identification of other *cis–trans* isomers present in complex flavonoid mixtures.

Keywords: *Maytenus ilicifolia*; Flavonoids; Proanthocyanidins; Negative ESI-MS; Reversed-phase chromatography; Fingerprint analysis

1. Introduction

Flavonoids are important constituents of plants, being present in the leaves, fruit, and roots. These are formed by three rings (A, B, and C), resulting in a 6-3-6 carbon framework. Based on the saturation and oxidation of heterocyclic C-ring, flavonoids are subdivided in flavan, flavanone, flavone, flavonol, dihydroflavonol, flavan-3-ol, flavan-4-ol, and flavan-3,4-diol [1]. The well-known flavonoids, belonging to the flavan-3-ol class, include catechin (*trans*) and epicatechin (*cis*), which differ from each other on the configuration of carbons 2 and 3, which are linked to a catechol ring and to a hydroxyl group, respectively (Fig. 1).

According to their structural diversity, the biological activities attributed to these compounds can be variable. For example, the biological properties attributed to the exudates of an invasive plant (*Centurea maculosa*), which secretes (±)-catechin. While the (−)-isomer showed phytotoxic activity, the (+)-isomer had activity against soil borne bacterial pathogens, whereas the phytotoxic (−)-catechin did not [2].

Other flavonoids structurally related to (epi)catechin included gallocatechin/epigallocatechin, and afzelechin/epiafzelechin. These compounds can be linked to a gallic acid residue, giving

Abbreviations: API, atmospheric pressure ionization; CID, collision-induced dissociation; COSY, correlation spectroscopy; (Epi)Afz, afzelechin or epiafzelchin; (Epi)Cat, catechin or epicatechin; (Epi)Gall, gallocatechin or epigallocatechin; ESI-MS, electrospray ionization mass spectrometry; GC–MS, gas chromatography–mass spectrometry; HMQC–TOCSY, heteronuclear multiple quantum correlation spectroscopy–total correlation spectroscopy; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; PAs, proanthocyanidins; PDA-UV, photodiode array-ultra-violet detector; R_t, retention time.

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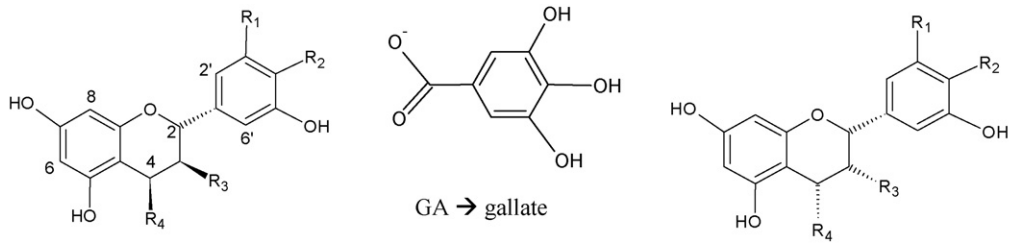

1 R_1, R_4 = H; R_2, R_3 = OH → (+)-Catechin
2 R_1, R_4 = H; R_2, R_3 = OH → (-)-Epicatechin
3 R_1, R_2, R_3 = OH; R_4 = H → (+)-Gallocatechin
4 R_1, R_2, R_3 = OH; R_4 = H → (-)-Epigallocatechin
5 R_1, R_2, R_4 = H; R_3 = OH → (+)-Afzelechin
6 R_1, R_2, R_4 = H; R_3 = OH → (-)-Epiafzelechin
7 R_3 = GA → gallate derivatives
8 R_3 = GA → gallate derivatives
9 R_1, R_3 = H; R_2, R_4 = OH → Luteoforol
10 R_1, R_3 = H; R_2, R_4 = OH → Epiluteoforol

Fig. 1. Representative structures of flavan-3-ols and flavan-4-ols.

rise to gallate derivatives (Fig. 1), or forming oligomers among themselves, giving rise to proanthocyanidins (PAs), known as condensed tannins. When linked via C4–C8 or C4–C6, they give rise to B-Type PAs, and if an additional ether linkage is present between C2 and C7, they are called A-Type PAs [3,4]. Luteoforol and epiluteoforol (Fig. 1) are also structurally similar to (epi)catechin, except for the presence of a hydroxyl group at C-4 rather than C-3, so they belong to the flavan-4-ol class.

Flavonoids are found in several natural teas, as in the well-known green tea of *Camellia sinensis*, and are used in popular medicine, with potential health benefit, such as antioxidants [5–7]. Green tea flavonoids are associated to the protection of cancer and heart diseases, and are also involved in anti-inflammatory processes [8–10].

Maytenus ilicifolia is a plant widely consumed as an infusion, in Brazilian folk medicine. It is employed in treatments of stomach ulcers and gastritis [11]. Moreover, a vasorelaxation activity has been recently attributed to compounds present in an aqueous extract of *M. ilicifolia* [12]. The governmental Brazilian drug agency (ANVISA) has approved the usage and commercialization of phytoterapics of *M. ilicifolia*, which are standardized by their tannin contents. Tannins are known to have cicatrizing properties due to their ability to promote protein precipitation, which gives rise to a protective layer that can improve the regenerative process [13,14].

Several metabolites with potential biological properties are present in teas from leaves of *Maytenus* spp., including flavonoids, triterpenes, and sesquiterpenes. Recent investigations have shown the presence of several types of flavonol-3-*O*-glycosides, such as rutin, quercitrin, hyperoside, isoquercitrin and, kaempferol and quercetin mono- di- tri- and tetra-glycosides [15–17].

We recently published a polysaccharide (arabinogalactan) present in the infusion of *M. ilicifolia* leaves with a potent anti-ulcer action, [18] and found that extracts containing flavonoids, had a protective affect on gastric mucosa via inhibition of H^+, K^+-ATPase activity and formation of nitric oxide [19]. Despite the identification of catechin and epicatechin, as well as galactitol, other components had not been identified at the time. We now investigate the presence of flavonoids and condensed tannins in bioactive extract of *M. ilicifolia* leaves, and using HPLC, ESI-MS/MS and NMR analyses, we described the composition and putative sequence of main tannins.

2. Experimental

2.1. Plant material

Leaves of *M. ilicifolia* Mart. ex Reissek (Celastraceae), collected in the region of Curitiba, PR (Southern Brazil), were donated by the *Central de Produção e Comercialização de Plantas Medicinais, Aromáticas e Condimentares do Paraná Ltda*. The plant was identified by Prof. Olavo Guimarães, from the Botany Department, Federal University of Paraná (UFPR), Curitiba, Brazil and is deposited in the Herbarium of UFPR as voucher no. 30842.

2.2. Extraction, fractionation of M. ilicifolia components

Defatted leaves (100 g) of *M. ilicifolia* were extracted thrice with water (500 ml) under reflux (100 °C), each for 3 h. The extracts were combined and concentrated under vacuum to low volume (200 ml), and then added to cold EtOH (600 ml) in order to precipitate macromolecules, similar to previously [12,19]. The EtOH-soluble compounds were concentrated to small volume and freeze-dried.

A portion of EtOH-soluble fraction (500 mg) was applied into a column (50 cm × 5 cm) packed with silica-gel 60G (Merck) and fractionated using different mixtures of $CHCl_3$–MeOH (ranging from 95:5, v/v to 100% of MeOH). The fractions collected were evaporated under nitrogen stream, at room temperature (∼25 °C) and chromatographed on silica-gel 60G TLC plates (Merck) using $CHCl_3$–MeOH–H_2O (65:25:4, v/v) as solvent. The plate was stained by MeOH–H_2SO_4 (9:1, v/v) at 100 °C, and the authentic catechin standard (Sigma Co.) was used as reference. Similar fractions were appropriated combined and were analyzed by HPLC.

2.3. HPLC analysis

HPLC analyses were performed in a Shimadzu apparatus equipped with SPA-10 UV/vis at 280 nm and ESI-MS as detectors, or Shimadzu Prominence using PDA-UV detection. The oven temperature was maintained at 30 °C, and the separation was carried out isocratically in a Synergy Fusion RP-C18 column, 250 mm × 4.6 mm, with particle size of 4 μm (Phenomenex), using H_2O:acetonitrile:acetic acid (89:10:1, v/v) as mobile phase, adapted from Soares et al. [20], at a flow rate of 0.8 ml min^{-1}. The samples were dissolved in MeOH (1 mg/ml) and 10 μl were used for injection.

2.4. ESI-MS analysis

Samples and standards (100 and 10 μg/ml, respectively) were solubilized in MeOH–H_2O (7:3, v/v) and submitted to positive- and negative API-ESI-MS investigation, recorded in a triple quadrupole, Quattro LC (Waters-Micromass), with nitrogen as nebuliser and desolvation gas. Offline analyses were performed by direct injection of samples, in methanolic solution, into the ESI-MS source with syringe-infusion pump at a flow rate of 10 μl min^{-1}, and online analyses were achieved by coupling HPLC and ESI-MS. Tandem-MS profiles were obtained by CID, using argon as collision gas. The negative ions of free flavonoids were obtained with capillary at 3.36 kV, and cone at 45 V, the fragmentation was achieved with the collision energy at 20 V. Positive ions were obtained with the following energies: capillary at 3.36 kV, cone 74 V and collision energy at 13.6 V. The energies used to obtain the negative ions of condensed tannins were: cone at 3.5 kV, capillary at 85 V and collision energy varying between 17 and 21 V.

2.5. NMR analysis

1D ^1H and ^{13}C, 2D COSY and 3D HMQC–TOCSY NMR spectroscopy were performed in a Bruker Avance DRX-400 spectrometer in a 5 mm inverse probe. The spectra were obtained using MeOD as solvent, at 30 °C, and the ^{13}C chemical shifts being measured in relation to Me_4Si ($\delta = 0$). In order to identify

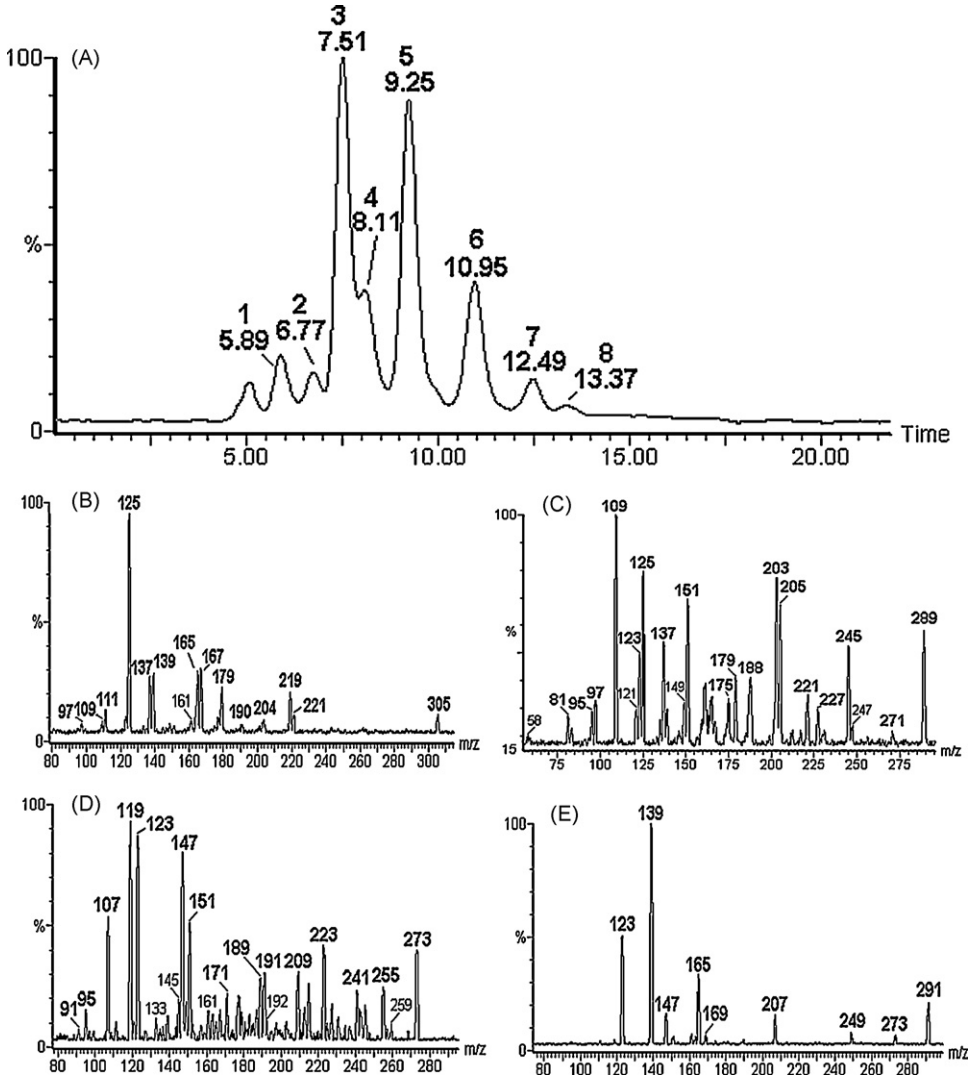

Fig. 2. (A) SEEA chromatogram of selected negative ions: peaks 1 and 2 at *m/z* 305, peaks 3–6 at *m/z* 289, and 7 and 8 at *m/z* 273. CID-fragmentation profiles with a negative ionization mode of peaks 1 and 2 (B); peaks 3–6 (C); peaks 7 and 8 (D). Fragmentation of peaks 3–6 at positive ionization mode (E).

the NMR chemical shifts, authentic standards of catechin and epicatechin (Sigma Co.) were used.

3. Results and discussion

3.1. Compounds present in the ethanolic fraction

The ethanolic fraction obtained from *M. ilicifolia* leaves (SEEA) was submitted to HPLC analysis, with UV detection at 280 nm [20]. Four major peaks on the chromatogram (peaks 3–6) were screened with PDA and showed the same UV absorbance curves with λ_{max} at 230 and 280 nm (Supplementary Fig. S-1 A and B) The sample was analyzed by online HPLC–ESI-MS, and the chromatogram (Fig. 2A) was scanned with negative ions at *m/z* 305 (peaks 1 and 2), *m/z* 289 (peaks 3–6), and *m/z* 273 (peaks 7 and 8). The ions correspond to (epi)galloctechin, (epi)catechin and (epi)afzelechin, respectively, and were confirmed by their CID-fragmentation profiles (Fig. 2B–D). Although tandem-MS is an important tool for identification of compounds with same molecular mass, the peaks 3–6 could not be distinguished by their CID profiles, even in both, positive and negative detection (Fig. 2C and E). Moreover, these peaks showed the same UV absorbance, suggesting compounds with very similar structures.

Peaks 3 and 5 were identified as catechin and epicatechin by comparison with authentic standards, although peaks 4 and 6 had similar characteristics as catechin and epicatechin, were not identified. Since the column used in HPLC was not suitable to resolve enantiomers, it was inferred that peaks 4 and 6 could correspond to the flavan-4-ols luteoforol, and epiluteoforol. Luteoforol was isolated from sorghum and strawberry leaves [21–23], and important properties are attributed to these class, particularly in regard to their antitumoral [2,24] and antibacterial activities [25].

Luteoforol is a 3′,4′,4,5,7-pentahydroxyflavan, and its structure differs from (epi)catechin by a single hydroxyl position, that would not be enough to afford differences in MS/MS spectra. However, other possibilities must be considered since there are a great variety of flavonol structures, such as isoflavonoids or the 5,7,2′,5′-tetrahydroxy-(2*R*,3*R*)-flavan-3-ol [26], which could result in similar ESI-MS detection. Since suitable standards were not available, further investigations are made necessary to confirm their identity.

Since SEEA chromatogram showed a highly complex mixture of compounds, it was submitted by chromatographic fractionation on silica-gel column. The fractions obtained were chromatographed on TLC plates and appropriately combined, giving rise to ten fractions. The presence of free flavonoids was detected in fraction 1 (F1), eluted with $CHCl_3$:MeOH (4:1, v/v) and tannins in fraction 2 (F2) eluted with $CHCl_3$:MeOH (7:3, v/v) which were submitted to HPLC–UV and HPLC–MS or offline ESI-MS and NMR analyses.

3.2. Analysis of F1

Offline ESI-MS was carried out in the negative and positive ionization modes. F1 gave, in the negative analysis, two main ions, characteristic of catechin or epicatechin, at *m/z* 289 $(M-1)^-$ and *m/z* 325 $(M+35)^-$ (Fig. 3A), which was confirmed by tandem-MS. Positive ESI-MS analysis gave only the ion at *m/z* 291 $(M+1)^+$. In our analytical conditions the negative ionization was more sensitive.

Since the ions found at *m/z* 289 and 291 could represent a mixture of isomers, and could not be distinguished by ESI-MS spectra, HPLC was used, giving only two peaks (Fig. 3B) with R_t values similar to catechin and epicatechin, respectively, confirming their presence in F1.

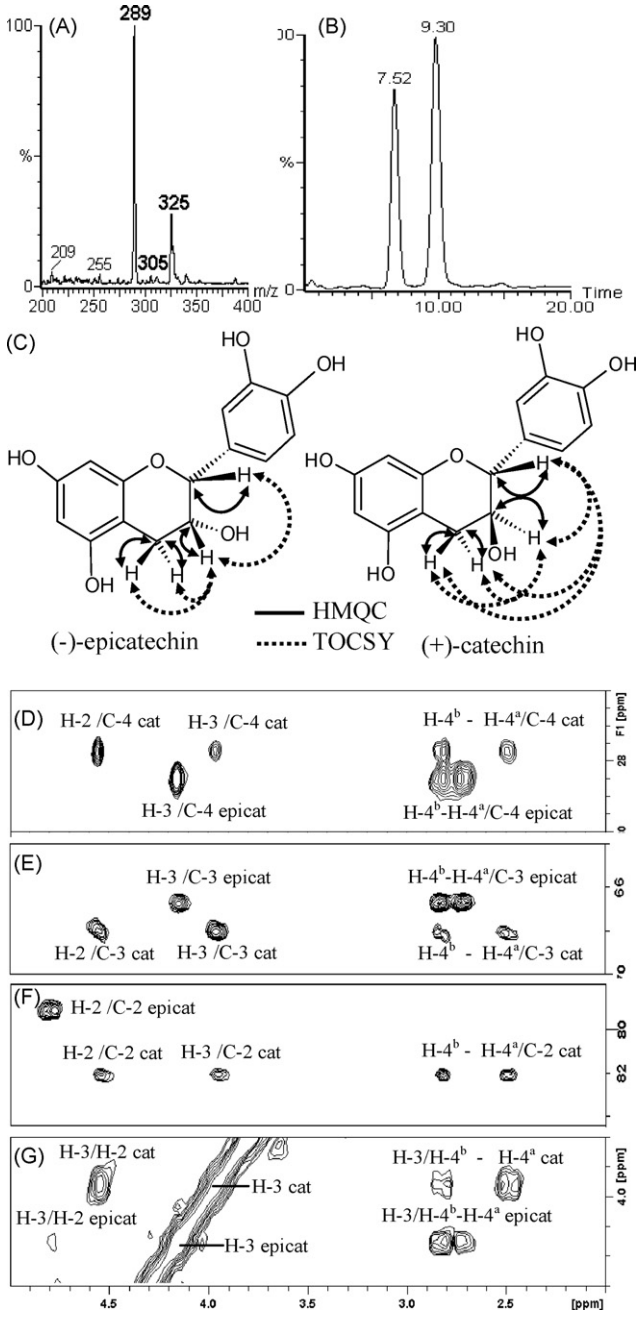

Fig. 3. (A) Offline ESI-MS of F1 with negative ionization mode and (B) HPLC chromatogram showing only catechin and epicatechin. (C) Structures of epicatechin and catechin showing connectivity among the C-2/H-2, C-3/H-3 and C-4/H-4^a-H-4^b. (D–F) Partial HMQC–TOCSY and (G) partial COSY spectrum, showing significant differences between catechin and epicatechin.

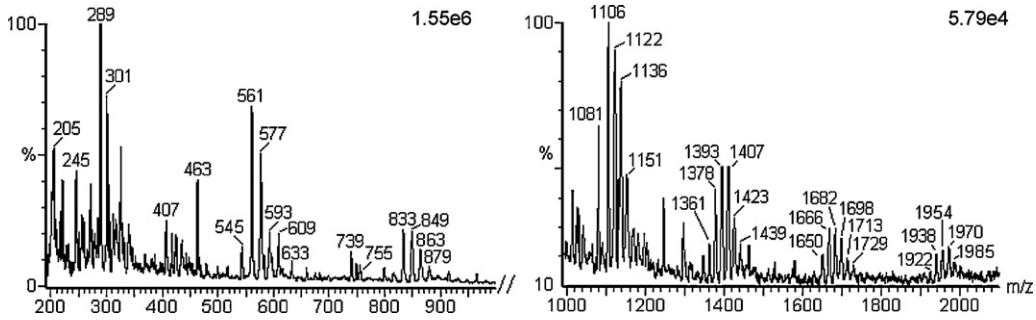

Fig. 4. Offline ESI-MS at full scan of F2 in the negative ionization mode.

According to Es-Safi et al. [4] the isomers catechin (*trans*) and epicatechin (*cis*) may be differentiated itself by ^{13}C NMR spectroscopy, since the chemical shift of C-2 varies from 76 ppm (epicatechin) to 84 ppm (catechin). As expected, we found similar variation at C-2, and also observed variations in chemical shifts of C-3 and C-4, as well as, in the chemical shifts of the protons linked to these carbons (Fig. 3C–G). These chemical shifts were confirmed by authentic standards.

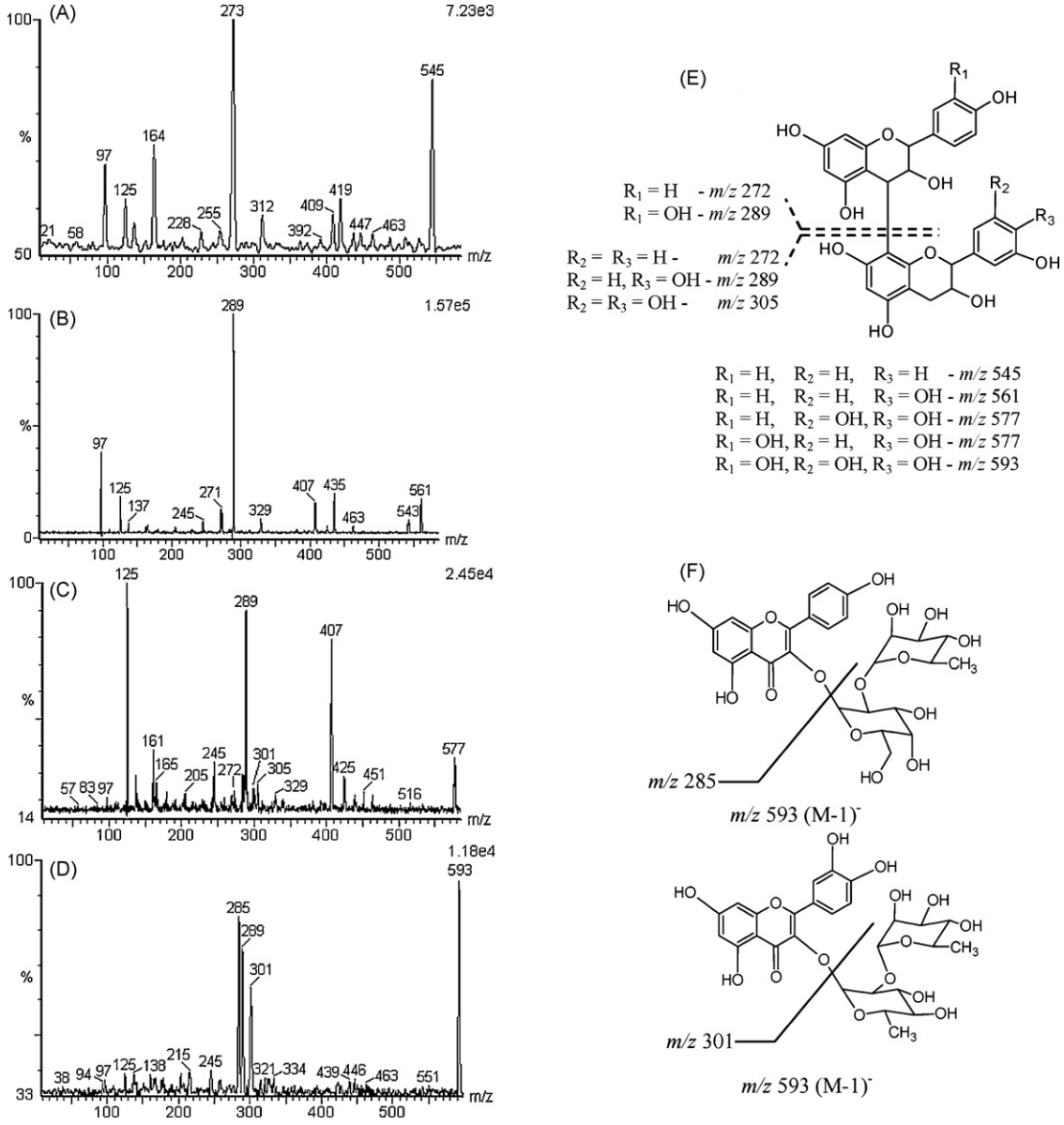

Fig. 5. (A–D) Tandem-MS of diflavonoids and (E) speculative structures with schematic representation of fragmentation. (F) Speculative structures of glycosides that present *m/z* 593 $(M-1)^-$ showing the removal of glycosyl units and formation of fragments at *m/z* 285 and 301, as observed in (D).

In the HMQC spectrum of F1, catechin had the chemical shifts arising at δ 4.55/82.1 (H-2/C-2), 3.96/68.1 (H-3/C-3) and 2.82-2.50/27.7 (H-4^{a}-H-4^{b}/C-4), while epicatechin had correlation signals at δ 4.78/79.1 (H-2/C-2), 4.15/66.7 (H-3/C-3), and 2.82-2.73/28.5 (H-4^{a}-H-4^{b}/C-4). Interestingly in the HMQC–TOCSY experiments the connectivity of H-2 with H-3/H-4^{a,b} of the epicatechin did not appear, different from the *trans*-configuration of catechin, which exhibits total connectivity. In the COSY experiment (Fig. 3G) a small signal of H-3/H-2 at δ 4.15/4.78 appeared in the spectrum, confirming the weak connectivity of H-2/H-3 of epicatechin, confirmed in spectra of standards (Supplementary Figs. S-2 and S-3).

3.3. ESI-MS analysis of F2

Offline ESI-MS on F2 provided many ions, in both negative (Fig. 4) and positive modes. However, negative detection proved

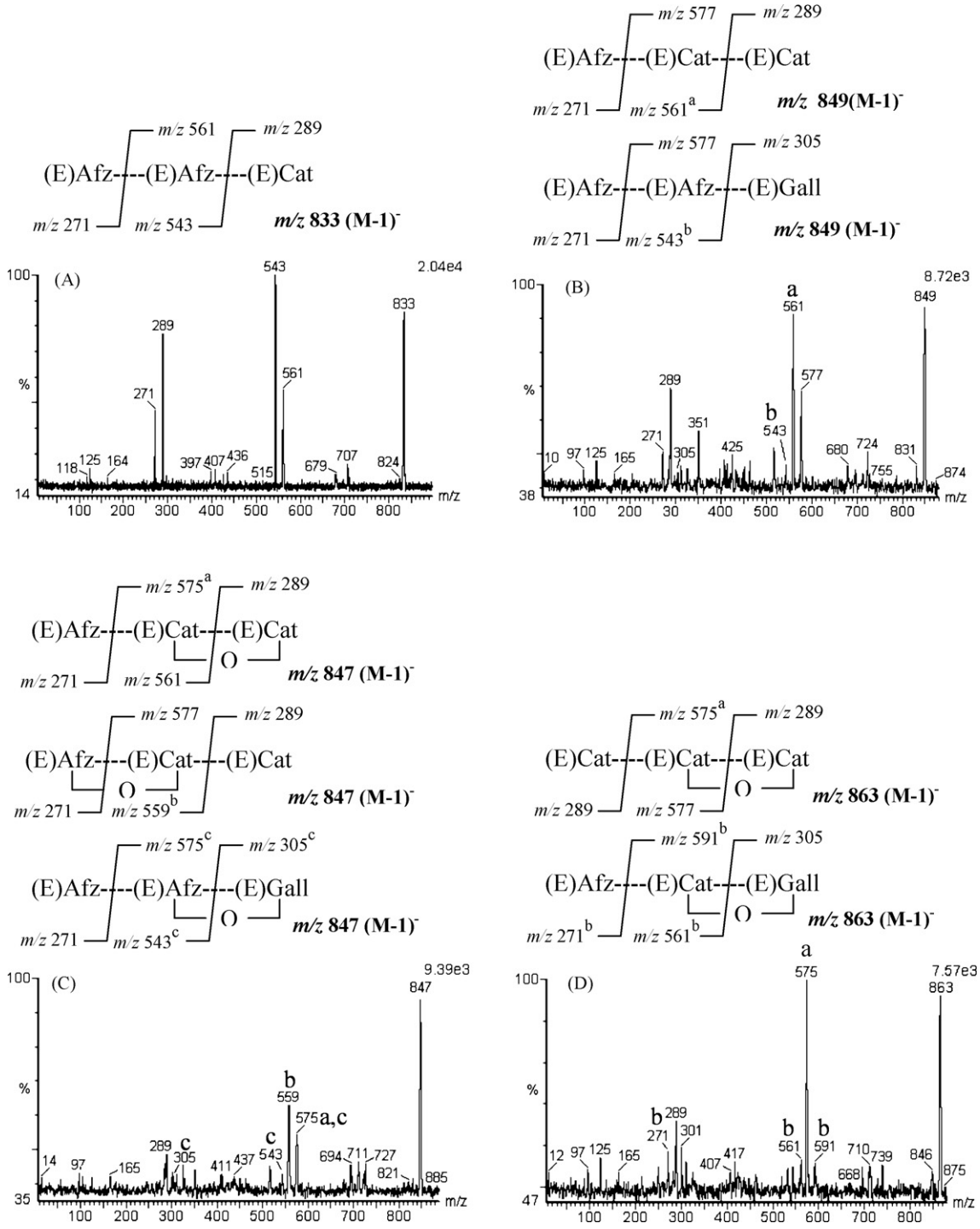

Fig. 6. (A and B) Tandem-MS of triflavonoids and (C and D) tandem-MS of triflavonoids containing ether linkage. [a–c]Key fragments for indication of tannin sequences.

to be more efficient rather than the positive mode, and thus was the main focus of discussion.

The presence of free (epi)catechin was found via ions at *m/z* 289, and quercetin at *m/z* 301 and flavonoid glycosides, at *m/z* 463, 609, 739, and 755, similar to previous data reported [15–17]. Condensed tannins were also detected, such as dimers, consisting of two (Epi)Afz units, at *m/z* 545, and (Epi)Afz–(Epi)Cat at *m/z* 561 (Fig. 5A and B). The ion found at *m/z* 577 corresponds to two units of (Epi)Cat, confirmed by a fragment at *m/z* 289, however even with their low intensities, fragments at *m/z* 271 and 305 were detected, indicating the presence of the dimer (Epi)Afz–(Epi)Gall (Fig. 5C and E).

The ion at *m/z* 593, indicates the diflavonoid (Epi)Cat–(Epi)Gall, although the CID-fragmentation was different from the expected, with three main ions at *m/z* 285, 289 and 301, attributed to kaempferol, (epi)catechin, and quercetin, respectively. We suggest that *m/z* 593 corresponds to a mixture of diflavonol, kaempferol-deoxyhexosyl-hexoside [15,16], and quercetin-deoxyhexosyl-deoxyhexoside, which appears as a new glycoside described for *M. ilicifolia* (Fig. 5D–F).

Other tannins with higher polymerization degree were shown by the ions at *m/z* 833 and 849, corresponding to (Epi)Afz–(Epi)Afz–(Epi)Cat and (Epi)Afz–(Epi)Cat–(Epi)Cat, respectively. A-Type PAs were shown by the ions at *m/z* 847, consisting of (Epi)Afz–(Epi)Cat–(Epi)Cat. The fragments with *m/z* 575 and 559 indicated the presence of ether linkage between two (Epi)Cat units and between (Epi)Afz and (Epi)Cat, respectively. The ion at *m/z* 863 is consistent with three condensed (Epi)Cat, and (Epi)Afz–(Epi)Cat–(Epi)Gall, having ether linkage mainly between two (Epi)Cat units (*m/z* 575), although the fragment at *m/z* 591 indicated that an ether linkage was also present between (Epi)Cat and (Epi)Gall (Fig. 6A–D).

Minor ions at *m/z* 1106, 1122, 1136 and 1151 were identified as arising from tetra-flavonoids, those at *m/z* 1136 and 1151 indicated A-Type PAs, the ions at *m/z* 1363, 1378, 1394, 1409,

Table 1
Compounds present in F2 identified by ESI-MS/MS at negative ionization mode

Compound	$(M-1)^-$	Main fragments
(Epi)catechin	289	203, 187, 179, 161, 151, 137, 125, 109
Quercetin	301	256, 245, 179, 164, 151, 135, 121, 109
Quercetin-hexoside	463	301, 271, 255, 179, 151
(E)Afz–(E)Afz	544	419, 273, 164, 97
(E)Afz–(E)Cat	561	543, 435, 407, 289, 271, 245, 125, 97
(E)Cat–(E)Cat	577	407, 289, 245, 161, 125
(E)Afz–(E)Gall	577	305, 301, 272, 125
(E)Cat–(E)Gall	593	289
Kaempferol-deoxyhexosyl-hexoside	593	285
Quercetin-deoxyhexosyl-deoxyhexoside	593	301
Quercetin-deoxyhexosyl-hexoside	609	301, 289
(E)Afz–(E)Afz–(E)Cat	833	*561, *543, 289, 271
(E)Afz–(E)Cat–(E)Cat (A-T) C-O-C	847	*575, 289
(E)Afz–(E)Cat–(E)Cat (A-T) A-O-C	847	*559, 289
(E)Afz–(E)Afz–(E)Gall (A-T) A-O-G	847	*543, 305
(E)Afz–(E)Cat–(E)Cat	849	*577, *561, 289, 271
(E)Afz–(E)Cat–(E)Gall (A-T) C-O-G	863	*591, *561, 289, 271
(E)Cat–(E)Cat–(E)Cat (A-T) C-O-C	863	*575, 289
(E)Cat–(E)Cat–(E)Gall (A-T) C-O-G	879	*591, *577, 289
(E)Afz–(E)Afz–(E)Afz–(E)Cat	1106	*833, *815, 561, 543, 289, 271
(E)Afz–(E)Cat–(E)Afz–(E)Cat	1122	849, 831, *561, 289, 271
(E)Afz–(E)Afz–(E)Cat–(E)Cat	1122	849, 831, *577, *543, 289, 271
(E)Afz–(E)Cat–(E)Cat–(E)Cat (A-T) C-O-C	1136	863, 848, 577, *575, *561, 289, 271
(E)Afz–(E)Afz–(E)Cat–(E)Gall (A-T) G-O-C	1136	863, 832, *591, 561, *543, 289, 271
(E)Afz–(E)Cat–(E)Gall–(E)Cat (A-T) G-O-C	1151	879, 863, *591, *561, 289, 271
(E)Cat–(E)Cat–(E)Cat–(E)Cat (A-T) C-O-C	1151	862, *577, *575, 289
(E)Gall–(E)Gall–(E)Afz–(E)Afz (A-T) G-O-C	1151	879, 845, *609, *543, 305, 271
(E)Cat–(E)Afz–(E)Afz–(E)Afz–(E)Afz	1378	*1090, 833, 817, 543, 289, 271
(E)Afz–(E)Afz–(E)Cat–(E)Afz–(E)Cat	1393	1121, 1103, *849, *833, 561, *543, 289
(E)Afz–(E)Afz–(E)Afz–(E)Cat–(E)Cat	1393	1121, *1103, 849, *577, 543, 289
(E)Afz–(E)Afz–(E)Afz–(E)Gall–(E)Cat (A-T) G-O-C	1407	1135, *862, *591
(E)Cat–(E)Afz–(E)Afz–(E)Cat–(E)Cat (A-T) C-O-C	1407	1118, *846, *832, *575
(E)Cat–(E)Afz–(E)Cat–(E)Cat–(E)Cat (A-T) C-O-C	1423	1133, *863, *847, *575, 289
(E)Afz–(E)Afz–(E)Cat–(E)Gall–(E)Cat (A-T) C-O-G	1423	1151, *1133, *833, *591, *543, 289
(E)Cat–(E)Cat–(E)Cat–(E)Cat–(E)Cat (A-T) C-O-C	1439	1151, *864
(E)Gall–(E)Cat–(E)Afz–(E)Cat–(E)Cat (A-T) G-O-C	1439	1151, *878, *849
(E)Gall–(E)Gall–(E)Afz–(E)Afz–(E)Cat (A-T) A-O-C	1439	*609, *559

(E)Afz → afzelechin or epiafzelechin; (E)Cat → catechin or epicatechin; (E)Gall → gallocatechin or epigallocatechin. A-T → A-Type proanthocianidin; A-O-C → ether linkage between (E)Afz and (E)Cat; C-O-C → ether linkage between (E)Cat and (E)Cat; G-O-C or C-O-G → ether linkage between (E)Gall and (E)Cat.
*Indicate key fragments for sequence identification.

1424, and 1440 were from penta-flavonoids (Supplementary Figs. S-4 and S-5). These compounds were further analyzed using their CID-fragments and the results are summarized in Table 1. Hexa-flavonoids were observed at *m/z* 1650, 1666, 1681, 1698, 1713, 1729, and those at *m/z* 1922, 1938, 1954, 1970, 1985 were consistent with hepta-flavonoids (Fig. 4), even though no CID-fragments could be observed.

Several pathways of positive fragmentation have been described in great detailing, initiated by water loss following retro-Diels-Alder (RDA) fission, heterocyclic ring fission (HRF), benzofuran-forming fission (BFF) and quinone methide (QM) fission [27]. Although the breakdown pathways are outside the scope of our work, we pointed to significant CID-fragments obtained at negative ionization, which could be considered the key for tannin sequencing, as well as allowing the localization of the units that belong to the ether linkage (C2–C7) present in A-Type PAs. These are also listed in Table 1.

With positive ionization, the only tannin observed in relative high amounts, corresponded to (Epi)Afz–(Epi)Cat, at *m/z* 563. Other dimers were observed with low abundance at *m/z* 547, corresponding to (Epi)Afz–(Epi)Afz and at *m/z* 579, from (Epi)Cat–(Epi)Cat. Trimers were also detected as giving *m/z* 835, 850 and 866, but higher PAs were not observed.

3.4. Online ESI-MS of F2

The chromatogram obtained by HPLC–UV showed the presence of four major peaks, detected at 280 nm, similar to the chromatogram of the original SEEA extract. The same peaks 3–6 were compared with authentic standards of catechin and epicatechin, confirming the identity of peaks 3 and 5. Minor peaks observed on chromatogram were not assigned.

Similarities between the F2 and SEEA chromatograms were confirmed by coupling HPLC to ESI-MS, which showed the same molecular ions in the four major peaks, at *m/z* 289 $(M-1)^-$ and 291 $(M+1)^+$. Tandem-MS was also used in order to confirm their fragmentation profiles, which were the same found in SEEA.

3.5. NMR spectroscopy of F2

1D, 2D and 3D NMR spectroscopy provided information on the configuration of the tannin units. The presence of *cis* and *trans* isomers in F2 was clear, since ^{13}C NMR signals of catechin were observed at δ 82.1, 68.1, and 27.7 and those of epicatechin at δ 79.1, 66.7, and 28.5. The chemical shifts signals of (epi)azfelechin and (epi)gallocatechin must be close to those of (epi)catechin, rendering analytical difficulty due to the presence of mixtures. However, we found an important ^{13}C signal at δ 37.3 which indicated a linked C-4 [28]. Other signals could not be identified, since further purification should be carried out for accurate analysis.

4. Conclusion

Negative ESI-MS provided a qualitative analysis useful in tannin sequencing, and in contrast with positive CID-fragmentation of Na^+ adducts, provides simple MS/MS spectra, in which were possible to observe the loss of units. Moreover, it appears that the presence of the catechol group influenced in negative ion detection, since the ion at *m/z* 561 that represents a dimer composed of (epi)afzlechin and (epi)catechin at the same molar ratio, when submitted to CID-fragmentation generates the ion at *m/z* 289, corresponding to (epi)catechin, with a higher intensity than ion at *m/z* 271 from (epi)afzelechin (Fig. 5B). This occurred even in cases when the (epi)catechin is the minor component of tannins (Fig. 6A).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2007.12.008.

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