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Short communication

Identification of flavonols in leaves of *Maytenus ilicifolia* and *M. aquifolium* (Celastraceae) by LC/UV/MS analysis

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Abstract

A comparative analysis of the flavonoid components of the leaves of two medicinal plants known in Brazil as "espinheira santa", namely, *Maytenus ilicifolia* Mart. ex Reiss. and *M. aquifolium* Mart. (Celastraceae), and a hybrid plant, *M. aquifolium* × *M. ilicifolia*, has been carried out using high-performance liquid chromatography coupled with photodiode array UV detection and mass spectrometry. One methoxyflavonoid glycoside and 18 flavonol-3-*O*-glycosides were identified in the extracts on the basis of their on-line UV spectra (measured in the absence and presence of shift reagents) and multiple stage mass spectral data. Fingerprint analysis of the flavonoid extracts revealed significant differences in the profiles of the two *Maytenus* species, while the hybrid plant contained flavonoids found in both parent species.

Keywords: LC/UV/MS; Flavonols; Phytomedicines; *Maytenus aquifolium*; *Maytenus ilicifolia*

1. Introduction

A number of species of medicinal plants are known in Brazil under the popular name of "espinheira santa". Two of these species, *Maytenus aquifolium* Mart. and *M. ilicifolia* Mart. ex Reiss. (Celastraceae), are somewhat difficult to differentiate on account of their similar morphologies. The leaves of both of these plants, however, exhibit anti-ulcerogenic and analgesic activities, and the phytochemical and toxicological studies of Souza-Formigoni et al. [1] and Gonzalez et al. [2] suggest that flavonoids, tannins and triterpenes are the likely active components. The triterpenoids of *Maytenus* species have been the focus of attention of a number of Japanese research groups in the search for biologically active compounds within the Brazilian flora [3].

The growing interest in *M. aquifolium* and *M. ilicifolia*, coupled with the increasing utilisation of these species by the pharmaceutical industry, has accentuated the importance of developing analytical methods for use in the production of standardised preparations of *Maytenus*-derived phytomedicines. The gas chromatographic (GC) analysis of friedelanol-type triterpenes [4] has been employed in the quality control of commercial phytopharmaceuticals produced from *Maytenus* spp. on the basis of pharmacological evidence suggesting a correlation between the marker triterpenoids and anti-ulcerogenic activity [5]. However, a high-performance thin layer chromatographic (HPTLC) method for fingerprinting *Maytenus* flavonoids has been developed, and its application to the quality assurance of plant material proposed [6]. Recent studies have, moreover, indicated that *Maytenus* flavonoids exhibit anti-ulcerogenic activities [7].

The present paper describes a comprehensive and comparative on-line LC/UV/MS analysis of the flavonoids of leaves of *M. aquifolium* and *M. ilicifolia*, and of a *M. aquifolium* × *M. ilicifolia* hybrid. By combining efficient LC separation and electrospray ionisation – multiple stage mass spectrometry (ESI–MS^n) with UV detection, performed both with and without the post-column addition of shift reagents, the on-line identification of minor flavonoids in *Maytenus* extracts could be readily achieved. Most of the compounds described in this paper have not been reported in the previous studies of *M. aquifolium* and *M. ilicifolia* that were carried out using conventional phytochemical isolation procedures involving laborious work-up

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procedures and subsequent purification of target compounds [7–9].

2. Experimental

2.1. Chemicals

All chemicals and solvents were of reagent or HPLC grade. The flavonoid standards rutin, hyperoside, isoquercitrin, quercetin and quercitrin were purchased from Carl Roth (Karlsruhe, Germany): sodium hydroxide, aluminium chloride and sodium acetate were obtained from Sigma–Aldrich Chemie (Steinheim, Germany).

2.2. Plant material

Leaves of authenticated specimens of *M. aquifolium* (voucher number HPMU-0755 deposited at the Herbarium of Universidade de Ribeirão Preto, UNAERP, Ribeirão Preto, SP, Brazil) and of *M. ilicifolia* (voucher number HPMU-0266) were kindly supplied by Dr. Ana Maria Soares Pereira (UNAERP). The *M. aquifolium* × *M. ilicifolia* hybrid was derived from the spontaneous crossing of specimens of *M. aquifolium* and *M. ilicifolia* ("parent plants") that had been cultivated in close proximity on the farm of the Pluridisciplinar Center of Chemical, Biological and Agricultural Research – CPQBA, Campinas State University – UNICAMP, Campinas, SP, Brazil. The initial hybrid plant produced seeds that were harvested and cultivated to generate progeny exhibiting morphological characteristics of both parent plants. Leaves from the parent plants and from the hybrid plant were collected in January 2000 and were the kind gift of Dr. Pedro Mellilo Magalhães (CPQBA – UNICAMP).

2.3. Sample preparation

Leaves were air-dried at 40 °C, ground to a powder and sieved. Leaf powder (1.0 g; 0.5 to 1.0 mm particle size) was extracted with 10 mL of methanol: water (1:1) for 30 min at 50 °C with constant magnetic stirring. The extract was filtered, extracted with 5 mL chloroform and the two phases separated. The chloroform layer (containing the plant pigments) was discarded, while the hydromethanolic layer was filtered though a 0.5 μm Fluorpore membrane (Millipore, Bedford, MA, USA) and submitted to LC analysis.

2.4. Chromatographic conditions

Analyses were carried out on a Hewlett–Packard (HP; Palo Alto, CA, USA) HP-1100 liquid chromatographic system consisting of binary pumps, an inline degasser, an autosampler and an HP-1040A photodiode array (PAD) detector coupled to an HP-85 personal computer. UV–vis spectra were recorded in the range 200–450 nm. Mass spectral data were obtained using a Finnigan MAT (San Jose, CA, USA) LCQ ion-trap mass spectrometer with an electrospray interface (ESI-IT-MS).

HPLC separations were performed using a Waters (Milford, MA, USA) Symmetry C_{18} analytical column (250 mm × 4.6 mm i.d.; 5 μm particle size) protected by a Waters Novapack RP C_{18} guard column. The mobile phase consisted of 0.5% formic acid in deionised water (A) and 0.5% formic acid in acetonitrile: methanol (50:50, v/v) (B). The gradient conditions were: 25% B to 40% B in 20 min, 40% B to 50% B in 5 min, 50% to 100% B in 5 min followed by 100% B in 5 min, all at a flow rate of 1 mL/min. The mobile phase was returned to the initial conditions and the column was re-equilibrated during a 10 min post-run procedure. The column oven temperature was maintained at 40 °C. All standards and samples were dissolved in methanol and 10 μL aliquots were injected for analysis.

The post-column derivatisation method was based on a previously reported protocol [10] and carried out using an Eldex Labs (Menlo Pak, CA, USA) model A-30-5-2 pump and a reaction coil (500 mm × 0.5 mm i.d.). Shift reagents (0.02 mol/L sodium hydroxide, 0.3 mol/L aluminium chloride or 0.5 mol/L sodium acetate) were added to the effluent from the chromatograph at a flow rate of 0.2 mL/min. The reaction coil was normally maintained at 20 °C but heated to 60 °C when aluminium chloride was the derivatising agent.

Mass spectra were acquired in the negative ion mode. The instrument parameters were optimized prior to sample analysis using rutin as standard and were: capillary temperature 225 °C, capillary voltage −57 V, cone voltage −35 V, spray voltage −2.8 KV and nebuliser gas (nitrogen) flow 4 L/min. In the ESI-IT-MS^n experiments, the most abundant ion or fragment ion was automatically selected as the precursor ion for the next stage, and fragmentation was continued through to MS^4. Product ion spectra were recorded in the range 150–1000 U. Collisions were performed by applying 30% of energy level. IT-MS are presented in % and not in eV since the voltages applied varied according to the *m/z* values of the precursor ions. At these conditions, the lower limit of detection was 2 ng/mL for quercetin.

3. Results and discussion

Comparison of the spectral data obtained from leaves of authenticated specimens of *M. aquifolium* and *M. ilicifolia* with those derived from the parent plants of the hybrid (see Section 2.2) revealed that for each species of *Maytenus*, the fingerprints of the various samples were identical. Therefore, in order to simplify the following discussion, the data reported in this paper relate to the parent plants and to the *Maytenus* hybrid.

The chromatogram of the hybrid plant displayed eleven peaks associated with flavonoids, while those of the parent plants, *M. aquifolium* and *M. ilicifolia*, presented nine and 11 peaks, respectively (Fig. 1). Additionally, most of the flavonoid peaks observed in the fingerprint of the hybrid plant were identical [with respect to retention time (t_R) and UV/PAD spectra] to those found in the parent plants. Only one peak in the chromatogram of the hybrid plant (peak 1) appeared not to be related to any of the peaks observed in the fingerprints of *M. aquifolium* or *M. ilicifolia*.

The flavonol peaks could be assigned unambiguously from their characteristic UV spectra, which displayed two absorption bands at *ca.* 250 and 350 nm [11] (Table 1; Fig. 1). The values

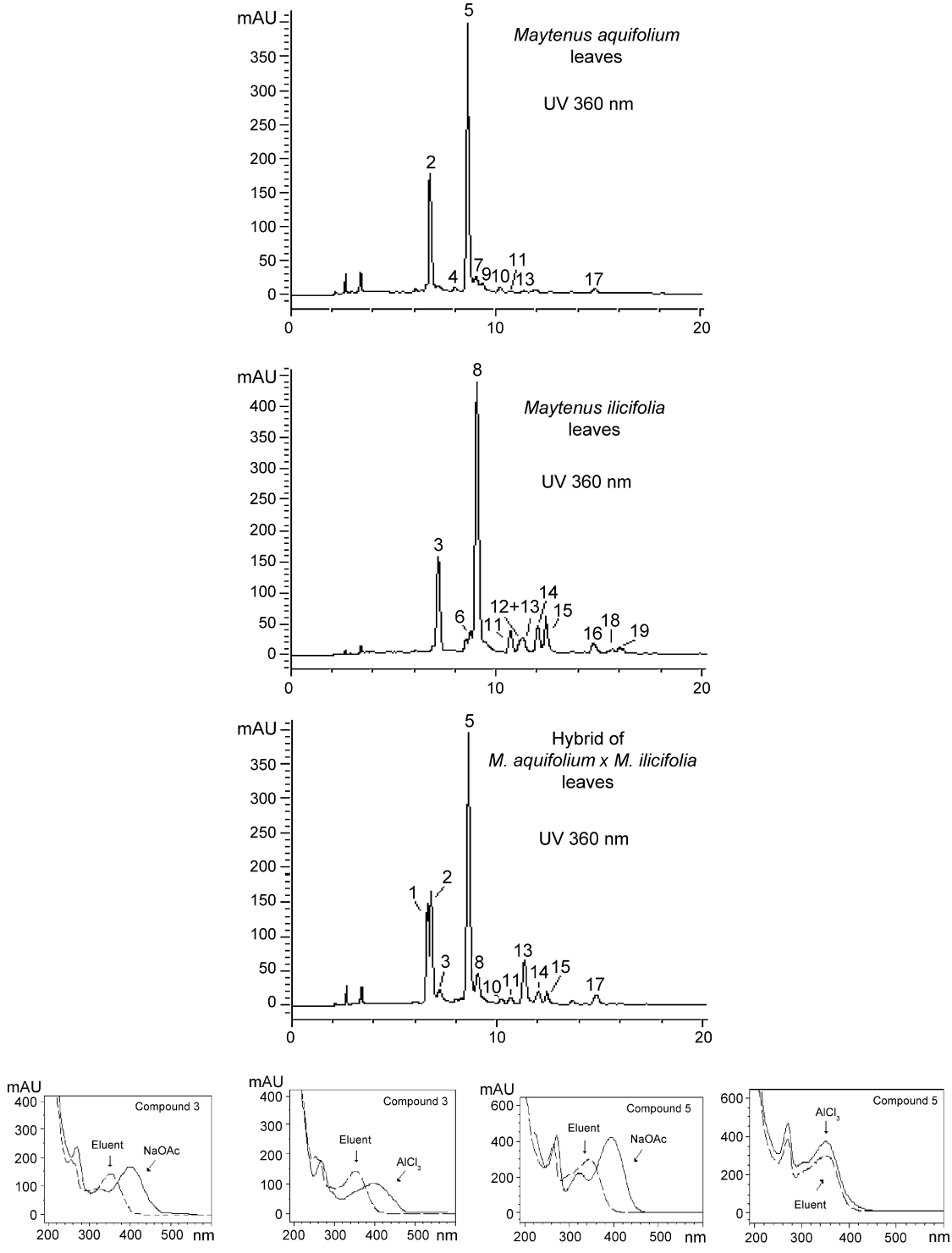

Fig. 1. LC/UV/PAD chromatograms of extracts of *Maytenus aquifolium*, *M. ilicifolia* and the hybrid *M. aquifolium* × *M. ilicifolia*, together with representative UV/PAD spectra. For the key to peak identification, see Table 3: for chromatographic protocols, see Section 2.4.

of λ_{max} ranged between 358–365 and 258–268 nm for peaks in Bands I and II, respectively, indicating that the flavonols in *Maytenus* extracts were substituted in the 3-OH position. The UV/PAD spectra of the *Maytenus* flavonoids suggested the presence of flavonol-3-*O*-glycosides, a finding that is compatible with previous reports [7–9]. The remaining peaks, shown without numbers in the chromatograms of Fig. 1, were associated with simple phenolic compounds that were not the focus of the present study.

Four flavonoids were conclusively identified by comparison of their t_R and UV/PAD spectra with those of flavonoid standards analysed under identical chromatographic conditions, namely, rutin (13), hyperoside (14), isoquercitrin (15), and quercitrin (19).

Table 1
On-line UV data of the *Maytenus* flavonoids identified in this study, determined in the absence and presence of shift reagents

Peak	UV spectra (nm)		Sodium acetate-shifted UV spectra (nm)		Aluminium chloride + sodium hydroxide-shifted UV spectra (nm)	
	Band I	Band II	Band I	Band II	Band I	Band II
1	355	255	405, 325 sh	275	390	275
2	355	255	405, 325 sh	275	390	275
3	358	258	403, 325 sh	266	400	265
4	350	265	410, 325 sh	270	410	270
5	350	265	400, 325 sh	275	350	265
6	350	270	400	275	390	280
7	350	265	395	275	350	265
8	358	260	398	275	358	260
9	350	265	410	270	350	265
10	345	260	395, 325 sh	270	390	270
11	350	265	395, 325 sh	275	350	265
12	355	265	400, 325 sh	270	400	270
13	350	265	400	270	390	275
14	360	260	395, 325 sh	275	400	270
15	358	260	403, 325 sh	275	410	270
16	350	265	395, 320 sh	275	350	265
17	350	265	390, 325 sh	265	350	265
18	350	265	395, 325 sh	275	350	265
19	350	265	400, 325 sh	275	410	275

sh: shoulder.

For the remaining constituents, LC/UV with post-column addition of shift reagents was utilised in order to determine substitution pattern, including the presence of oxygenated groups and the position of the sugar, or other component, linked to the aglycone [11]. Although the derivatisation technique was originally developed for use in conjunction with conventional UV analysis of flavonoids employing methanol as solvent [12], it has also been shown to be appropriate for acidic solvents, such as the formic acid:acetonitrile:methanol mixture utilised in the LC analysis of *Maytenus* extracts. All phenolic groups, except for those in the *peri* position to a keto function, are affected by the presence of strong base (sodium hydroxide), while Al^{3+} forms a complex with *ortho*-dihydroxy groups or keto groups having a hydroxyl in the *peri* position. Sodium acetate deprotonates the more acidic phenolic groups (i.e. 3, 7 and 4′-OH groups) producing shifts mainly in Band I. However, a shift in Band II suggests the presence of a free 7-OH group, which contributes to the observed shift and also results in an additional shoulder in Band I indicating the presence of further free OH groups in positions 3′ and/or 4′, as exemplified by quercetin derivatives. Therefore, the Band II shifts of 35–60 nm detected for *Maytenus* flavonoids following addition of sodium acetate (Table 1; Fig. 1) confirmed the presence of free 7-OH groups in all of the compounds under investigation. For a number these flavonoids, addition of aluminium chloride gave rise to shifts ranging from 35 to 60 nm in Band I indicating the presence of *ortho*-dihydroxy groups in ring B (i.e. quercetin derivatives). Flavonoids for which these shifts were absent were considered to be kaempferol or isorhamnetin derivatives.

Whilst UV data allowed the partial determination of structure, conclusive structural information could be obtained from the LC/MS analyses. Preliminary results indicated that the *Maytenus* flavonoids were more extensively fragmented by ESI-MS in the negative rather than the positive ion mode. Full single-stage ESI-MS of the flavonoid glycosides present in various *Maytenus* extracts yielded the deprotonated molecular ions, $[M\text{-}H]^-$, as shown in Table 2. These $[M\text{-}H]^-$ ions were used as precursors in the LC/ESI-IT-MS^n analyses (Table 3; Fig. 2) producing ions corresponding to the deprotonated aglycones, $[A\text{-}H]^-$, generated by the loss of sugar units. Thus, the ESI-IT-MS^2 spectra allowed the aglycones quercetin (*m/z* 301, $[A\text{-}H]^-$ or *m/z* 300, $[A\text{-}2H]^-$), kaempferol (*m/z* 285) and isorhamnetin

Table 2
Base peaks observed in the full MS analyses of the extracts of two *Maytenus* species and of a *M. aquifolium* × *M. ilicifolia* hybrid

Peak	$[M\text{-}H]^-$ (*m/z*)	*Maytenus aquifolium*	*Maytenus ilicifolia*	Hybrid plant
1	917			+
2	917	+		+
3	755	+		+
4	771	+		
5	901	+		+
6	609		+	
7	740	+		
8	740		+	+
9	931	+		
10	755	+		+
11	593	+	+	+
12	593		+	
13	609	+	+	+
14	463		+	+
15	463		+	+
16	447		+	
17	725	+		+
18	563		+	
19	447		+	

+: peak detected.

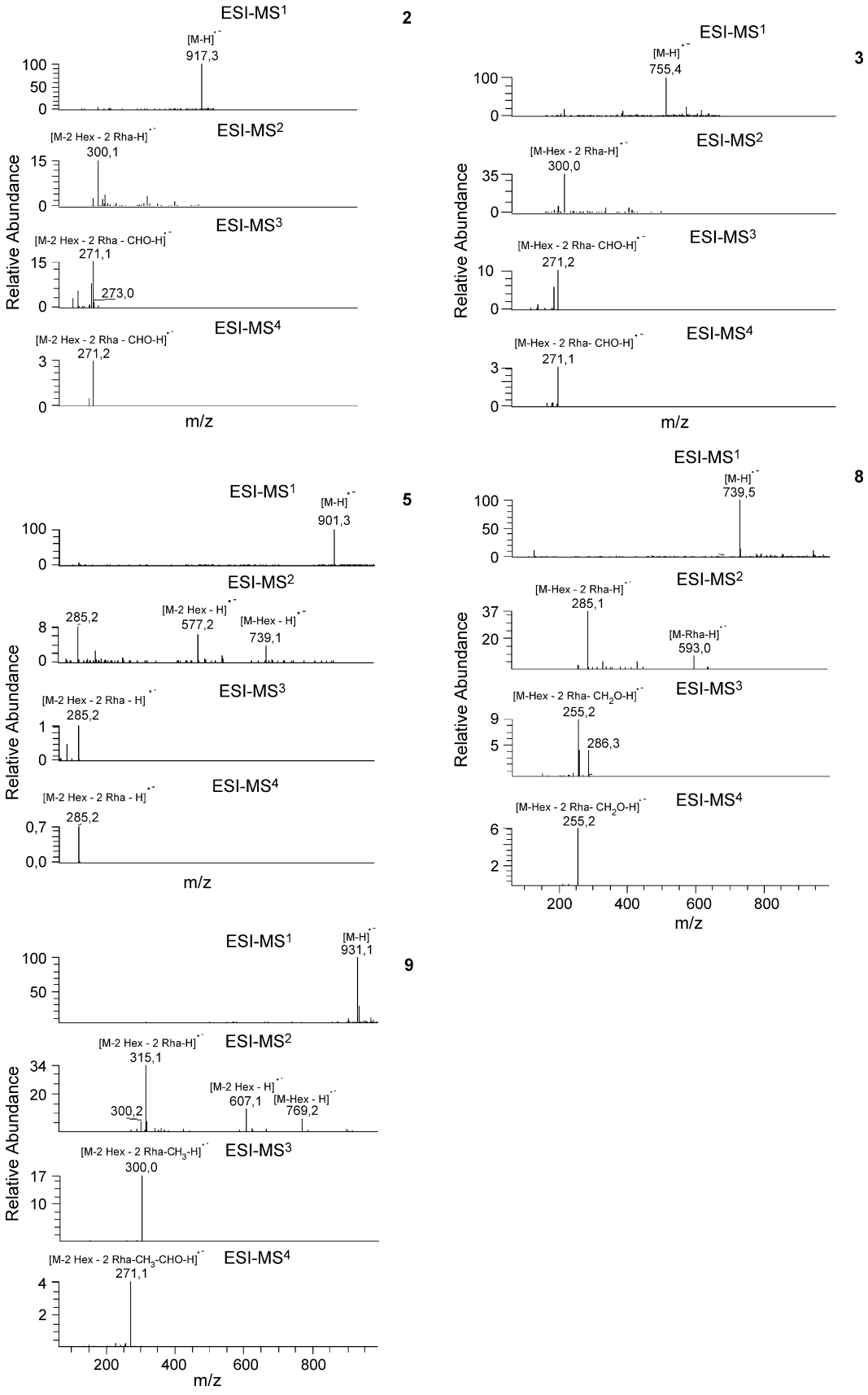

Fig. 2. Representative ESI-MS^4 spectra of quercetin-di-(rhamnohexoside) (2), quercetin-di-(rhamno)-hexoside (3), kaempferol-di-(rhamnohexoside) (5), kaempferol-di-(rhamno)-hexoside (8) and isorhamnetin-di-rhamnohexoside (9).

Table 3
Precursor ions and their corresponding fragments obtained by LC-ESI-IT-MS^n (negative ion mode) analyses of the extracts of two *Maytenus* species and of a *M. aquifolium* × *M. ilicifolia* hybrid

Peak	t_R (min)	R_1	R_2	[M-H]$^-$	Fragments			Identification
					MS^2	MS^3	MS^4	
1	6.5	2 Hexose–2 Rha	OH	917	300	271	271	Quercetin-di-(rhamnohexoside)[a]
2	6.8	2 Hexose–2 Rha	OH	917	300	271	271	Quercetin-di-(rhamnohexoside)[a]
3	7.2	Hexose–2 Rha	OH	755	300	271	271	Quercetin-di-(rhamno)-hexoside[a]
4	8.0	2 Hexose–Rha	OH	771	300	271	271	Quercetin-rhamno-di-(hexoside)[a]
5	8.6	2 Hexose–2 Rha	H	901	285	285	285	Kaempferol-di-(rhamnohexoside)[a]
6	8.7	Hexose–Rha	OH	609	301	271	271	Quercetin-rhamno-hexoside[a]
7	9.0	2 Hexose–2 Rha	H	901	285	–	–	Kaempferol-di-(rhamnohexoside)[a]
8	9.1	Hexose–2 Rha	H	740	285	255	255	Kaempferol-di-(rhamno)-hexoside[a]
9	9.4	2 Hexose–2 Rha	OCH_3	931	315	300	271	Isorhamnetin-di-rhamnohexoside[a]
10	10.2	Hexose–2 Rha	OH	755	300	271	271	Quercetin-di-(rhamno)-hexoside[a]
11	10.7	Hexose–Rha	H	593	285	255	255	Kaempferol-rhamno-hexoside[a]
12	11.2	Hexose–Rha	H	593	285	255	255	Kaempferol-rhamno-hexoside[a]
13	11.3	Glu–Rha	OH	609	301	179	151	Rutin[b]
14	12.0	Gal	OH	463	301	179	151	Hyperoside[b]
15	12.4	Glu	OH	463	301	179	151	Isoquercitrin[b]
16	14.7	Hexose	H	447	285	255	255	Kaempferol-hexoside[a]
17	14.8	Hexose–Pentose–Rha	H	725	285	255	255	Kaempferol-rhamno-hexo-pentoside[a]
18	15.7	Pentose–Rha	H	563	285	255	255	Kaempferol-rhamno-pentoside[a]
19	16.0	Rha	H	447	301	179	151	Quercitrin[b]

[a] Compound tentatively identified from spectral data R_1 and R_2 refer to Fig. 3.
[b] Compound conclusively identified by comparison with authentic standard.

(*m*/*z* 315) to be readily identified. The molecular mass values of the sugar units were calculated from the difference in mass of the [M-H]$^-$ and the [A-H]$^-$ ions, such that a difference of 162 U indicated a hexose (possibly glucose or galactose), 146 U indicated rhamnose, and 132 U indicated a pentose (possibly xylose or arabinose).

The spectra obtained from the ESI-IT-MS^n experiments also revealed some specific information associated with the fragmentation of rings A and C (Fig. 3), which should result in the formation of X$^-$ and X$^-$–CO ions, respectively, at *m*/*z* values of 179 and 151. The elimination of Y (122 U, MS^3), followed by loss of CO (28 U, MS^4), was observed for some quercetin glycosides, such as rutin. On other hand, the X$^-$ ion, which corresponds to the cleavage of ring C, was not observed for kaempferol or for various other derivatives of quercetin, which only showed losses of CHO$^-$ (29 U, neutral radical) and CH_2O (30 U, neutral loss). In addition, the spectrum of 9 showed a loss of 15 U (CH_3 group) from the [A-H]$^-$ ion suggesting the presence of a methoxylated flavonoid. These data are compatible with those reported for the analysis of quercetin, kaempferol and isorhamnetin standards by negative mode APCI-MS-MS [13].

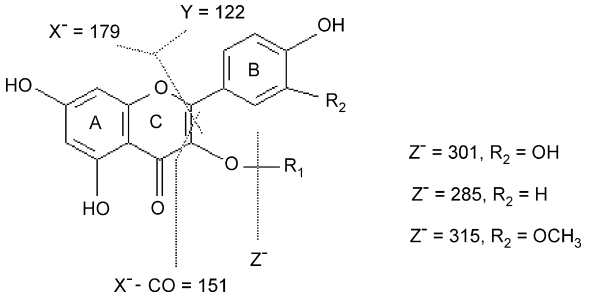

Fig. 3. Proposed ESI-IT-MS^n fragmentation pathways for derivatives of quercetin, kaempferol and isorhamnetin.

The peak identities originally proposed on the basis of LC/UV spectral studies (Fig. 1) were confirmed by consideration of *m*/*z* and t_R data. In addition, full identification of some of the *Maytenus* flavonoids was verified by comparison of MS data derived from the extracts with those obtained from standard compounds (Table 3). Nineteen different flavonoids were detected in the *Maytenus* extracts, nine of which were quercetin derivatives, nine were kaempferol derivatives, and one was an isorhamnetin derivative.

Vilegas et al. [7–9] have previously reported the presence of flavonoid tetrasaccharides in extracts of *M. aquifolium*, and the major peaks 2 and 5 may be attributed, respectively, to quercetin and kaempferol tetrasaccharides. As shown in Fig. 1 and Table 3, it was possible in the present study to identify the presence of rutin (13) and six other flavonoids in *M. aquifolium*, namely, two triglycosylated quercetin derivatives (4 and 10), two triglycosylated kaempferol derivatives (7 and 17), one diglycosylated kaempferol derivative (11), and one tetraglycosylated isorhamnetin derivative (9). Eleven glycosylated flavonoids were determined in *M. ilicifolia*, the two major components being quercetin-di-rhamno-hexoside (3) and kaempferol-di-rhamno-pentoside (8), neither of which were detected in *M. aquifolium*. *M. ilicifolia* also showed the presence of a diglycosylated quercetin derivative (6) and rutin (13), together with four diglycosylated kaempferol derivatives (11, 12, 16 and 18), two monoglycosylated quercetin derivatives (14 and 15) and quercitrin (19). The presence in *M. ilicifolia* of rutin, quercitrin and the monoglycosylated flavonoids 14 and 15 has not been previously reported [7].

The *M. aquifolium* × *M. ilicifolia* hybrid showed eleven glycosylated flavonoids, ten of which were present in the extracts of the parent plants, and one, quercetin-di-(rhamnohexoside) (1), was exclusive to the hybrid plant (Table 2). As the UV and MS^n data of compounds 1 and 2 are identical (albeit with different t_R), it is possible that they differ only in the hexose substituents (galactose or glucose). The chromatographic profile of the hybrid plant suggests a predominance of flavonoids derived from *M. aquifolium*, together with a larger amount of rutin in comparison with either of the parent plants. However, the analyses of further plant samples must be carried out before final quantitative conclusions can be drawn.

4. Conclusions

The combination of on-line UV-PAD data, in the presence and absence of shift reagents, and ESI-IT-MS^n spectral information, has enabled a comprehensive comparison of the flavonoid profiles of leaves of two species of *Maytenus* and a hybrid plant to be carried out. Nineteen flavonoids could be identified through on-line analysis of the complex extracts obtained using only small amounts of plant material. Moreover, in comparison with the HPTLC method previously reported for the analysis of *Maytenus*-derived plant drugs [6], the LC/UV/MS method has been shown to be a powerful tool for the rapid fingerprint analysis of plant materials used in the phytomedicines industry. Moreover, most of the flavonoids presently identified were not reported in previous studies conducted using conventional phytochemical procedures [7–9], illustrating the importance of LC-hyphenated techniques in structural elucidation. The structural data reported herein are currently being applied in our continuing studies on the quantitative analysis of *Maytenus* flavonoids by HPLC with the purpose of assessing the chemical quality of phytomedicines.

Acknowledgments

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