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# Characterization of phenolic compounds in *Helichrysum melaleucum* by high-performance liquid chromatography with on-line ultraviolet and mass spectrometry detection

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***Helicrysum melaleucum* is a medicinal plant traditionally used in the islands of the Macaronesia region for the treatment of respiratory diseases. In this work, the phenolic compounds of *Helicrysum melaleucum* plants collected in different geographical locations of Madeira Island and their morphological parts (total aerial parts, leaves, flowers and stems) were extracted and analyzed separately by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-DAD/ESI-MS$^n$). A total of 68 compounds were characterized based mainly on their UV and mass spectra. These included derivatives of *O*-glycosylated flavonoids (flavonol and flavones type), quinic acid, caffeic acid, lignans and polyphenols. The flowers were found to be the morphological part with higher variety of phenolic compounds. The large differences in the phenolic composition of plants collected from different geographical locations allowed the identification of a few components, such as pinoresinol and methoxylated flavone derivatives, likely to be useful as geographical markers. Also, these results promote further comparison of the bioactivities of the different samples analyzed. This paper marks the first report on the chemical analysis of *Helichrysum melaleucum* species.**


There are more than 500 species of *Helichrysum* genus distributed around the world. Plants of this *genus* have been found to possess several biological activities, such as antimicrobial, antiallergic, antioxidant, anti-inflammatory, cough relief, cold and wounds.[1]

In Madeira Archipelago (Portugal) there are some *Helichrysum* species used in traditional medicine. Several of them are imported and four are endemic species. *Helichrysum melaleucum* Rchb. Ex Holl is one of these endemic subspecies and, according to folk medicine, the leaves and the flowers heads are used for the treatment of bronchitis and pharingitis while infusions of the flowers are used as cardiotonic and cough relief remedy.[2] This particular plant only grows on the north coast of Madeira Island. It is very common in locations near the sea and rare in high altitude locations (up to 1200 m).

The biological activities of *Helichrysum* plants have been attributed to several classes of compounds such as flavonoids, α-pyrones, coumarins and terpenoids, detected in different morphological parts of the plant.[3]

To our knowledge, there is no report establishing a relation between the phenolic composition of *Helichrysum melaleucum* and its biological activities. In previous work by our group, the phenolic composition of *Helichrysum devium* was established using a high-performance liquid chromatography diode-array detection/electrospray ionization tandem mass spectrometry (HPLC-DAD/ESI-MS$^n$) method. The most abundant phenolic compounds were found to be hydroxycinnamic derivatives, in particular quinic acid derivatives.[4] Quinic acids were also found in other *Helichrysum* plants showing strong antioxidant activity.[1]

Phenolic compounds are a large class of low molecular weight secondary plant metabolites, which are fundamental for plant normal development and an important key in their defence mechanisms. The great interest in this class of compounds is a result of their important biological activities such as antioxidant activity, protection against cancer, cardiovascular and neurodegenerative diseases. They can also be used as natural antioxidants in food processing in order to prevent lipid peroxidation.[5]

The main classes of phenolic compounds are phenolic acids and flavonoids. The major subclasses of flavonoids are flavonols, flavones, isoflavones, flavanones, catechins, aurones, anthocyanins and chalcones.

In plant cells, flavonoids usually occur as glycosides and are divided into two groups, according to the site of glycoside substitution on the flavonoid structure: *O*-glycosides) and *C*-glycosides. Besides glycosylation, flavonoids can occur in more modified forms, due to additional hydroxylation, methylation and acetylation.[6]

Reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with UV diode-array detector (DAD) is widely used for separation and detection of phenolic compounds from complex samples including natural sources like plant extracts. Coupling a tandem mass spectrometry detector (LC/MS/MS) with electrospray

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ionization (ESI) or atmospheric pressure chemical ionization (APCI) has proved to be a powerful tool for the unequivocal characterization and structural identification of phenolic compounds.

The use of ESI as ionization source operating in the negative mode has proved to be more efficient and selective for the detection of phenolic compounds like flavonoids glycosides.[6,7] Also, it allows for the detection of minor components, difficult to detect by other means. With multi-stage tandem mass spectrometry ($MS^n$) analysis, it is possible to obtain structural information and also to exclude the presence of interferences, which is not possible with UV detection.

In this paper, a HPLC-DAD/ESI-$MS^n$ method was used to separate and identify the phenolic compounds present in the methanolic extracts of *Helichrysum melaleucum*. Given the use of different morphological parts of this plant (leaves, flowers, stems and total aerial parts) for different medicinal purposes, it is important to perform a screening of their phenolic profile. In addition, a comparison of the phenolic composition was made for plants collected in different geographical environments: São Vicente (located at sea level, just across the road from the beach) and Fajã da Nogueira (about 1000 m altitude).

This report is the first exhaustive study on the phenolic composition of methanolic extracts of different morphological parts from *Helichrysum melaleucum*.

## EXPERIMENTAL

### Chemical and standards

HPLC grade acetonitrile ($CH_3CN$) (Lab-Scan, 99%), ultrapure water (Milli-Q, Waters) and formic acid (analytical grade) were used for mobile phase preparation in the LC/MS analysis. The methanol used for extraction of *Helichrysum devium* was AR grade, purchased from Fisher. Eluents prepared for LC/MS analysis were additionally filtered through 0.45 μm (Millipore) membranes.

Apigenin (>99%), quercetin (>99%), ferulic acid (>99%) and coumaric acid (>99%) were purchased from Extra-Synthese and 5-*O*-caffeoylquinic acid (99%), kaempferol (>99%) and ellagic acid (>99%) from Acros Organics. Stock solutions of these compounds (100 μg/mL) were prepared in ethanol and further analyzed by LC-DAD/ESI-$MS^n$.

### Plant material and sample preparation

Samples of *Helichrysum melaleucum* were collected in the wild in two different locations of the north cost of Madeira: São Vicente and Fajã da Nogueira. The plant material collected in São Vicente consisted of total aerial parts and individually separated leaves, flowers and stems. The amount of plant material collected at Fajã da Nogueira was very small, so only the total aerial parts were analyzed. The plants were authenticated by taxonomist Fátima Rocha and a voucher was deposited in the Madeira Botanical Garden herbarium collection.

Dried and powdered plant material (100 g) was exhaustively extracted by maceration with methanol (1 L), at room temperature for 24 h.

In all cases the solutions were filtered and concentrated to dryness under reduced pressure in a rotary evaporator (40°C). Stock solutions with concentrations (m/v) of 5 mg/mL were prepared by dissolving dried extract in initial HPLC mobile phase ($CH_3CN/H_2O$ (20:80)).

These solutions were filtered through 0.45 μm micropore membranes prior to use and 10 μL were injected for LC-DAD/ESI-$MS^n$ analysis. Three independent assays were performed for each sample.

### Liquid chromatography

The HPLC analysis was performed on a Dionex ultimate 3000 series instrument coupled to a binary pump, a diode-array detector (DAD), an autosampler and a column compartment. The wavelength range was set at 210–520 nm and was monitored at 280 nm. Samples were separated on a Phenomenex Gemini $C_{18}$ column (5 μm, 250 × 3.0 mm i.d.; Phenomenex) with a sample injection volume of 10 μL. The mobile phase consisted of $CH_3CN$ (A) and water/formic acid (100:0.1, v/v) (B). A gradient program was used as follows: 20% A (0 min), 25% A (10 min), 25% A (20 min), 50% A (40 min), 100% A (42–47 min), 20% A (49–55 min). The mobile phase flow rate was 0.4 mL/min; the chromatogram was recorded at 280 nm and spectral data for all peaks were accumulated in the range of 190–400 nm. Column temperature was controlled at 30°C.

### Mass spectrometry

For LC/ESI-$MS^n$ analysis, a model 6000 ion trap mass spectrometer (Bruker Esquire, Bremen, Germany) fitted with an ESI source was used. Data acquisition and processing were performed using Esquire control software. Negative ion mass spectra of the column eluate were recorded in the range *m/z* 100–1000 at a scan speed of 13000 Da/s. High-purity nitrogen ($N_2$) was used both as drying gas at a flow of 10.0 mL/min and as a nebulizing gas at a pressure of 50 psi. The nebulizer temperature was set at 365°C and a potential of +4500 V was used on the capillary. Ultra-high-purity helium (He) was used as collision gas at a pressure of $1 \times 10^{-5}$ mbar and the collision energy was set at 40 V.

The acquisition of $MS^n$ data was made in *auto* $MS^n$ mode, with an isolation width of 4.0 *m/z*. For $MS^n$ analysis, the mass spectrometer was scanned from 10 to 1000 *m/z* with a fragmentation amplitude of 1.0 V ($MS^n$ up to $MS^4$) and two precursor ions.

## RESULTS AND DISCUSSION

As mentioned before, *Helichrysum melaleucum* was collected in the wild in two different geographical locations. To simplify the identification of extracts, samples from São Vicente were denominated as SV and the one from Fajã da Nogueira as FN.

It must be mentioned that in Fajã da Nogueira, located at 1000 m altitude, *H. melaleucum* is a rare specimen and it was not possible to collect enough plant material to separate into different morphological parts, as it was done for the plants collected in São Vicente.

The base peak chromatogram (BPC) profiles of the methanolic extracts from plants from SV and FN are shown





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in Figs. 1 and 2. To a large extent, the compounds could be well separated and no relevant variation was observed in the three independent assays performed for each sample.

When a reference pure standard was available, the compound was identified by comparing the HPLC retention time, UV and mass spectra with those obtained for the standard. However, since the access to pure reference compounds was limited and the characterization of several compounds detected based only on information provided by the UV spectra is not possible, the structures of unknown compounds were proposed based mainly on the $MS^n$ fragmentation pattern.

A preliminary analysis of the UV spectra of all compounds allowed the identification of hydroxycinnamic acid derivatives and flavonoid derivatives. The first group showed characteristic absorption bands at 230–240 and 320–340 nm, together with a shoulder around 300–310 nm. In the group of flavonoids, flavonol glycosylated derivatives showed two maximum absorptions at 250–270 and 320–360 nm. Flavones showed two absorptions at 230–250 and 330–360 nm.

More than 68 different compounds were detected and 55 of them were characterized. Their $MS^n$ fragmentation ions and UV absorptions are presented in Tables 1 and 2 and their chemical structures are given in Fig. 3.

Most of the detected phenolic compounds gave deprotonated molecular ions, $[M–H]^-$, of high abundance that allowed for $MS^n$ analysis. Usually, the base peak in a full MS spectrum was assigned as the $[M–H]^-$ ion.

When two or more isomers were detected, their identification was made based on previous reports of *Helichrysum* species, comparison of HPLC retention times and relative intensity of characteristics fragments.[4]

Compounds were numbered by their order of elution since some of them were not found in all samples.

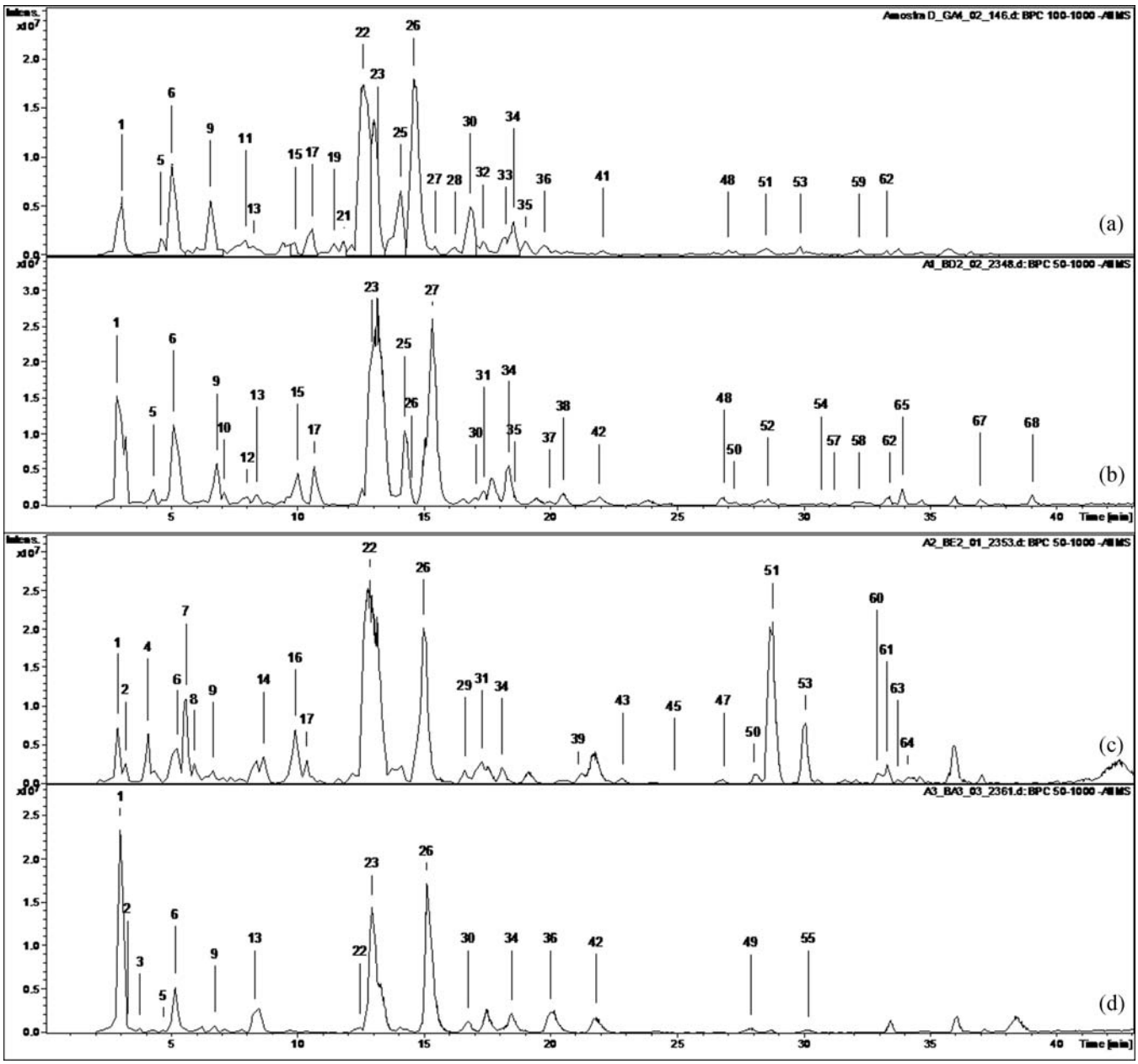


**Figure 1.** LC-DAD/ESI-$MS^n$ analysis of the methanolic extracts of *Helichrysum melaleucum* from São Vicente (SV) – LC/MS negative ion ESI-MS base peak chromatogram (BPC): (a) total aerial parts; (b) leaves; (c) flowers; and (d) stems.





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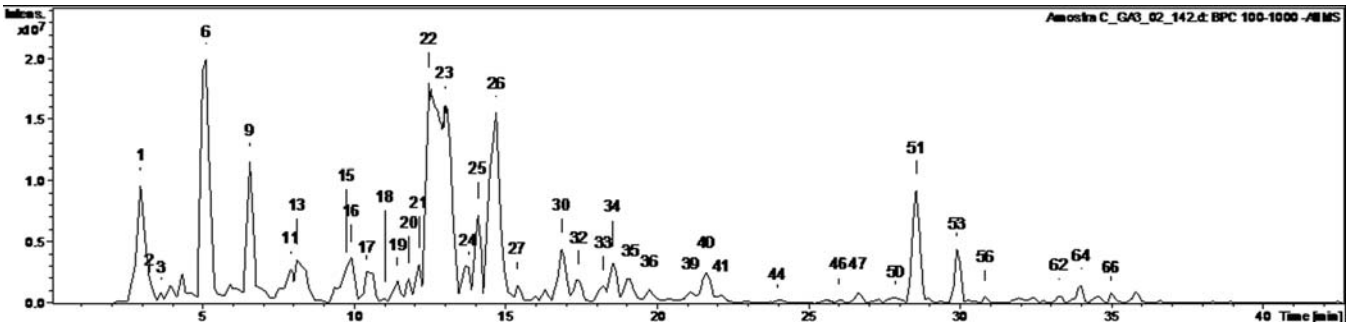


**Figure 2.** LC-DAD/ESI-MS$^n$ analysis of the methanolic extract of *Helichrysum melaleucum* total aerial parts from Fajã da Nogueira (FN) – LC/MS negative ion ESI-MS base peak chromatogram (BPC).

## Identification of flavonoids

The occurrence of flavonoids in *Helichrysum* species has been reported previously[4] with aglycones belonging to two subtypes, flavonols and flavones. In this study, several flavonoids were detected in their glycosylated form and/or esterified with acyl groups. Free aglycones were found in trace amounts in some samples.

MS$^n$ fragmentation of the ion $[M–H]^-$ gave the deprotonated aglycone ion ($Y_0^-$) by the loss of the sugar residue.

The nature of the glycoside groups was identified based on the neutral losses of hexoside, caffeoyl, rhamnoside, coumaroyl and malonyl moieties (−162, −162, −146, −146 and −86 Da, respectively).

The nomenclature proposed by Ma *et al*.[8] for MS$^n$ fragment ions of flavonoids was adopted in this work. For free aglycones, the $^{i,j}A^-$ and $^{i,j}B^-$ labels correspond to ions containing intact A- and B-rings, respectively, in which i and j indicate the C-ring bonds that have been broken. For conjugated aglycones, $Y_0^-$ is used to refer to the aglycone fragment [M–H–glycoside]$^-$.

Compounds **4** (retention time ($t_R$) = 4.0 min) and **8** ($t_R$ = 5.9 min) were only detected in the SV flowers methanolic extract. Compound **4** ($t_R$ = 4.0 min) displayed a $[M–H]^-$ ion at *m/z* 609 which easily loses two residues of 162 Da each, by MS$^n$ fragmentation, forming fragment ions at *m/z* 447 (base peak) and 285 (ca. 30% of base peak). The fragmentation of the ion at *m/z* 285 gave a fragment ion at *m/z* 255 (loss of 30 Da, $[Y_0^–CH_2OH]^-$) which is characteristic of kaempferol (comparison made with a standard solution of kaempferol). The two fragments with 162 Da could be attributed either to a caffeoylhexoside or a dihexoside residue. However, a fragment ion at *m/z* 323 was not observed, indicating the presence of a caffeoylhexoside residue; moreover, it is known that acylated flavonoids appear at higher retention time. Based on these assumptions, the two 162 Da residues were identified as being hexoside residues.

Given that the MS$^2$ spectrum base peak does not correspond to the deprotonated aglycone ion and based on the rules described by Ablajan *et al*.[9] it is clear that the two hexoside residues are not linked in the same position of the aglycone skeleton. It is known that for flavonols, like kaempferol, the 3-OH and 7-OH positions are the more favoured positions for glycosylation to occur. Thus, compound **4** was identified as kaempferol-3,7-*O*-dihexoside.

Compound **8** ($t_R$ = 5.9 min) gave a $[M–H]^-$ ion at *m/z* 651. In the MS$^2$ spectrum a fragment ion was observed at *m/z* 447, due to the loss of 206 Da, which indicates a hexoside residue (162 Da) linked to an acetyl group (44 Da). The MS$^3$ spectrum of the ion at *m/z* 447 gave a fragment ion at *m/z* at 284 as base peak and a low intensity ion at *m/z* 285 (18.3% of the base peak). The further MS$^n$ fragmentation gave a fragment ion at *m/z* 255, consistent with the MS$^n$ data of kaempferol. As mentioned above, the favoured glycosylation positions for kaempferol are 3-OH and 7-OH. In the MS$^3$ spectrum, the aglycone radical ion is more abundant than the deprotonated aglycone ion, which corresponds to an aglycone substituted in position 3-OH.[10] Therefore, compound **8** was identified as kaempferol-3-*O*-hexoside-7-*O*-acetylhexoside.

In the FN total aerial parts methanolic extract, at a retention time of 9.8 min, there are two overlapping peaks (compounds **15** and **16**). However, their deprotonated molecular ions, $[M–H]^-$, could be clearly identified and presented enough intensity to be subjected to further MS$^n$ fragmentation.

Compound **15** displayed a $[M–H]^-$ ion at *m/z* 493 and its MS$^2$ spectrum showed the aglycone ion ($Y_0^-$) at *m/z* 331, as base peak, suggesting the presence of a hexoside residue. This ion, under MS$^n$ fragmentation, gave a radical fragment $[Y_0^–CH_3]^{\cdot-}$ at *m/z* 316, consistent with literature data for mearnsetin.[11]

Compound **16** yielded a $[M–H]^-$ ion at *m/z* 463 and its analysis by MS$^2$ fragmentation resulted in the aglycone ion ($Y_0^-$) at *m/z* 301, probably due to a hexoside residue (162 Da). The MS$^n$ fragmentation of the ion at *m/z* 301 gave fragment ions at *m/z* 151 ($^{1,2}A^–CO$), 179 ($[^{1,2}A^–H]^-$), 255 ($[M–H–H_2O–CO]^-$) and 271 ($[M–H–CH_2O]^-$), originating from a retro-Diels-Alder (RDA) reaction. MS$^n$ data are very similar to those from the fragmentation of a standard solution of quercetin (data not shown), so quercetin should be the aglycone of compound **16**.

It is known that, despite the fact that any of the hydroxyl groups of the flavonoid aglycone can be glycosylated, certain positions are favoured. For flavonols the 3-OH and 7-OH positions are regular glycosylation sites.[6] Even so, based only on MS$^n$ data of these two compounds, neither the nature of the hexoside residue nor the sugar linkage position to the aglycone could be determined. Thus, compounds **15** and **16** were preliminary characterized as mearnsetin-*O*-hexoside and quercetin-*O*-hexoside, respectively. They were also





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**Table 1.** Characterization of phenolic compounds of the methanolic extract of total aerial parts, leaves, flowers and stems from *Helichrysum melaleucum* from São Vicente (SV) by LC-DAD/ESI-$MS^n$

| No. | $t_R$ (min) | UV $\lambda_{max}$ (nm) | $[M-H]^-$ *m/z* | LC-DAD/ESI-$MS^n$ *m/z* (% base peak) | Assigned identity | Morphological part |
|---|---|---|---|---|---|---|
| 1 | 2.9 | 268 | 683 | $MS^2$ [683]: 342 (10.7), 341 (100)<br>$MS^3$ [683→341]: 179 (100), 161 (11.7), 131 (20.7), 119 (20.9), 113 (15.7)<br>$MS^4$ [683→341→179]: 143 (40.9), 119 (43.7), 101 (100) | Caffeic acid-*O*-hexoside | Leaves<br>Flowers<br>Stems |
| 2 | 3.0 | 261 | 191 | $MS^2$ [191]: 173 (21.6), 171 (14.0), 127 (89.5), 109 (100)<br>$MS^3$ [191→127]: 109 (100) | Quinic acid | Total aerial parts<br>Flowers<br>Stems |
| 3 | 3.5 | - | 317 | $MS^2$ [317]: 207 (34.2), 225 (100), 125 (55.8), 107 (10.3)<br>$MS^3$ [317→225]: 165 (100) | Unknown | Stems |
| 4 | 4.0 | 265, 345 | 609 | $MS^2$ [609]: 449 (11.8), 448 (24.6), 447 (100), 285 (30.5)<br>$MS^3$ [609→447]: 327 (28.6), 285 (75.0), 284 (100), 255 (74.1)<br>$MS^4$ [609→447→284]: 256 (32.3), 255 (100) | Kaempferol-3,7-di-*O*-hexoside | Flowers |
| 5 | 4.6 | 289 | 341 | $MS^2$ [341]: 179 (100), 135 (22.7)<br>$MS^3$ [341→179]: 135 (100) | Caffeic acid-*O*-hexoside | Total aerial parts<br>Leaves Stems |
| 6* | 5.0 | 242, 300, 325 | 353 | $MS^2$ [353]: 191 (100)<br>$MS^3$ [353→191]: 173 (100), 127 (38.5), 111 (25.3), 93 (45.0)<br>$MS^4$ [353→191→173]: 155 (83.8), 93 (100) | 5-*O*-Caffeoylquinic acid | Total aerial parts<br>Leaves<br>Flowers<br>Stems |
| 7 | 5.5 | - | 481 | $MS^2$ [481]: 445 (100), 179 (11.7), 161 (10.3)<br>$MS^3$ [481→445]: 221 (100), 179 (83.2), 131 (85.8)<br>$MS^4$ [481→445→179]: 113 (49.5), 101 (100) | Caffeic acid derivative | Flowers |
| 8 | 5.9 | 244, 284, 324 | 651 | $MS^2$ [651]: 489 (63.0), 448 (16.5), 447 (100), 327 (11.0), 285 (26.0)<br>$MS^3$ [651→447]: 285 (18.3), 284 (100), 255 (26.6), 174 (14.4)<br>$MS^4$ [651→447→284]: 256 (48.1), 255 (100) | Kaempferol-3-*O*-hexoside-7-*O*-acetylhexoside | Flowers |
| 9 | 6.5 | 238, 302, 321 | 515 | $MS^2$ [515]: 353 (100), 335 (27.8), 191 (26.0), 179 (53.7)<br>$MS^3$ [515→353]: 191 (100), 179 (50.4), 135 (11.3)<br>$MS^4$ [515→353→191]: 173 (30.2), 127 (100), 111 (84.0), 109 (11.1) | 1,3-*O*-Dicaffeoylquinic acid | Total aerial parts<br>Leaves<br>Flowers<br>Stems |
| 10 | 7.2 | 282, 340 | 463 | $MS^2$ [463]: 302 (14.3), 301 (100)<br>$MS^3$ [463→301]: 283 (100), 257 (45.1), 227 (47.4), 192 (61.8), 175 (48.4), 165 (45.8)<br>$MS^4$ [463→301→283]: 229 (100) | Ellagic acid-*O*-hexoside | Leaves |
| 11 | 7.9 | 244, 302, 327 | 367 | $MS^2$ [367]: 191 (16.1), 179 (100), 161 (8.6), 135 (50.1)<br>$MS^3$ [367→179]: 135 (100) | Caffeic acid derivative | Total aerial parts |
| 12 | 8.0 | 318 | 533 | $MS^2$ [533]: 371 (100), 353 (40.3), 191 (21.3)<br>$MS^3$ [533→371]: 353 (100), 191 (60.3), 179 (13.8), 135 (53.3)<br>$MS^4$ [533→371→353]: 191 (100), 179 (33.8), 134 (30.7) | Caffeic acid hexoside derivative | Leaves |
| 13 | 8.2 | 305 | 337 | $MS^2$ [337]: 191 (100)<br>$MS^3$ [337→191]: 173 (73.6), 127 (100), 111 (53.7)<br>$MS^4$ [337→191→127]: 171 (13.8), 109 (8.6) | 5-*O*-*p*-Coumaroylquinic acid | Total aerial parts<br>Leaves<br>Stems |
| 14 | 8.6 | 229, 300, 321 | 677 | $MS^2$ [677]: 516 (16.5), 515 (100)<br>$MS^3$ [677→515]: 324 (10.8), 323 (100), 191 (34.5), 179 (36.9)<br>$MS^4$ [677→515→323]: 161 (100) | Dicaffeoylquinic acid hexoside | Flowers |
| 15 | 9.8 | 258, 300, 344 | 493 | $MS^2$ [493]: 331 (100), 316 (21.6)<br>$MS^3$ [493→331]: 316 (100)<br>$MS^4$ [493→331→316]: 287 (100), 271 (31.7), 255 (15.7), 166 (55.9) | Mearnsetin-*O*-hexoside | Total aerial parts<br>Leaves |
| 16 | 9.9 | 256, 353 | 463 | $MS^2$ [463]: 301 (100), 300 (17.7)<br>$MS^3$ [463→301]: 271 (28.7), 255 (17.8), 179 (67.9), 151 (100)<br>$MS^4$ [463→301→151]: 107 (100), 83(17.5) | Quercetin-*O*-hexoside | Flowers |

(*Continues*)





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**Table 1.** (Continued)

| No. | $t_R$ (min) | UV $\lambda_{max}$ (nm) | $[M-H]^-$ $m/z$ | LC-DAD/ESI-$MS^n$ $m/z$ (% base peak) | Assigned identity | Morphological part |
|---|---|---|---|---|---|---|
| 17 | 10.6 | 255, 272, 343 | 477 | $MS^2$ [477]: 316 (17.2), 315 (100), 300 (30.2)<br>$MS^3$ [477→315]: 301 (16.8), 300 (100), 271 (1.9)<br>$MS^4$ [477→315→300]: 283 (22.9), 272 (44.3), 255 (86.0), 216 (100) | Isorhamnetin-*O*-hexoside | Total aerial parts<br>Leaves<br>Flowers |
| 19 | 11.6 | 212, 296 | 547 | $MS^2$ [547]: 515 (90.0), 385 (25.4), 353 (100), 335 (12.1), 191 (20.6)<br>$MS^3$ [547→353]: 191 (100), 179 (7.4)<br>$MS^4$ [547→353→191]: 173 (40.4), 127 (100), 111 (32.9), 109 (27.5) | 1,5-*O*-Dicaffeoylquinic acid derivative | Total aerial parts |
| 21 | 12.1 | 286, 324 | 353 | $MS^2$ [515]: 353 (100), 335 (14.7), 191 (37.9), 179 (21.4), 173 (33.2)<br>$MS^3$ [515→353]: 191 (51.0), 179 (74.0), 173 (100), 135 (20.9)<br>$MS^4$ [515→353→173]: 155 (77.0), 111 (100) | 3,4-*O*-Dicaffeoylquinic acid | Total aerial parts |
| 22 | 12.5 | 243, 300, 323 | 515 | $MS^2$ [515]: 353 (100), 335 (4.8), 191 (37.9), 173 (4.9)<br>$MS^3$ [515→353]: 191 (100)<br>$MS^4$ [515→353→191]: 173 (70.2), 127 (100), 111 (41.8), 109 (91.3) | 1,5-*O*-Dicaffeoylquinic acid | Total aerial parts<br>Flowers<br>Stems |
| 23 | 13 | 242, 300, 328 | 515 | $MS^2$ [515]: 353 (100), 191 (12.9), 179 (2.2)<br>$MS^3$ [515→353]: 191 (100), 179 (32.2), 173 (2.5), 135 (13.3)<br>$MS^4$ [515→353→191]: 173 (63.7), 127 (100), 111 (15.9) | 3,5-*O*-Dicaffeoylquinic acid | Total aerial parts<br>Leaves<br>Stems |
| 25 | 14.0 | 273, 337 | 461 | $MS^2$ [461]: 446 (59.5), 299 (100), 284 (46.5), 283 (43.5)<br>$MS^3$ [461→299]: 297 (9.1), 285 (11.1), 284 (100)<br>$MS^4$ [461→299→284]: 256 (98.4), 255 (100), 228 (67.7), 227 (46.7), 200 (58.1), 163 (82.7) | Hispidulin-*O*-hexoside | Total aerial parts<br>Leaves |
| 26 | 14.6 | 244, 300, 328 | 601 | $MS^2$ [601]: 557 (40.5), 515 (80.0), 439 (27.6), 395 (100), 233 (33.1)<br>$MS^3$ [601→395]: 335 (3.3), 233 (100), 173 (26.9)<br>$MS^4$ [601→395→233]: 173 (100), 155 (2.0) | Malonyl-3,4-*O*-dicaffeoylquinic acid | Total aerial parts<br>Leaves<br>Flowers<br>Stems |
| 27 | 15.4 | - | 445 | $MS^2$ [445]: 282 (13.6), 281 (100), 163 (12.1), 137 (25.0)<br>$MS^3$ [445→281]: 137 (100), 113 (3.1) | Unknown | Total aerial parts |
| 28 | 16.1 | 235, 327 | 601 | $MS^2$ [601]: 557 (28.7), 515 (6.3), 396 (16.1), 395 (100), 233 (27.4)<br>$MS^3$ [601→395]: 233 (100), 173 (34.0), 135 (1.5)<br>$MS^4$ [601→395→233]: 173 (100) | 4,5-*O*-Dicaffeoylquinic acid derivative | Total aerial parts |
| 29 | 16.6 | 285, 346 | 489 | $MS^2$ [489]: 286 (23.9), 285 (100)<br>$MS^3$ [489→285]: 257 (100), 255 (20.2), 229 (63.1), 195 (40.4)<br>$MS^4$ [489→285→257]: 240 (11.8), 167 (100), 163 (64.7) | Kaempferol-*O*- acetylhexoside | Flowers |
| 30 | 16.9 | 245, 300, 325 | 601 | $MS^2$ [601]: 557 (16.6), 515 (32.9), 395 (100), 233 (47.2)<br>$MS^3$ [601→395]: 335 (5.6), 233 (100), 173 (24.2)<br>$MS^4$ [601→395→233]: 173 (100) | Malonyl-4,5-*O*-dicaffeoylquinic acid | Total aerial parts<br>Leaves<br>Flowers<br>Stems |
| 31 | 17.1 | - | 499 | $MS^2$ [499]: 354 (15.6), 353 (100), 191 (14.1)<br>$MS^3$ [499→353]: 191 (100)<br>$MS^4$ [499→337→191]: 173 (64.7), 171 (74.4), 93 (100) | Coumaroyl 5-*O*-caffeoylquinic acid | Leaves<br>Flowers |
| 32 | 17.3 | 223, 311 | 499 | $MS^2$ [499]: 353 (23.3), 337 (100), 191 (24.2)<br>$MS^3$ [499→337]: 191 (100), 173 (3.4), 163 (8.4)<br>$MS^4$ [499→337→191]: 173 (39.2), 137 (43.8), 127 (100), 111 (58.3) | 4-*O*-caffeoyl-5-*O*-coumaroylquinic acid | Total aerial parts |
| 33 | 18.1 | - | 625 | $MS^2$ [625]: 473 (100)<br>$MS^3$ [625→473]: 341 (100), 293 (27.2), 233 (46.6), 179 (31.1) | Caffeic acid derivative | Flowers |

(*Continues*)





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**Table 1.** (Continued)

| No. | $t_R$ (min) | UV $\lambda_{max}$ (nm) | $[M-H]^-$ $m/z$ | LC-DAD/ESI-$MS^n$ $m/z$ (% base peak) | Assigned identity | Morphological part |
|---|---|---|---|---|---|---|
| | | | | $MS^4$ [625→473→341]: 239 (45.5), 179 (100) | | |
| 34 | 18.4 | - | 625 | $MS^2$ [625]: 474 (17.9), 473 (100), 341 (5.9), 293 (14.8), 233 (3.7)<br>$MS^3$ [625→473]: 342 (20.0), 341 (100), 326 (21.7), 293 (58.6), 233 (29.1), 191 (16.2)<br>$MS^4$ [625→473→341]: 239 (68.5), 209 (14.8), 197 (15.0), 179 (100), 150 (17.1) | Caffeic acid derivative | Total aerial parts<br>Leaves<br>Stems |
| 35 | 18.9 | 243, 329 | 529 | $MS^2$ [529]: 368 (21.0), 367 (100), 353 (18.1), 191 (20.0), 179 (4.2)<br>$MS^3$ [529→367]: 191 (100)<br>$MS^4$ [529→367→191]: 173 (58.2), 155 (10.7), 127 (100), 109 (10.1) | 1-Caffeoyl-5-ferruoylquinic acid | Total aerial parts<br>Leaves |
| 36 | 19.8 | - | 457 | $MS^2$ [457]: 273 (3.6), 261 (20.9), 260 (100), 259 (8.6), 231 (8.7), 151 (5.8)<br>$MS^3$ [457→260]: 245 (34.1), 231 (100), 179 (73.4), 138 (16.7)<br>$MS^4$ [457→260→231]: 151 (100) | Unknown | Total aerial parts<br>Stems |
| 37 | 19.8 | - | 499 | $MS^2$ [499]: 353 (100), 191 (16.2)<br>$MS^3$ [499→353]: 191 (100)<br>$MS^4$ [499→353→191]: 127 (100) | Coumaroyl 1-*O*-caffeoyl quinic acid | Leaves |
| 38 | 20.4 | - | 819 | $MS^2$ [819]: 787 (41.1), 518 (24.1), 517 (100)<br>$MS^3$ [819→517]: 337 (100), 314 (48.2), 309 (28.9), 190 (55.5)<br>$MS^4$ [819→517→337]: 309 (20.1), 305 (42.2), 191 (100) | 5-*O*-*p*-Coumaroylquinic acid derivative | Leaves |
| 39 | 21.1 | - | 609 | $MS^2$ [609]: 323 (27.1), 286 (18.5), 285 (100), 179 (19.4)<br>$MS^3$ [609→285]: 213 (53.5), 151 (76.4), 107 (100) | Kaempferol-*O*-caffeoylhexoside | Flowers |
| 41 | 21.8 | - | 529 | $MS^2$ [529]:368 (13.4), 367 (100), 349 (9.0), 179 (7.0), 161 (10.1)<br>$MS^3$ [529→367]: 191 (45.8), 179 (100); 173 (23.4), 161 (86.5), 135 (86.9), 133 (15.5)<br>$MS^4$ [529→367→179]: 136 (14.4), 135 (100) | Caffeic acid derivative | Total aerial parts |
| 42 | 22.0 | - | 457 | $MS^2$ [457]: 329 (70.4), 260 (100), 231 (40.9), 165 (31.6)<br>$MS^3$ [457→260]: 231 (91.5), 180 (81.0), 179 (36.2), 97 (100) | Unknown | Leaves<br>Stems |
| 43 | 22.8 | - | 193 | $MS^2$ [193]: 178 (100)<br>$MS^3$ [193→178]: 163 (100)<br>$MS^4$ [193→178→163]: 135 (100) | Ferulic acid | Flowers |
| 45 | 24.9 | - | 483 | $MS^2$ [483]: 338 (63.3), 337 (100), 191 (44.0), 163 (13.0)<br>$MS^3$ [483→337]: 191 (100), 173 (15.3), 163 (39.6) | 1,5-di-*O*-*p*-Coumaroylquinic acid. | Flowers |
| 47 | 26.6 | - | 625 | $MS^2$ [625]: 579 (21.2), 464 (21.2), 463 (62.8), 445 (36.5), 301 (100), 179 (21.0)<br>$MS^3$ [625→301]: 255 (56.1), 179 (91.3), 151 (100) | Quercetin-*O*-dihexoside | Flowers |
| 48 | 26.9 | - | 425 | $MS^2$ [425]: 179 (100), 135 (36.0)<br>$MS^3$ [425→179]: 135 (100) | Caffeic acid derivative | Total aerial parts<br>Leaves<br>Flowers |
| 49 | 27.7 | - | 487 | $MS^2$ [487]: 457 (46.8), 290 (100), 275 (69.5), 195 (27.0)<br>$MS^3$ [487→290]: 276 (64.2), 275 (15.8), 260 (48.7), 259 (100), 97 (50.1)<br>$MS^4$ [487→290→259]: 180 (100) | Unknown | Stems |
| 50 | 28.0 | - | 711 | $MS^2$ [711]: 668 (33.8), 667 (100)<br>$MS^3$ [711→667]: 625 (29.6), 505 (100), 487 (56.7), 365 (31.7), 301 (96.6), 300 (30.5)<br>$MS^4$ [711→667→505]: 463 (23.3), 445 (38.8), 301 (100), 300 (47.7), 273 (34.1), 179 (29.1) | Quercetin-7-*O*-hexoside-3-*O*-(malonyl)hexoside | Leaves<br>Flowers |
| 51 | 28.4 | 243, 300, 328 | 593 | $MS^2$ [593]: 447 (10.7), 307 (3.1), 286 (15.9), 285 (100)<br>$MS^3$ [593→285]: 267 (52.2), 257 (40.5), 255 (22.2), 229 (17.8), 169 (34.1), 151 (100), 107 (44.6)<br>$MS^4$ [593→285→151]: 107 (100) | Kaempferol-*O*-coumaroyl hexoside | Total aerial parts<br>Flowers |
| 52 | 28.6 | - | 409 | $MS^2$ [409]: 164 (24.9), 163 (100), 119 (52.2)<br>$MS^3$ [409→163]: 119 (100) | Coumaric acid derivative | Leaves |

(*Continues*)





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**Table 1.** (Continued)

| No. | $t_R$ (min) | UV $\lambda_{max}$ (nm) | $[M-H]^-$ $m/z$ | LC-DAD/ESI-$MS^n$ $m/z$ (% base peak) | Assigned identity | Morphological part |
|---|---|---|---|---|---|---|
| 53 | 30.0 | 286, 312 | 593 | $MS^2$ [593]: 447 (19.4), 285 (100), 255 (11.8)<br>$MS^3$ [593→285]: 257 (100), 255 (19.4), 241 (17.1), 213 (21.7), 151 (35.6)<br>$MS^4$ [593→285→257]: 229 (54.2), 163 (100) | Kaempferol-*O*-coumaroyl hexoside | Flowers |
| 54 | 30.1 | - | 491 | $MS^2$ [491]: 473 (20.8), 330 (49.9), 329 (100), 315 (25.8), 314 (32.3)<br>$MS^3$ [491→329]: 315 (12.7), 314 (100), 299 (23.4)<br>$MS^4$ [491→329→314]: 300 (61.8), 299 (100), 271 (35.8) | 3′,4-Dihydroxy-5,6-dimethoxy-7-*O*-glucoside flavone | Leaves |
| 55 | 30.1 | - | 543 | $MS^2$ [543]: 513 (20.9), 483 (20.0), 440 (39.1), 439 (33.3), 438 (100), 358 (35.8), 261 (14.7)<br>$MS^3$ [543→438]: 423 (100), 405 (14.5), 357 (15.6) | Unknown | Stems |
| 57 | 31.2 | - | 835 | $MS^2$ [835]: 804 (24.5), 803 (100), 771 (54.1)<br>$MS^3$ [835→803]: 772 (10.5), 771 (35.0), 667 (27.8), 661 (100)<br>$MS^4$ [835→803→661]: 639 (60.6), 638 (100), 637 (54.5), 620 (76.0) | Unknown | Leaves |
| 58 | 31.5 | 233, 316, 334 | 375 | $MS^2$ [375]: 343 (21.4), 316 (27.4), 300 (21.9), 299 (100), 284 (66.2)<br>$MS^3$ [375→299]: 285 (18.3), 284 (100), 269 (13.0), 241 (11.8)<br>$MS^4$ [375→299→284]: 269 (100), 241 (99.0) | Dihydroxydimethoxy flavone derivative | Leaves |
| 59 | 32.3 | - | 681 | $MS^2$ [681]: 519 (91.0), 515 (67.0), 353 (100), 327 (6.8), 191 (12.0)<br>$MS^3$ [681→353]: 191 (100), 179 (3.3), 135 (1.1)<br>$MS^4$ [681→353→191]: 173 (50.3), 171 (38.4), 127 (100), 111 (17.7) | Dicaffeoylquinic acid derivative | Total aerial parts |
| 60 | 32.8 | - | 329 | $MS^2$ [329]: 311 (26.4), 275 (25.4), 201 (100), 171 (47.8), 155 (22.3)<br>$MS^3$ [329→201]: 165 (51.9), 156 (100), 155 (28.2), 151 (24.1) | Unknown | Flowers |
| 61 | 33.0 | - | 613 | $MS^2$ [613]: 459 (11.0), 447 (100)<br>$MS^3$ [613→447]: 327 (28.4), 285 (48.1), 284 (100), 255 (46.7), 151 (15.0)<br>$MS^4$ [613→447→284]: 256 (49.3), 255 (100), 227 (12.3) | Kaempferol-*O*-hexoside derivative | Flowers |
| 62 | 33.3 | - | 327 | $MS^2$ [327]: 309 (18.4), 291 (53.5), 229 (100), 211 (71.9), 209 (27.2), 171 (68.7), 165 (21.7)<br>$MS^3$ [327→229]: 211 (100), 209 (33.3), 155 (25.3), 125 (38.8)<br>$MS^4$ [327→229→211]: 183 (100) | Unknown | Total aerial parts<br>Leaves |
| 63 | 33.7 | - | 785 | $MS^2$ [785]: 666 (26.3), 665 (100), 545 (86.5), 519 (12.3)<br>$MS^3$ [785→665]: 545 (100)<br>$MS^4$ [613→447→284]: 256 (49.3), 255 (100), 227 (12.3) | Unknown | Flowers |
| 64 | 34.0 | 267, 332 | 269 | $MS^2$ [269]: 227 (100), 225 (78.1), 223 (27.3), 151 (58.5), 149 (29.2)<br>$MS^3$ [269→225]: 181 (100) | Apigenin | Flowers |
| 65 | 33.9 | - | 803 | $MS^2$ [803]: 772 (48.5), 771 (100)<br>$MS^3$ [803→771]: 753 (59.7), 744 (52.5), 743 (100), 563 (18.4), 412 (11.2)<br>$MS^4$ [803→771→743]: 725 (100), 563 (28.0), 502 (27.1), 412 (12.8) | Unknown | leaves |
| 67 | 36.9 | - | 599 | $MS^2$ [599]: 438 (14.4), 437 (100), 275 (38.1), 173 (7.8)<br>$MS^3$ [599→437]: 335 (3.3), 275 (100), 173 (23.3)<br>$MS^4$ [599→437→275]: 173 (100), 101 (13.1) | Unknown | Total aerial parts<br>Leaves |
| 68 | 39.0 | 233, 281 | 329 | $MS^2$ [329]: 314 (100), 299 (18.8)<br>$MS^3$ [329→314]: 299 (99.9), 271 (100)<br>$MS^4$ [329→314→271]: 272 (27.0), 271 (100) | 1,2,6-Trihydroxy-7,8-dimethoxy-3-methylanthraquinone | Leaves |

*Comparison with a reference standard.
Their UV spectra have not been properly observed due to low intensity.





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**Table 2.** Characterization of phenolic compounds of the methanolic extract of total aerial parts from *Helichrysum melaleucum* from Fajã da Nogueira (FN) by LC-DAD/ESI-MS$^n$

| No. | $t_R$ (min) | UV $\lambda_{max}$ (nm) | $[M-H]^-$ *m/z* | LC-DAD/ESI-MS$^n$ *m/z* (% base peak) | Assigned identity |
|---|---|---|---|---|---|
| 1 | 2.9 | 224, 278 | 683 | MS$^2$ [683]: 342 (10.7), 341 (100)<br>MS$^3$ [683→341]: 179 (100), 161 (11.7), 131 (20.7), 119 (20.9), 113 (15.7)<br>MS$^4$ [683→341→179]: 143 (40.9), 119 (43.7), 101 (100) | Caffeic acid–*O*-glucoside |
| 2 | 3.3 | 259 | 191 | MS$^2$ [191]: 173 (96.0), 171 (15.0), 127 (100), 111 (75.7)<br>MS$^3$ [191→127]: 155 (61.5), 127 (100), 111 (54.3) | Quinic acid |
| 3 | 4.4 | - | 317 | MS$^2$ [317]: 225 (100), 207 (21.9), 165 (34.9), 153 (41.6), 125 (38.9)<br>MS$^3$ [317→225]: 207 (97.9), 189 (24.2), 165 (77.2), 153 (12.2), 149 (23.8), 125 (100), 101 (11.6) | Unknown |
| 6* | 5.0 | 242, 300, 325 | 353 | MS$^2$ [353]: 191 (100), 179 (2.3)<br>MS$^3$ [353→191]: 173 (93.1), 127 (100), 109 (37.9)<br>MS$^4$ [353→191→127]: 109 (100) | 5-*O*-Caffeoylquinic acid |
| 9 | 6.5 | 243, 300, 320 | 515 | MS$^2$ [515]: 353 (100), 335 (29.1), 191 (30.4), 179 (56.7)<br>MS$^3$ [515→353]: 191 (100), 179 (60.9), 135 (14.1)<br>MS$^4$ [515→353→191]: 173 (83.7), 127 (86.1), 111 (100), 109 (40.7) | 1,3-*O*-Dicaffeoylquinic acid |
| 11 | 7.9 | 243, 300, 326 | 367 | MS$^2$ [367]: 191 (11.7), 179 (100), 161 (9.8), 135 (60.8)<br>MS$^3$ [367→179]: 135 (100) | Caffeic acid derivative |
| 13 | 8.1 | 212, 305 | 337 | MS$^2$ [337]: 191 (100)<br>MS$^3$ [337→191]: 173 (28.3), 171 (14.7), 127 (100), 109 (11.5)<br>MS$^4$ [337→191→127]: 109 (100) | 5-*O*-*p*-Coumaroylquinic acid |
| 15 | 9.8 | 256, 352 | 493 | MS$^2$ [493]: 331 (100), 330 (18.1), 316 (16.8)<br>MS$^3$ [493→331]: 316 (100), 315 (22.7)<br>MS$^4$ [493→331→316]: 287 (100), 271 (38.4), 270 (43.0), 166 (33.4) | Mearnsetin-*O*-hexoside |
| 16 | 9.8 | - | 463 | MS$^2$ [463]: 301 (100), 300 (23.0), 179 (2.8)<br>MS$^3$ [463→301]: 271 (29.0), 179 (83.9), 151 (100), 107 (8.4)<br>MS$^4$ [463→301→151]: 107 (100) | Quercetin-*O*-hexoside |
| 17 | 10.6 | 254, 271, 340 | 477 | MS$^2$ [477]: 316 (12.4), 315 (100), 300 (29.3)<br>MS$^3$ [477→315]: 301 (19.3), 300 (100), 271 (1.6)<br>MS$^4$ [477→315→300]: 283 (50.5), 272 (100), 255 (82.0), 216 (64.1), 214 (59.8) | Isorhamnetin-*O*-hexoside |
| 18¥ | 11.1 | - | 519 | MS$^2$ [519]: 503 (49.3), 491 (24.1), 357 (100), 151 (2.7)<br>MS$^3$ [519→357]: 342 (5.6), 327 (1.0), 151 (100); 136 (44.7)<br>MS$^4$ [519→357→151]: 136 (100) | Pinoresinol-4-*O*-hexoside |
| 19 | 11.4 | 300, 324 | 547 | MS$^2$ [547]: 515 (80.1), 385 (28.3), 353 (100), 335 (4.1), 191 (15.9)<br>MS$^3$ [547→353]: 191 (100), 179 (7.4), 173 (2.0)<br>MS$^4$ [547→353→191]: 173 (40.4), 127 (100), 111 (32.9), 109 (27.5) | 1,5-*O*-Dicaffeoylquinic acid derivative |
| 20 | 11.8 | 298, 324 | 547 | MS$^2$ [547]: 515 (78.6), 385 (24.6), 353 (100), 335 (14.4)<br>MS$^3$ [547→353]: 191 (100), 179 (4.3), 135 (2.2)<br>MS$^4$ [547→353→191]: 173 (100), 155 (59.1), 127 (86.1), 111 (54.0), 109 (48.3) | 1,5-*O*-Dicaffeoylquinic acid derivative |
| 21 | 12.1 | 246, 300, 323 | 515 | MS$^2$ [515]: 353 (100), 335 (6.8), 191 (8.7), 179 (25.8), 173 (36.9)<br>MS$^3$ [515→353]: 191 (53.2), 179 (61.9), 173 (100), 135 (13.6)<br>MS$^4$ [515→353→173]: 137 (15.5), 127 (33.3), 111 (100), 109 (31.0) | 3,4-*O*-Dicaffeoylquinic acid |
| 22 | 12.5 | 243, 300, 326 | 515 | MS$^2$ [515]: 353 (100), 335 (4.5), 191 (41.6),<br>MS$^3$ [515→353]: 191 (100)<br>MS$^4$ [515→353→191]: 173 (27.2), 155 (24.3), 127 (100), 109 (43.9) | 1,5-*O*-Dicaffeoylquinic acid |
| 23 | 13.0 | 243, 295, 325 | 515 | MS$^2$ [515]: 353 (100), 191 (11.9)<br>MS$^3$ [515→353]: 191 (100), 179 (25.9), 135 (8.7)<br>MS$^4$ [515→353→191]: 173 (24.1), 127 (100), 111 (73.5), 109 (15.5) | 3,5-*O*-Dicaffeoylquinic acid |

(*Continues*)





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**Table 2.** (Continued)

| No. | $t_R$ (min) | UV $\lambda_{max}$ (nm) | $[M-H]^-$ $m/z$ | LC-DAD/ESI-$MS^n$ $m/z$ (% base peak) | Assigned identity |
|---|---|---|---|---|---|
| 24 | 13.7 | 245, 300, 324 | 601 | $MS^2$ [601]: 557 (30.7), 515 (99.5), 395 (100)<br>$MS^3$ [601→395]: 353 (27.2), 335 (97.8), 233 (99.6), 203 (46.2), 173 (29.8), 179 (100)<br>$MS^4$ [601→395→233]: 173 (100), 191 (7.2) | Malonyl-1,4-*O*-dicaffeoylquinic acid |
| 25 | 14.0 | 273, 333 | 461 | $MS^2$ [461]: 446 (54.6), 299 (100), 284 (61.2), 283 (41.8)<br>$MS^3$ [461→299]: 297 (10.3), 284 (100)<br>$MS^4$ [461→299→284]: 256 (100), 240 (90.0), 228 (76.4), 227 (100), 212 (24.0), 166 (15.6), 163 (20.1) | Hispidulin-7-*O*-hexoside |
| 26 | 14.5 | 244, 300, 327 | 601 | $MS^2$ [601]: 557 (37.0), 515 (86.2), 395 (100), 233 (38.5)<br>$MS^3$ [601→395]: 335 (3.4), 233 (100), 173 (25.7)<br>$MS^4$ [601→395→233]: 173 (100) | Malonyl-3,4-*O*-dicaffeoylquinic acid |
| 27 | 15.2 | 255, 324 | 445 | $MS^2$ [455]: 285 (75.1), 281 (100), 137 (20.5)<br>$MS^3$ [455→281]: 137 (100) | Unknown |
| 30 | 16.9 | 235, 300, 325 | 601 | $MS^2$ [601]: 557 (26.7), 515 (46.7), 395 (100), 233 (36.8)<br>$MS^3$ [601→395]: 335 (6.3), 233 (100), 173 (26.4)<br>$MS^4$ [601→395→233]: 173 (100) | Malonyl-4,5-*O*-dicaffeoylquinic acid |
| 32 | 17.5 | 219, 315 | 499 | $MS^2$ [499]: 353 (18.3), 337 (100), 191 (7.2)<br>$MS^3$ [499→337]: 191 (100), 173 (37.2), 163 (50.1)<br>$MS^4$ [499→337→191]: 173 (74.8), 153 (96.8), 127 (100) | 4-*O*-Caffeoyl-5-*p*-coumaroylquinic acid |
| 33 | 18.1 | - | 457 | $MS^2$ [457]: 329 (38.8), 261 (26.5), 260 (100)<br>$MS^3$ [457→260]: 231 (100), 179 (35.5)<br>$MS^4$ [457→260→231]: 151 (100) | Unknown |
| 34 | 18.4 | - | 625 | $MS^2$ [625]: 474 (20.2), 473 (100), 293 (18.8)<br>$MS^3$ [625→473]: 341 (100), 293 (58.6), 233 (46.5), 179 (15.5)<br>$MS^4$ [625→473→341]: 239 (71.4), 197 (17.8), 179 (100), 164 (16.6) | Caffeic acid derivative |
| 35 | 18.9 | 241, 300, 324 | 529 | $MS^2$ [529]: 368 (23.2), 367 (100), 353 (22.1), 191 (21.2)<br>$MS^3$ [529→367]: 191 (100)<br>$MS^4$ [529→367→191]: 173 (87.9), 134 (58.1), 127 (100), 109 (47.7) | 1-Caffeoyl-5-ferruoylquinic acid |
| 36 | 19.8 | - | 457 | $MS^2$ [457]: 261 (17.6), 260 (100), 231 (8.6)<br>$MS^3$ [457→260]: 246 (35.0), 231 (100), 179 (47.9)<br>$MS^4$ [457→260→231]: 151 (100) | Unknown |
| 39 | 20.9 | 274, 328 | 609 | $MS^2$ [609]: 447 (15.8), 323 (44.0), 285 (100), 221 (8.6)<br>$MS^3$ [609→285]: 257 (100), 239 (23.8), 229 (25.5), 197 (21.9), 151 (65.3)<br>$MS^4$ [609→285→257]: 255 (71.8), 240 (72.2), 229 (100) | Kaempferol-*O*-caffeoylhexoside |
| 40 | 21.5 | 262, 314 | 609 | $MS^2$ [609]: 464 (16.9), 463 (100), 301 (29.7)<br>$MS^3$ [609→463]: 301 (100), 300 (17.2)<br>$MS^4$ [609→463→301]: 271 (27.5), 255 (13.5), 179 (100), 151 (97.5) | Quercetin-*O*-rhamnosylhexoside |
| 41 | 21.7 | - | 529 | $MS^2$ [529]:368 (16.4), 367 (100), 179 (10.7), 161 (9.8)<br>$MS^3$ [529→367]: 191 (26.7), 179 (100); 173 (15.8), 161 (72.9), 135 (72.5)<br>$MS^4$ [529→367→179]: 135 (100) | Caffeic acid derivative |
| 44 | 23.8 | 253, 330 | 609 | $MS^2$ [609]: 464 (19.6), 463 (100), 301 (33)<br>$MS^3$ [609→463]: 301 (100), 300 (31.0)<br>$MS^4$ [609→463→301]: 271 (37.9), 257 (7.3), 255 (24.2), 179 (100), 151 (72.6) | Quercetin-*O*-coumaroylhexoside |
| 46 | 26.0 | - | 529 | $MS^2$ [529]: 367 (100), 179 (20.8)<br>$MS^3$ [529→367]: 191 (17.6), 179 (100), 161 (61.7), 135 (69.2)<br>$MS^4$ [529→367→179]: 135 (100) | Caffeic acid derivative |
| 47 | 26.6 | 253, 330 | 625 | $MS^2$ [625]: 463 (57.1), 445 (24.2), 323 (14.3), 301 (100), 300 (5.1)<br>$MS^3$ [625→301]: 273 (5.5), 271 (4.8), 255 (9.3), 179 (68.2), 151 (100), 107 (8.2)<br>$MS^4$ [625→301→151]: 107 (100) | Quercetin-*O*-dihexoside |
| 50 | 27.8 | - | 711 | $MS^2$ [711]: 667 (100)<br>$MS^3$ [711→667]: 625 (40.2), 505 (100), 487 (56.2), 301 (88.9), 179 (10.1)<br>$MS^4$ [711→667→505]: 463 (23.3), 301 (100), 300 (59.6), | Quercetin-7-*O*-hexoside-3-*O*-(malonyl)hexoside |

(*Continues*)





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**Table 2.** (Continued)

| No. | $t_R$ (min) | UV $\lambda_{max}$ (nm) | $[M-H]^-$ *m/z* | LC-DAD/ESI-$MS^n$ *m/z* (% base peak) | Assigned identity |
|---|---|---|---|---|---|
| 51 | 28.4 | 266, 313 | 593 | $MS^2$ [593]: 447 (10.7), 307 (5.5), 285 (100)<br>$MS^3$ [593→285]: 257 (90.9), 255 (40.0), 241 (32.7), 229 (39.4), 169 (34.1), 151 (100)<br>$MS^4$ [593→285→151]: 107 (100) | Kaempferol 7-*O*-coumaroylhexoside |
| 53 | 29.7 | 266, 313 | 593 | $MS^2$ [593]: 447 (11.2), 307 (2.9), 257 (3.8), 285 (100)<br>$MS^3$ [593→285]: 255 (65.6), 241 (25.8), 229 (39.4), 151 (100), 107 (27.3)<br>$MS^4$ [593→285→151]: 107 (100) | Kaempferol 4′-*O*-coumaroylhexoside |
| 56[¥] | 30.6 | 268, 322 | 475 | $MS^2$ [475.4]: 460 (4.7), 355 (3.7), 314 (15.5), 313 (100), 298 (18.4)<br>$MS^3$ [475.4→313]: 298 (100)<br>$MS^4$ [475.4→313→298]: 283 (100), 255 (77.4) | 5-Hydroxyl-6,8-dimethoxy-7-hexoside flavone |
| 62 | 33.2 | - | 327 | $MS^2$ [327]: 291 (43.8), 229 (100), 221 (17.3), 211 (74.4), 193 (21.3), 171 (35.1)<br>$MS^3$ [327→229]: 211 (100), 209 (48.6), 183 (20.3), 165 (19.9), 127 (14.1), 125 (19.7)<br>$MS^4$ [327→229→210]: 164 (100) | Unknown |
| 64 | 33.8 | 275, 334 | 269 | $MS^2$ [269]: 227 (62.1), 225 (100), 201 (49.2), 151 (56.7), 149 (91.7)<br>$MS^3$ [269→226]: 183 (40.7), 181 (100), 117 (39.5) | Apigenin |
| 66[¥] | 34.9 | - | 547 (100)<br>343 (84.3) | $MS^2$ [547]: 344 (15.5), 343 (100)<br>$MS^2$ [343]: 329 (18.8), 328 (100), 313 (14.9)<br>$MS^3$ [547→343]: 329 (15.9), 328 (100), 313 (14.4)<br>$MS^3$ [343→328]: 314 (12.8), 313 (100), 285 (6.8)<br>$MS^4$ [547→343→328]: 314 (12.0), 313 (100), 285 (4.9)<br>$MS^4$ [343→328→313]: 298 (40.8), 285 (100), 270 (80.1) | 5-Hydroxy-7,8,6′-trimehoxy-2′-hexoside(acetyl) flavone |

*Comparison with a reference standard.
Their UV spectra have not been properly observed due to low intensity.
[¥]Geographical markers for *Helichrysum melaleucum* species form Fajã da Nogueira.

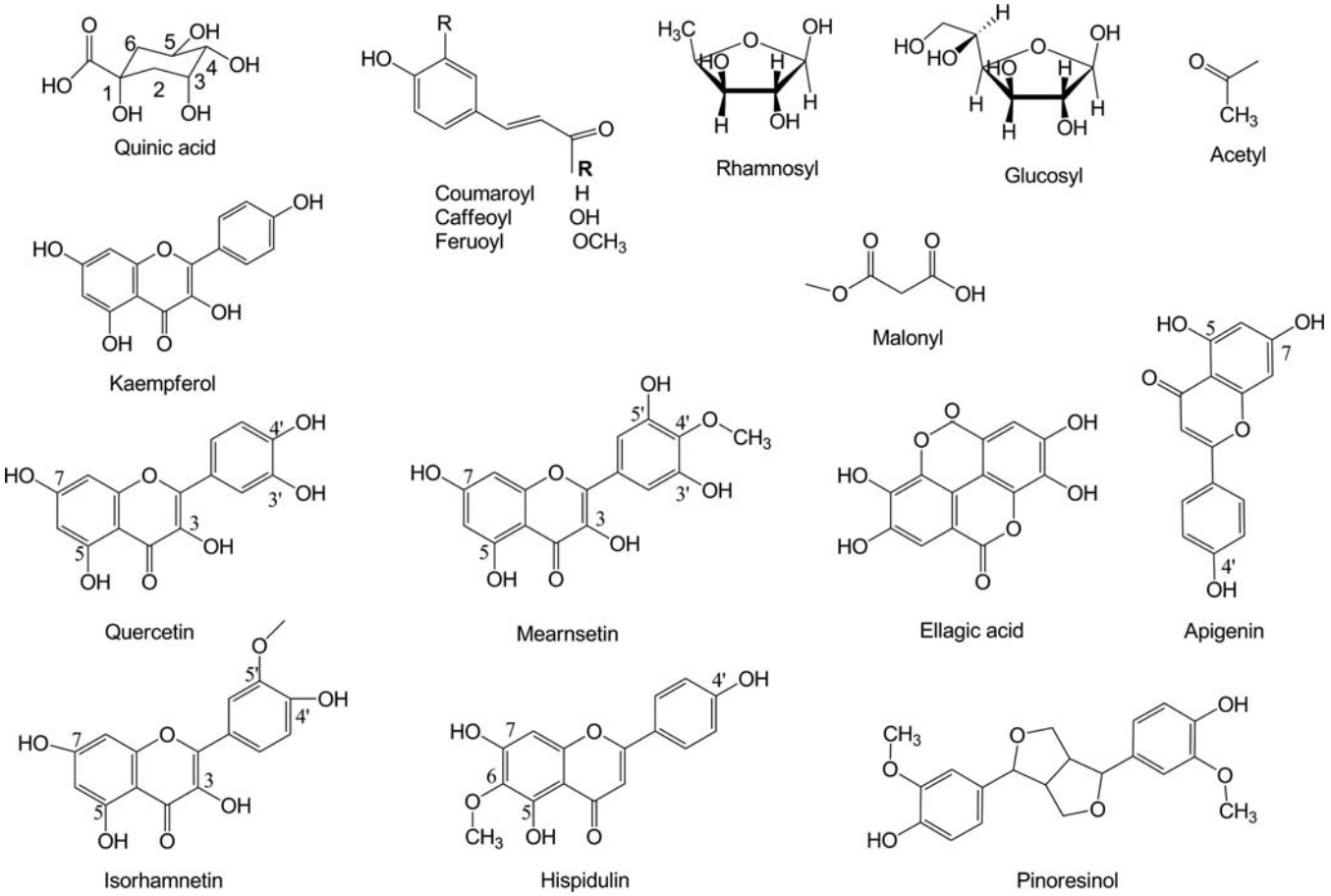


**Figure 3.** Chemical structures of phenolic compounds detected in *Helichrysum melaleucum* species.



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found only in the total aerial parts, leaves and flowers methanolic extracts of *H. melaleucum* from SV. Quercetin-*O*-hexoside was detected in the flower extracts, while mearnsetin-*O*-hexoside was identified in the total aerial parts and leaves extracts.

Compound **17** ($t_R = 10.6$ min) was tentatively identified as isorhamnetin-*O*-hexoside. This compound gave a deprotonated molecular ion $[M–H]^-$ at *m/z* 477 and its $MS^2$ spectrum showed an aglycone ion ($Y_0^-$) at *m/z* 315 due to the loss of 162 Da, suggesting the presence of a hexoside residue. $MS^n$ fragmentation of the ion at *m/z* 315 was very similar to that of isorhamnetin reported in our previous studies on *Helichrysum devium*, where this compound was detected only in the flowers extract.[4,11,12]

For *Helichrysum melaleucum*, the compound was not detected in the stems extract, but was present in all the other extracts analyzed.

Compound **25** ($t_R = 14.0$ min) showed a $[M–H]^-$ ion at *m/z* 461 and its $MS^2$ spectrum exhibited a fragment ion at *m/z* 299 as base peak, suggesting the presence of a hexoside moiety (loss of 162 Da). A weak ion at *m/z* 446 was also detected (ca. 50% of base peak) which corresponds to the loss of a methyl group (15 Da) from the $[M–H]^-$ ion. This ion at *m/z* 299 corresponds to the aglycone ion ($Y_0^-$) which under $MS^3$ fragmentation easily lost a methyl group (15 Da), producing a fragment ion at *m/z* 284 (Fig. 4).

The $MS^n$ fragmentation of the ion at *m/z* 284 yielded several fragments at *m/z* 228 ($[Y_0^- – H – CH_3 – 2CO]^-$); 240 ($[Y_0^- – H – CH_3 – CO_2]^-$); 256 ($[Y_0^- – H – CH_3 – CO]^-$); 212 ($[Y_0^- – H – CH_3 – CO_2 – CO]^-$); and 167 ($^{1,3}A^-$), originating from a RDA reaction (Scheme 1). According to these data, the aglycone was identified as being hispidulin, a 6-methoxyflavone.[13]

For flavones like hispidulin, the 7-OH position is the most regular and common glycosylation site.[6] Therefore, compound **25** was identified as hispidulin-7-*O*-hexoside. This compound was detected in SV and FN total aerial parts and also in the SV leaves extract.

Compound **29** ($t_R = 16.6$ min) exhibited a $[M–H]^-$ ion at *m/z* 489 which, under fragmentation, eliminated a neutral fragment of 204 Da forming the aglycone ion ($Y_0^-$) at *m/z* 285. $MS^n$ fragmentation of this ion gave the characteristic fragments of kaempferol (*m/z* 257, 255 and 229). The loss of 204 Da can be associated with an acetylhexoside moiety. The linkage position of this moiety is difficult to establish only based on $MS^n$ data, but it is known that flavonols glycosylated at the 3-OH position present a radical aglycone ion ($[Y_0^– H]^-$) with a high relative abundance.[10] Nevertheless, this radical fragment was not detected in order to confirm the 3-OH position and compound **29** was classified as kaempferol-*O*-acetylhexoside; it was only detected in SV flowers extract.

Compound **39** ($t_R = 20.9$ min) yielded a $[M–H]^-$ ion at *m/z* 609. The $MS^2$ spectrum of this ion showed a fragment ion at *m/z* 285, as base peak, due to the loss of 324 Da, and also a fragment ion at *m/z* 447 (loss of 162 Da). According to this, it is possible to infer that there is a combined loss of two residues of 162 Da.

The fragment ion at *m/z* 285 corresponds to the aglycone ion ($Y_0^-$) and its $MS^3$ spectrum showed ions at *m/z* 229 ($[Y_0^– 2CO]$), 151 ($^{1,3}A^-$) and, as base peak, a fragment ion at *m/z* 257 ($[Y_0^– CO]^-$). These RDA fragments are consistent with those found for a standard solution of kaempferol, as mentioned before.

Since the $MS^2$ spectrum base peak ion (*m/z* 285) corresponds to the aglycone ion, the two substituent groups must be attached to the same kaempferol hydroxyl group. Further evidence for this type of substitution is that the fragments $[Y_0^3 – H]^-$ and $[Y_0 – 2H]^-$, characteristic ions for di-*O*-glycosides, were not detected.[9]

The two substituent groups of kaempferol can be either a moiety composed of two hexosides residues or one hexoside residue esterified with a caffeoyl group. The last hypothesis was confirmed by the presence of a fragment ion at *m/z* 323

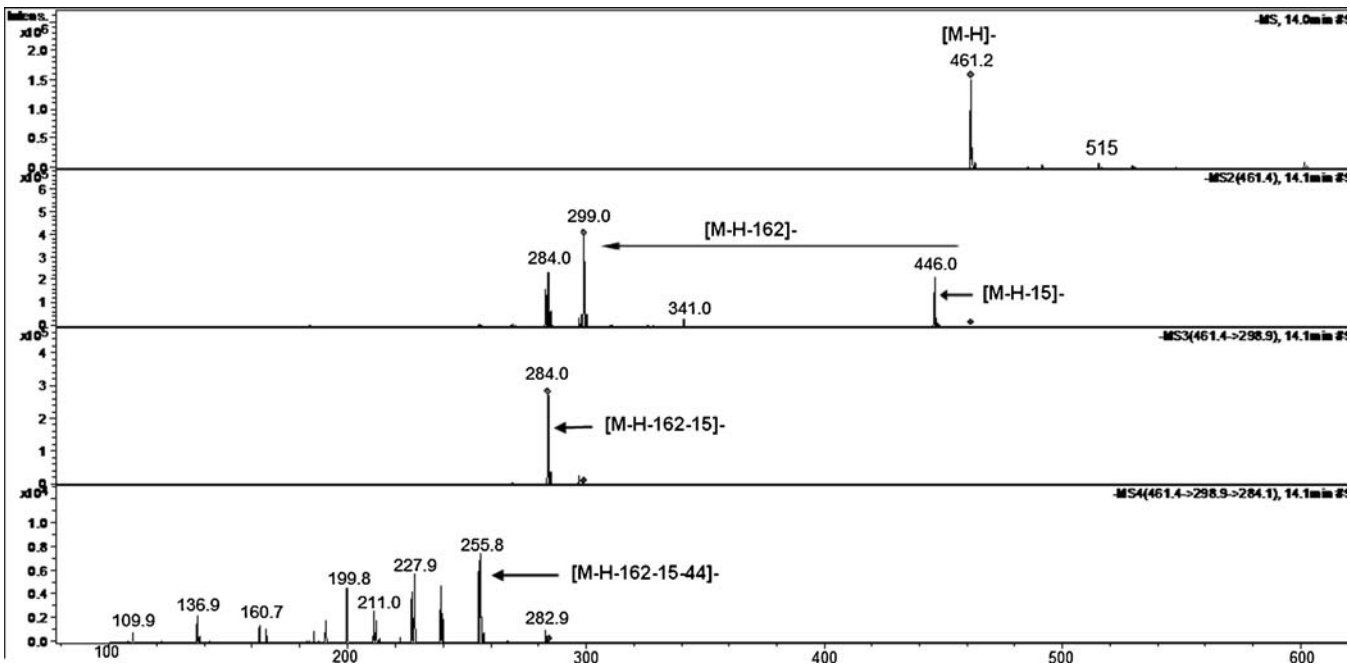


**Figure 4.** ESI-$MS^n$ negative mode analysis of compound **25**. Sequential fragmentation, $MS^n$ (n = 2–4) of the ion at *m/z* 461.





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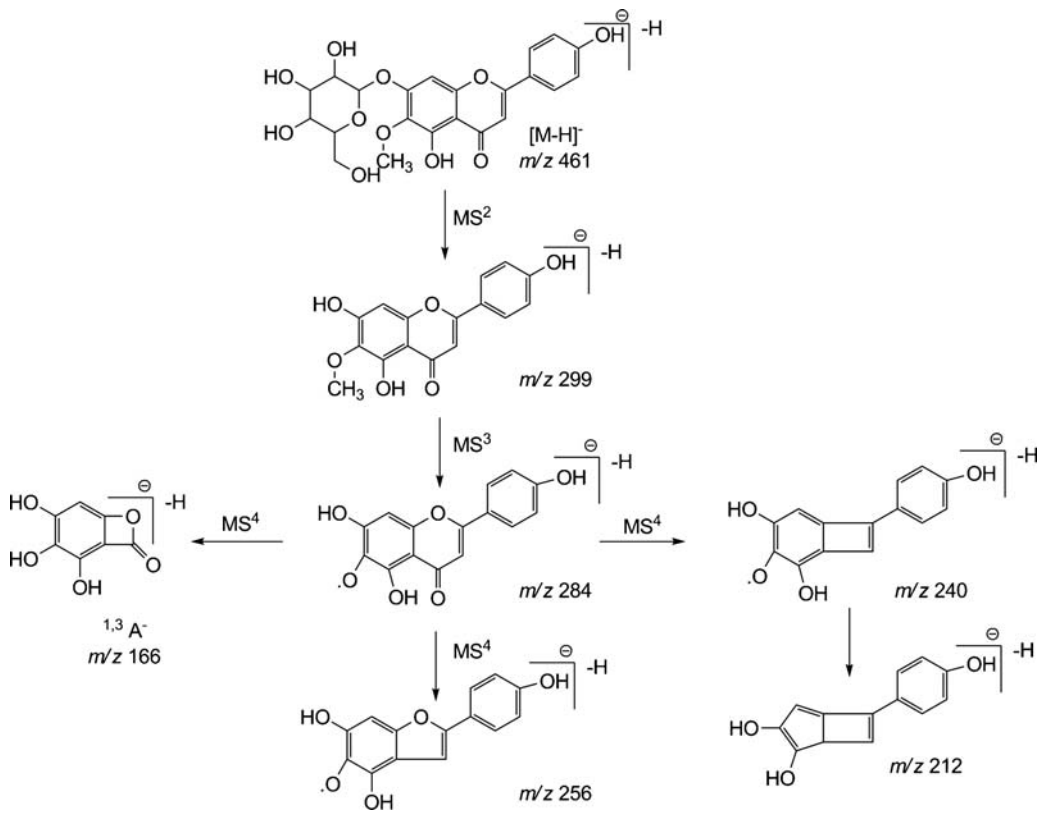


**Scheme 1.** Proposed fragmentation pathway for compound **25**.

(ca. 44% of base peak) assigned as [caffeoylhexose–H]$^-$ and a [caffeic acid–H]$^-$ ion at *m/z* 179;[12] the long retention time is also an indication of the presence of an acyl group, rather than a dihexoside group.

Since the aglycone radical ion was not detected, it is possible to infer that the aglycone is not substituted at the 3-OH position. Thus, compound **39** was classified as kaempferol-*O*-caffeoylhexoside. It was detected only in the FN total aerial parts extract and in the SV flowers extract.

Another two compounds, **40** ($t_R = 21.5$ min) and **44** ($t_R = 23.8$ min), with a [M–H]$^-$ ion at *m/z* 609, were identified in the FN total aerial parts extract, although they have a different MS$^n$ fragmentation pathway of that found for compound **39**, which gave also a deprotonated molecular ion [M–H]$^-$ at *m/z* 609.

The main fragment ions observed in the MS$^n$ fragmentation experiments appeared at *m/z* 463 ([M–H–146]$^-$); 301 ($Y_0^-$); 300 ([$Y_0^-$–H]$^-$); 151 ($^{1,2}A^-$-CO); 179 ([$^{1,2}A^-$–H]); and 271 ([M–H–$CH_2O$]$^-$). These two compounds were detected in our previously work[4] and were identified as quercetin-*O*-rhamnosylhexoside (compound **40**) and quercetin-*O*-coumaroylhexoside (compound **44**).

Compound **47** ($t_R = 26.6$ min) was detected in the FN total aerial parts and in the SV flowers extract. This compound exhibited a [M–H]$^-$ ion at *m/z* 625 and, in the MS$^2$ spectrum, the loss of 324 Da was observed, corresponding to two hexoside moieties linked in the same position of the aglycone. Further MS$^n$ fragmentation of the ion at *m/z* 301 led to the identification of the aglycone as quercetin. Thus, compound **47** was identified as quercetin-*O*-dihexoside.

Compound **50** ($t_R = 27.8$ min) showed a [M–H]$^-$ ion at *m/z* 711 and was identified as being quercetin-7-*O*-hexoside-3-*O*-(malonyl)hexoside by comparison of its MS$^n$ fragmentation behaviour with that found in literature data.[4] It was present in trace amounts in all extracts with the exception of SV total aerial parts and stems extracts.

Compounds **51** ($t_R = 28.4$ min) and **53** ($t_R = 29.7$ min) have the same [M–H]$^-$ ion at *m/z* 593. The MS$^2$ spectra of both compounds were identical and gave a [M–H–146–162]$^-$ ion at *m/z* 285 (base peak) and a [M–H–146]$^-$ ion at *m/z* 447 (ca. 10% of base peak). The neutral loss of 146 Da is common for coumaroyl moieties[14] which was confirmed with a [coumaroylhexoside–H]$^-$ ion at *m/z* 307.

The aglycone was identified as kaempferol based on the principal RDA reaction fragment ions. Full characterization of these compounds was achieved by comparison of the MS$^n$ fragmentation behaviour with that described in our previous work with similar compounds.[4] Thus, compounds **51** and **53** were identified as kaempferol 7-*O*-coumaroylhexoside and kaempferol 4′-*O*-coumaroylhexoside, respectively. However, the occurrence of these two compounds is not the same in all extracts. For example, compound **51** was identified in all extracts with the exception of the SV total aerial parts and stems extracts. Compound **53** was detected in the FN and SV total aerial parts and SV flowers extracts.

Compound **54** ($t_R = 30.1$ min) exhibited a [M–H]$^-$ ion at *m/z* 491 and was only detected in the SV leaves extract. Its MS$^2$ fragmentation produced a fragment ion at *m/z* 329, probably due to the loss of a hexoside residue (162 Da). The sequential MS$^n$ fragmentation allowed the identification of two losses of 15 Da each, due to the presence of two methoxyl groups. This fragmentation behaviour is consistent with that described before for 3′,4′-dihydroxy-5,6-dimethoxy-7-*O*-hexoside flavone.[15]





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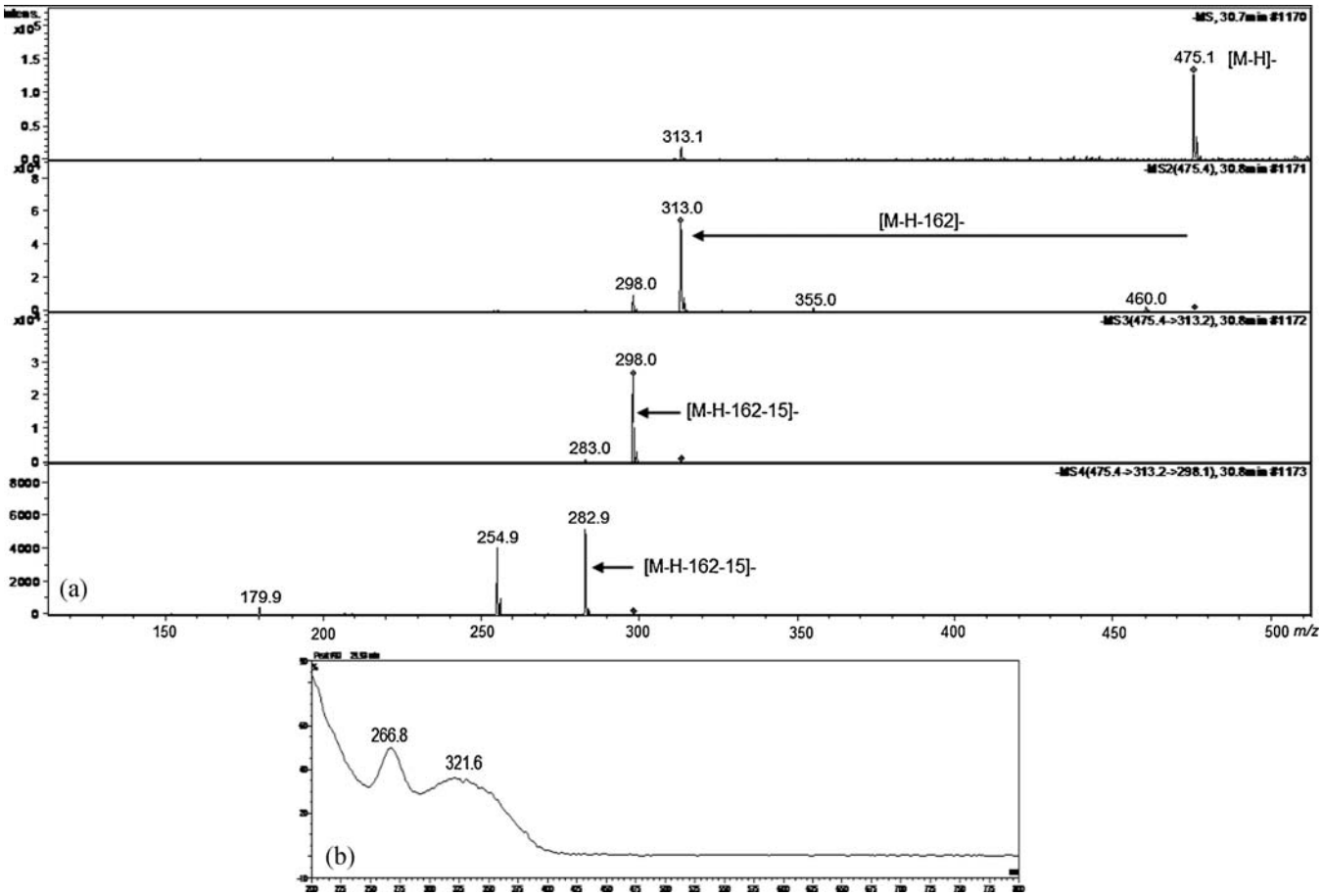


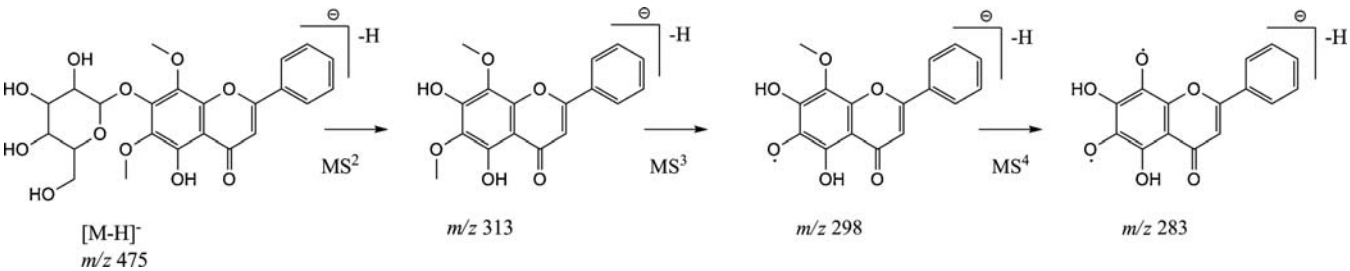

**Figure 5.** (a) ESI-$MS^n$ negative mode analysis of compound **56**. Sequential fragmentation, $MS^n$ (n = 2–4) of the ion at *m/z* 475. (b) UV spectrum of compound **56**.

Compound **56** ($t_R = 30.6$ min) was only found in the FN total aerial parts extract and showed a $[M–H]^-$ ion at *m/z* 475 (Fig. 5(a)). The $MS^2$ fragmentation of this ion produced a base peak at *m/z* 313, attributed to the loss of 162 Da, suggesting the presence of a hexoside residue. $MS^n$ fragmentation of the ion at *m/z* 313 gave ions at *m/z* 298 and 283, due to two consecutive losses of 15 Da, probably due to two methyl groups. Based on the $MS^n$ data it was possible to identify a flavone skeleton.

This compound exhibited a low-intensity but clear band I absorption at 322 nm (Fig. 5(b)) which is characteristic of flavones (band I at 315–322 nm) with 6-oxygenation but without B-ring oxygenation.[7] Furthermore, the band II at 267 nm, with a bathochromic shift, is also characteristic of a flavones with a 7-hydroxylated and 6,8-methoxylated A-ring.[7] Scheme 2 presents the proposed fragmentation pathway for compound **56** that was tentatively characterized as 5-hydroxy-6,8-dimethoxy-flavone-7-*O*-hexoside.

Compound **58** ($t_R = 31.5$ min) displayed a $[M–H]^-$ ion at *m/z* 375 and was only detected in SV leaves extract. The base peak in the $MS^2$ spectrum is a fragment ion at *m/z* 299 formed by the loss of 76 Da. Under $MS^n$ fragmentation, this $MS^2$ ion at *m/z* 299 easily lost two fragments of 15 Da resulting in fragment ions at *m/z* 284 and 269. This behaviour is characteristic of flavones with two methoxyl and two hydroxyl groups located on the aglycone. Despite the fact that the UV spectrum of this compound showed the same bands as compound **56**, based only on this information, complete identification of compound **58** was not achieved and it was tentatively characterized as a dihydroxydimethoxy flavone derivative.

Compound **61** ($t_R = 33.0$ min) gave a $[M–H]^-$ ion at *m/z* 613. In the $MS^2$ spectrum, it is possible to observe a loss of 166 Da,




**Scheme 2.** Proposed fragmentation pathway for compound **56**.



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forming the ion at *m/z* 447. The nature of this fragment could not be determined. The $MS^3$ spectrum of the ion at *m/z* 447 gave a fragment ion at *m/z* 285, as base peak, suggesting the presence of a hexoside residue (162 Da). The ion at *m/z* 285 corresponds to the aglycone ion ($Y_0^-$) and its fragmentation gave RDA characteristic fragments of kaempferol at *m/z* 257 and 255. So, compound **61** was identified as a kaempferol-*O*-hexoside derivative, probably substituted with an acyl group which will explain the long retention time.

Compound **64** ($t_R = 34.0$ min) displayed a $[M–H]^-$ ion at *m/z* 269 and, by $MS^n$ fragmentation, fragment ions were detected at *m/z* 225($[M–H–CO_2]^-$); 201 ($[M–H–C_3O_2]^-$); 151 ($^{1,3}A^-$); and 149 ($^{1,4}B + 2H$). This fragmentation behaviour matches that of a standard solution of apigenin and agrees with the literature data for this compound.[4] This compound was only found in the FN total aerial parts and in SV flowers extract. Normally, the presence of free aglycones indicates the presence of their glycosylated forms, but no glycosylated apigenin was detected.

The peak that occurs at 34.9 min showed two intense ions at *m/z* 547 (base peak) and 343 (84.3% of base peak). $MS^n$ fragmentation of the ion at *m/z* 547 gave the aglycone ion ($Y_0^-$) at *m/z* 343 (loss of 204 Da), probably combined loss of a hexoside (162 Da) and an acetyl group (42 Da).

Further fragmentation of the aglycone ion at *m/z* 343 showed the elimination of three methyl groups (15 Da) originating ions at *m/z* 328, 313 and 298. Comparing this results with those obtained by Han *et al.*,[15] the aglycone was identified as 5,2′-dihydroxy-7,8,6′-trimethoxyflavone (skullcap flavon I).

The 204 Da residue can be located in two -OH positions: 5-OH and 2′-OH. It is well known that 5-*O*-glycosides are rare for compounds with a carbonyl group at position 4, since the 5-OH group participates in hydrogen bonding with the adjacent 4-C=O group. So, compound **66** was identified as being 5-hydroxy-7,8,6′-trimethoxy-2′-hexoside (acetyl) flavone.

## Hydroxycinnamic derivatives

It was possible to detect a total of 20 hydroxycinnamic acid derivatives in the five analyzed samples from *Helichrysum melaleucum*. For all compounds, the deprotonated molecular ion, $[M–H]^-$, was formed with sufficient intensity to undergo $MS^n$ fragmentation. The loss of the substituent groups is always referred in respect of this ion.

The linkage position of acyl groups on the quinic acid structure can be established based on the main fragment ions from $MS^n$ fragmentation of $[M–H]^-$ ions. Acyl groups linked to the 4-OH position gave a [caffeic acid–H]$^-$ ion at *m/z* 173 as base peak. When the acyl group is connected to the 3-OH or 5-OH position, the [quinic acid–H]$^-$ ion at *m/z* 191 appears as the base peak and the [caffeic acid–H]$^-$ ion at *m/z* 179 is more significant for 3-OH compounds.[16]

The quinic acid derivatives found were identified based on these assumptions and on the hierarchical key for the identification by LC/$MS^n$ of quinic acid derivatives proposed by Clifford *et al.*[16]

Compound **2** ($t_R = 3.3$ min) gave a fragment ion $[M–H]^-$ at *m/z* 191 and was identified as quinic acid. This identification was based on the main $MS^n$ fragment ions detected at *m/z* 127 ($[M–CO–2H_2O]^-$) and *m/z* 173 ($[M–CO–2H_2O]^-$) which correspond to literature reports for quinic acid.[17] It was possible to detect the presence of quinic acid in all extracts with the exception of the SV leaves extract.

### *Monocaffeoylquinic acid (monoCQA)*

5-*O*-Caffeoylquinic acid (compound **6**, $t_R = 5.0$ min) was identified by comparison of the HPLC retention time, UV and mass spectra with those of a reference standard. It displayed a $[M–H]^-$ ion at *m/z* 353 and its $MS^2$ spectrum gave a [quinic acid–H]$^-$ ion at *m/z* 191 as base peak and a weak [caffeic acid–H]$^-$ ion at *m/z* 179. The occurrence of this compound in the *Helichrysum* genus is very common and it was also reported for *Helichrysum devium*.[4]

### *Dicaffeoylquinic acid (diCQA)*

Several dicaffeoylquinic acids (diCQA) isomers were detected in *Helichrysum melaleucum*.

Compounds **9** ($t_R = 6.5$ min)), **21** ($t_R = 12.1$ min)), **22** ($t_R = 12.5$ min) and **23** ($t_R = 13.0$ min) all showed $[M–H]^-$ ions at *m/z* 515 and $MS^2$ fragmentation of these ions gave a $[M–H–162]^-$ ion at *m/z* 353, indicating the presence of more than one caffeoyl group attached to different quinic acid OH groups. However, $MS^3$ and $MS^4$ fragment ions produced from the $MS^2$ fragmentation were different for the four compounds. Based on the obtained results (Tables 1 and 2) these compounds were assigned as 1,3-*O*-dicaffeoylquinic acid (**9**), 3,4-*O*-dicaffeoylquinic acid (**21**), 1,5-*O*-dicaffeoylquinic acid (**22**) and 3,5-*O*-dicaffeoylquinic acid(**23**). The full explanation concerning the characterization of these isomers is given in our previous work.[4] These four compounds were found in the SV and FN total aerial parts extract and compound **9** was detected in all extracts. The occurrence of the other compounds in the other extracts is variable.

Compounds **19** ($t_R = 11.4$ min) and **20** ($t_R = 11.8$ min) yielded a $[M–H]^-$ ion at *m/z* 547. Their $MS^2$ spectra showed a fragment ion at *m/z* 353, as base peak, and an intense fragment ion at *m/z* 515 (ca. 80% of base peak). $MS^n$ fragmentation of the ion at *m/z* 353 gave common fragments to those obtained for caffeoylquinic acid fragmentation. For example, the $MS^3$ spectrum displayed fragment ions at *m/z* 191 (base peak) and 179 (<10% of base peak), which indicates a quinic acid substituted at position 1-OH or 5-OH.[16] This conclusion was achieved taking into account the presence of weak fragment ions characteristic of that compound, namely the $MS^2$ ion at *m/z* 335 (ca. 4% of base peak) and the $MS^3$ ion (ca. 2% of base peak). Nevertheless, based only on $MS^n$ data, it was not possible to completely identify the structures of these two compounds. Thus, compounds **19** and **20** were tentatively characterized as a 1,5-*O*-dicaffeoylquinic acid derivatives. Compound **19** was found in SV and FN total aerial parts and compound **20** was only detected in the FN total aerial parts extract.

Compound **14** ($t_R = 8.6$ min) was only detected in the SV leaves methanolic extract and it displayed a $[M–H]^-$ ion at *m/z* 677. In the $MS^2$ spectrum, a loss of 162 Da, probably a hexoside residue, was observed forming a base peak at *m/z* 515, which is characteristic for dicaffeoylquinic acid derivatives. However, the further $MS^n$ fragmentation led to a fragmentation behaviour very different from





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those isomers. For example, in the $MS^3$ spectrum the most intense peak was a fragment ion at *m/z* 323 (loss of 192 Da) and in the $MS^4$ spectrum the base peak corresponds to a fragment ion at *m/z* 161 (loss of 162 Da). However, despite the fact that common fragments of dicaffeoylquinic acids were detected, compound **14** was not completely characterized being assigned as a dicaffeoylquinic acid hexoside.

Compound **59** ($t_R = 32.3$ min) gave a $[M–H]^-$ ion at *m/z* 681. Fragmentation of this ion gave fragment ions at *m/z* 353 (base peak); 515 (67.0% of base peak); and 191 (12.0% of base peak), which led to the identification of a dicaffeoylquinic acid derivative. However, based only on these $MS^n$ data it was not possible to fully characterize compound **59** that was only detected in the SV total aerial parts extract.

### *Coumaroylcaffeoylquinic acid*

Compound **13** occurred at a retention time of 8.1 min and exhibited a deprotonated molecular ion $[M–H]^-$ at *m/z* 337. $MS^n$ fragmentation showed characteristic fragments of the *p*-coumaric acid at *m/z* 163 and 110 and a fragment ion at *m/z* 191 as base peak. This compound was identified as 5-*O*-*p*-coumaroylquinic acid according to the $MS^n$ fragmentation behaviour and by referring to the hierarchical key for the identification by LC/$MS^n$ of quinic acid derivatives proposed by Clifford *et al*.[18] It was detected in all extracts with exception of the SV flowers methanolic extract.

Compounds **31** ($t_R = 17.1$ min), **32** ($t_R = 17.5$ min) and **37** ($t_R = 19.8$ min) exhibited a $[M–H]^-$ ion at *m/z* 499. However, their $MS^n$ fragmentation patterns are quite different. $MS^2$ spectra of compounds **31** ($t_R = 17.1$ min) and **37** ($t_R = 19.8$ min) gave a fragment ion at *m/z* 353 (loss of 146 Da) indicating the presence of a coumaroyl group. The $MS^3$ spectra of the ion at *m/z* 353 displayed only one fragment ion at *m/z* 191, suggesting the presence either of a 1-OH or 5-OH caffeoylquinic acid (CQA) derivative.[16] 5-CQA is more hydrophobic than 1-CQA, so 5-CQA derivatives should appear at a lower retention time than 1-CQA derivatives. Based only on $MS^n$ data the linkage position of the coumaroyl group could not be determined. Therefore, compounds **31** and **37** were characterized as coumaroyl 5-*O*-caffeoyl quinic acid and coumaroyl 1-*O*-caffeoyl quinic acid, respectively.

For compound **32** ($t_R = 17.5$ min), the ion at *m/z* 499 easily lost a caffeoyl moiety (162 Da) to form in the $MS^2$ spectrum a base peak ion at *m/z* 337. The $MS^3$ spectrum was similar to that described above for 5-*O*-*p*-coumaroylquinic acid (compound **13**). The caffeoyl group must therefore be linked to the 4-OH position of quinic acid since an intense fragment ion at *m/z* 173 was detected in the $MS^3$ spectrum. It is known that the residues connected to the 5-OH position are more easily lost than those at the 4-OH position; however, that situation was not observed for this compound. Therefore, compound **32** was identified as 4-*O*-caffeoyl-5-*O*-*p*-coumaroylquinic acid.

Compound **45** ($t_R = 24.9$ min) exhibited a $[M–H]^-$ ion at *m/z* 483. The $MS^2$ spectrum gave a fragment ion at *m/z* 337, which corresponds to the loss of 146 Da. A second loss of 146 Da was observed in the $MS^3$ spectrum forming the fragment ion at *m/z* 191. Comparing these results with literature data[18,19] it is possible to infer that compound **45** is a di-*p*-coumaroylquinic acid.

According to Clifford *et al*.,[18] if the fragmentation of the ion at *m/z* 337 leads to a fragment ion at *m/z* 191, the linkage position of the *p*-coumaroyl group should be assigned to the 5-OH group. This type of fragmentation was observed for compound **13**. The other *p*-coumaroyl group should be connected to the 1-OH position, which is more easily expelled forming the 5-*O*-*p*-coumaroylquinic acid residue. Therefore, compound **45** was identified as 1,5-di-*O*-*p*-coumaroylquinic acid.

Compound **38** ($t_R = 20.4$ min) showed a $[M–H]^-$ ion at *m/z* 819 and was only detected in the SV leaves extract. The $MS^2$ spectrum showed a fragment ion at *m/z* 517 (loss of 302 Da) and the base peak in the $MS^3$ spectrum corresponds to a fragment ion at *m/z* 337 (loss of 150 Da). Fragmentation of this ion at *m/z* 337 gave characteristic ions of 5-*p*-coumaroylquinic acid. The available $MS^n$ data were not sufficient to identify the other residues. So, compound **38** was assigned as a 5-*O*-*p*-coumaroylquinic acid derivative.

### *Malonylcaffeoylquinic acid*

Three malonylcaffeoylquinic acid isomers, compounds **24** ($t_R = 13.7$ min), **26** ($t_R = 14.5$ min) and **30** ($t_R = 16.9$ min), were identified in *Helichrysum melaleucum*. They gave a $[M–H]^-$ ion at *m/z* 601 and their $MS^2$ spectra gave fragment ions at *m/z* 557 and 515, suggesting the presence of a malonyl moiety in their structures (loss of 44 and 86 Da). A fragment ion at *m/z* 395 was the base peak in the $MS^2$ spectra and corresponds to the loss of 206 Da from the deprotonated molecular ion. In our recent studies, fragmentation of these compounds was analyzed and their identification was achieved.[4] So, compounds **24**, **26** and **30** were characterized as malonyl-1,4-*O*-dicaffeoylquinic acid, malonyl-3,4-*O*-dicaffeoylquinic acid and malonyl-4,5-*O*-dicaffeoylquinic acid, respectively. These three compounds were detected in all extracts, with the exception of compound **24** which was found only in the FN extract.

### *Caffeoylferuloylquinic acid*

Compound **35** ($t_R = 18.9$ min) was detected in the SV and FN total aerial parts and in the SV leaves extracts. This compound showed a $[M–H]^-$ ion at *m/z* 529 which under $MS^n$ fragmentation produced fragment ions at *m/z* 367 [feruloylquinic acid–H]$^-$ and 191 [quinic acid–H]$^-$ and was thus characterized as a caffeoylferuoylquinic acid (CFQA) isomer. Using the hierarchical key developed by Clifford *et al*.,[18] compound **35** was identified 1-*O*-caffeoyl-5-*O*-feruoylquinic acid. This compound was present in all extracts with the exception of the SV flowers and stems methanolic extracts.

### *Caffeic acid derivatives*

Compounds **1** ($t_R = 2.8$ min) and **5** ($t_R = 4.6$ min) were characterized as caffeic acid-*O*-hexoside.

Compound **5** yielded a $[M–H]^-$ ion at *m/z* 341 and its $MS^2$ spectrum showed a base peak at *m/z* 179, resulting from the loss of 162 Da, which indicates the presence of a hexoside residue. The ion at *m/z* 179 is formed probably due to the presence of a caffeic acid residue. With no further information and comparing with literature data,[14] where the same fragmentation pattern was observed, compound **5** was assigned as a caffeic acid *O*-hexoside.





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Compound **1** originated a $[M–H]^-$ ion at *m/z* 683 as base peak, and an intense fragment ion at *m/z* 341. By means of $MS^2$ fragmentation, it was possible to deduce that the ion at *m/z* 683 is a dimer of the ion at *m/z* 341. $MS^n$ fragmentation of the $MS^2$ ion, at *m/z* 341, led to the identification of a similar pattern to compound **5**.

Another caffeic acid hexoside derivative (compound **12**) was found at a retention time of 8.0 min in the SV leaves extract. This compound showed a $[M–H]^-$ ion at *m/z* 533. The base peak in the $MS^2$ spectrum is a fragment ion at *m/z* 371, due to the loss of 162 Da (hexoside moiety). The sequential $MS^n$ fragmentation and the detection of fragment ions at *m/z* 353 and 179 led to the identification of a caffeic acid residue.

Five more caffeic acid derivatives were found in *Helichrysum melaleucum* extracts. They gave very different $MS^n$ patterns but all had in common the fragment ion at *m/z* 179 [caffeic acid–H]$^-$.

Compound **7** ($t_R = 5.5$ min) exhibited a $[M–H]^-$ ion at *m/z* 481. The $MS^2$ spectrum gave a fragment ion at *m/z* 445 due to the loss of 36 Da. In the $MS^3$ spectrum, the base peak is a fragment ion at *m/z* 221, but it showed also an intense fragment ion at *m/z* 179 (83.2% of base peak). In the $MS^4$ experiment only this ion was fragmented forming a fragment ion at *m/z* 101. Based on $MS^n$ data compound **7** was tentatively characterized as a caffeic acid derivative.

Compound **11** ($t_R = 7.9$ min) showed a $[M–H]^-$ ion at *m/z* 367 and its fragmentation produced the fragment ion at *m/z* 179 as base peak. The $MS^3$ spectrum displayed a fragment ion at *m/z* 135 which corresponds to a loss of 44 Da (probably decarboxylation).[14]

Compound **34** ($t_R = 18.4$ min) exhibited a $[M–H]^-$ ion at *m/z* 625. The $MS^n$ experiments gave fragment ions at *m/z* 473, 341 and 179. This behaviour is similar to that described previously[4] for a caffeic acid derivative.

Compound **41** ($t_R = 21.7$ min) presented a $[M–H]^-$ ion at *m/z* 529 and easily lost a 162 Da moiety (probably a hexoside) to form a base peak ion at *m/z* 367 in the $MS^2$ spectrum. The presence of this ion indicates a feruoylquinic residue, but with the $MS^n$ fragmentation the presence of a ferulic acid could not be confirmed. However, the base peak at *m/z* 179 in the $MS^3$ spectrum corresponds to a [caffeoyl–H]$^-$ ion indicating that compound **41** is also a caffeic acid derivative.

Compound **48** ($t_R = 26.9$ min) exhibited a $[M–H]^-$ ion at *m/z* 425 and the occurrence of a fragment ion at *m/z* 179, as base peak in the $MS^3$ spectrum, led to the identification of a caffeic acid derivative. Due to the long retention time of compound **48**, it is possibly conjugated with another hydrophobic group.

## Other compounds

Three other compounds that do not belong to the subclasses presented above were also identified.

Compound **10** ($t_R = 7.2$ min) was only detected in the SV leaves methanolic extract. This compound exhibited a $[M–H]^-$ ion at *m/z* 463 and its fragmentation by $MS^2$ experiments showed a loss of 162 Da, probably due to a hexoside residue, forming a fragment ion at *m/z* 301. Further $MS^n$ fragmentation of this ion gave intense ions at *m/z* 283, 257 and 229, which are similar to those obtained for a standard solution of ellagic acid and described in literature data.[20] Ellagic acid belongs to the polyphenols, more precisely to hydroxybenzoic acids that are commonly *O*-glycosylated. Hence, compound **10** was assigned as ellagic acid-*O*-hexoside.

Compound **18** ($t_R = 11.1$ min) belongs to the class of lignans and was only found in the FN total aerial parts extract. This compound exhibited a $[M–H]^-$ ion at *m/z* 519. The $MS^2$ spectrum of this ion showed a fragment at *m/z* 357, indicating the loss of 162 Da, probably a hexoside moiety. The $MS^3$ spectrum of the ion at *m/z* 357 exhibited, as base peak, a fragment ion at *m/z* 151 that is assigned as a cleavage of a tetrahydrofuran ring.[12] In addition, fragment ions at *m/z* 342 and 327 were observed, indicative of successive losses of 15 Da from methyl groups. Based on these $MS^n$ data compound **18** was identified as pinoresinol-4-*O*-hexoside. It should be mentioned that natural furofuran lignans may exist as different stereoisomers but their configuration could not be assigned by $MS^n$ experiments.

Compound **43** ($t_R = 22.8$ min) was identified as ferulic acid. This compound exhibited a $[M–H]^-$ ion at *m/z* 193 and its $MS^n$ fragmentation showed fragment ions at *m/z* 178 (loss 15 Da), 163 (loss $2 \times 15$ Da) and 135 (loss $2 \times 15$Da + 28 Da). This fragmentation pattern matches the one observed for a standard solution of ferulic acid. The only extract where it was possible to find this compound was in SV flowers.

Compound **52** ($t_R = 28.6$ min) showed a $[M–H]^-$ ion at *m/z* 409 and its $MS^2$ fragmentation gave a fragment ion at *m/z* 163 which indicates the presence of a coumaric acid moiety. The fragmentation of this ion at *m/z* 163 formed a fragment ion at *m/z* 119 (loss of 44 Da) and is similar to that of a standard solution of coumaric acid ($MS^n$ data not shown). However, based only on these data it was not possible to completely characterize compound **52**, which was tentatively assigned as a coumaric acid derivative.

Compound **68** ($t_R = 39.0$ min) exhibited a $[M–H]^-$ ion at *m/z* 329 and was only detected in the SV leaves methanolic extract. Under $MS^2$ fragmentation, the ion at *m/z* 329 gave a fragment ion at *m/z* 314 due to the loss of 15 Da. The $MS^3$ spectrum of this ion produced two very intense fragment ions at *m/z* 271 (base peak) and 299 (ca. 99.9% of base peak). Comparing these results with literature data,[21] compound **68** was identified as 1,2,6-trihydroxy-7,8-dimethoxy-3-methylanthraquinone.

## Unknown compounds

Other peaks were detected although the elucidation of their structures based only on the $MS^n$ data was not completely achieved.

Compounds **36** ($t_R = 19.8$ min) and **42** ($t_R = 21.9$ min) exhibited a $[M–H]^-$ ion at *m/z* 457. The $MS^n$ fragmentation behaviour was identical for both compounds. The $MS^2$ spectra showed a fragment ion at *m/z* 260 indicating the loss of 197 Da. Further fragmentation gave fragment ions at *m/z* 231 and 151. However, it was not possible to identify their structures.

Compound **67** ($t_R = 37.0$ min) gave a $[M–H]^-$ ion at *m/z* 599 and was only detected in the SV leaves extract. In the $MS^2$ fragmentation, two successive losses of 162 Da were observed, probably due to hexoside residues. Nevertheless, with no other information available, it was not possible to identify the nature of this compound.





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## CONCLUSIONS

Phenolic compounds present in *Helichrysum melaleucum* were analyzed, for the first time to our knowledge, by a LC-DAD/ESI-$MS^n$ method. By the analysis of the different morphological parts of plants collected in São Vicente (SV) it is possible to conclude that the flowers extract revealed a larger number of compounds, most of them flavonoids substituted with glycosides and/or acyl groups.

A comparison was made for the total aerial parts methanolic extracts collected in different geographical locations. Plants collected at higher altitude, Fajã da Nogueira (FN), showed a much higher variety of phenolic compounds. Despite belonging to the same subspecies, the phenolic compositions of these two extracts were significantly different and some substances, such as pinoresinol (compound **18**) and flavone derivatives (compounds **56** and **66**), can be used as geographical markers.

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