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Antioxidant potential of *Artemisia argentea* L'Hér alcoholic extract and its relation with the phenolic composition

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ABSTRACT

Artemisia argentea, known as losna or Madeira wormwood is used as aperitif drink with tonic effects. A reversed-phase high-performance liquid chromatography method (RP-HPLC) coupled with diode-array detection (DAD) and electrospray ionization mass spectrometry (ESI/MS) was used for the separation/characterization of phenolic compounds in *A. argentea*. A wide variety of components was found, mainly flavonoids (*O*- and *C*-glycosylated) and hydroxycinnamic acids derivatives. Five saponins, an uncommon type of compound in *Artemisia* species, were reported. Quantification of caffeoylquinic acids (CQA) was performed and 5-*O*-CQA and 3,5-*O*-diCQA were the major compounds (ca. 300 mg/100 g dried plant). Total phenolic content (TPC) and total flavonoid content (TFC) were established and four assays were used to measure the antioxidant capacity of the plant, revealing a high radical scavenging capacity and a weak reducing potential. Unlike other *Artemisia* subspecies, *A. argentea* is totally free of harmful components such as thujene, thujone or artemisia ketone.

1. Introduction

Artemisia argentea L'Hér belongs to the *Asteraceae* family and is a shrub endemic from Archipelago of Madeira (Portugal), very common in rocky areas.

The leaves are used in the local folk medicine mainly in aqueous infusions as vermifuge, stomachic, for treatment for stroke and as emmenagogue. The alcoholic infusion is used as a tonic, sudorific and aperitive (Rivera & Obón, 1995).

There are very few scientific publications concerning this plant. The composition of essential oil from the aerial parts (leaves and flowers, separately) has been investigated by GC and GC–MS (Figueiredo et al., 1994) and the main conclusion was that the essential oils from flowers and leaves were similar and composed of monoterpenes, mainly α-phellandrene. The aerial parts of *A. argentea* afforded some sesquiterpene lactones, namely arborescin and argentiolides A and B, deacetylargentiolide B and guaianolides (El-Emary, Makboul, & Hamed, 1986).

The composition of the more polar extracts, which correspond to those used locally, is not described in literature for this particular plant.

Alcoholic extracts and aqueous formulations contain mainly phenolic compounds, a group of low and medium molecular weight secondary metabolites that can embrace a great diversity of substances (Aligiannis et al., 2003). They can be divided in three main groups: flavonoids, phenolic acids and tannins.

Phenolic acids include mainly hydroxybenzoates or hydroxycinnamates derivatives. They differ according to the number and position of hydroxylation and methoxylation of the aromatic ring. There is a much higher quantity and diversity of hydroxycinnamates than hydroxybenzoates and they consist of *p*-coumaric, caffeic, ferulic and sinapic acids either glycosylated or esterified with quinic, shikimic or tartaric acids. In fruits and leaves, the main hydroxycinnamates result from the esterification of caffeic acid group(s) with quinic acid, the most frequent and abundant caffeoylquinic acid isomer being 5-*O*-caffeoylquinic acid (chlorogenic acid). In cereal grains, ferulic acid esters are the most common hydroxycinnamates.

Flavonoids can occur as glycosides: *O*-glycosides and/or *C*-glycosides and in some cases with additional methylation, acetylation and hydroxylation (Cuyckens & Claeys, 2004).

Phenolic compounds are associated with a high number of biological activities and one with special interest is the antioxidant capacity. The consumption of antioxidant compounds or foods with high levels of these compounds is associated to prevention and reduction of the risk of diseases associated to free radical reactions. The increase of degenerative diseases such as coronary heart disease, diabetes, cancer and age related diseases has required the urgency to found new natural sources of non-toxic antioxidant compounds (Katalinic et al., 2010).

In this work, the alcoholic extract composition of *A. argentea*, with special emphasis on the phenolic profile, was investigated by a HPLC-

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DAD-ESI/MS^n method. The major caffeoylquinic acids detected were also quantified by UV-DAD detection.

The antioxidant capacity of this plant was evaluated using radical scavenging methods (ABTS and DPPH), ferric reducing power (FRAP) and β-carotene assays.

2. Material and methods

2.1. Chemical and standards

The following reagents were purchased from Merck (Darmstadt, Germany): potassium persulfate (99%), sodium chloride (99.5%), disodium phosphate dodecahydrated (99%), glacial acetic acid (100%), sodium carbonate (p.a.) and ferrous sulfate heptahydrate (99%). 2,2-diphenyl-1-picrylhydrazyl (>95%), Trolox (≥99.8%, HPLC), 2,2′azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (≥99%, HPLC), 2,4,6-Tri(2-Pyridyl)-s-triazine (≥99.0%, TLC) β-carotene (≥97%, UV), Tween40 and Folin-Ciocalteu's phenol reagent were purchased from Fluka (Lisbon, Portugal). Potassium chloride (>99.5%), gallic acid (99%, HPLC), potassium acetate (p.a.), rutin (≥98%, HPLC) and ferric chloride hexahydrate (97%–100%) were purchased from Panreac (Barcelona, Spain); potassium dihydrogen phosphate (99.5%), aluminum chloride (98%) and sodium acetate trihydrate (pure) were purchased from Riedel-de Haën (Hanover, Germany).

All solvents used for plant extraction were AR grade, purchased from Fisher. LC–MS^n grade acetonitrile (LabScan, 99.9%) and ultra-pure water (Milli-Q water purification system, EUA) were used for HPLC analysis.

Stock solutions of standard (100 μg/mL) were prepared in ethanol for HPLC-DAD-ESI/MS^n identification and kept in a refrigerator at −20 °C until use. Standards: *p*-coumaric acid (>99%), caffeic acid (>99%), protocathecuic acid (>99%) from Extrasynthese and 5-*O*-caffeoylquinic acid (99%) from Acros Organics. 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid were obtained from Chengdo Biopurify Phytochemicals, Ltd China (Sichuan, China).

2.2. Plant material and sample preparation

Aerial parts of *A. argentea* L'Hér were purchased at a specialized medicinal plant market as bunches of dried plant material (aerial parts, composed of leaves and stems) in October 2010. They had been collected four weeks previously at Ponta do Sol, Madeira Archipelago (Portugal) and hanged to dry in a cool dark store. The plants were authenticated by taxonomist Fátima Rocha, from the Madeira Regional Secretary for the Environment and Agriculture.

For analysis, the stems were discarded and the dried leaves were ground to a fine powder in a mechanic grinder and extracted by ultrasound-assisted solvent extraction. Briefly, plant material (1 g) was extracted with methanol (25 mL) using a sonicator Bandelin Sonorex (Germany) at 35 kHz and 200 W for 60 min at room temperature. The solution was filtered and concentrated in a rotary evaporator (40 °C) and kept in the dark at 4 °C until tested.

2.3. HPLC-DAD-ESI/MS^n screening

A solution with concentration (w/v) of 5 mg/mL was prepared by dissolving the dried extract in the initial HPLC mobile phase (acetonitrile/water, 20/80, v/v).

This solution was filtered through 0.45 mm Nylon micropore membranes prior to use and 10 μL were injected for HPLC-DAD-ESI/MS^n analysis. Three independent assays were performed for each sample.

The HPLC analysis was done on a Dionex ultimate 3000 series instrument (California, EUA) coupled to a binary pump, a diode-array detector (DAD), an autosampler and a column compartment.

HPLC separation was carried out on a Phenomenex Gemini C_{18} column (5 μm, 250 × 3.0 mm i.d.; Phenomenex) at 30 °C. The mobile phase was acetonitrile (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) and 20% A (49–55 min). The mobile phase flow rate was 0.4 mL/min; the chromatogram was recorded at 280 nm and 320 nm and spectral data for all peaks were accumulated in the range of 190–400 nm.

For HPLC-ESI/MS^n analysis, a model 6000 ion trap mass spectrometer (Bruker Esquire, Bremen, Germany) fitted with an ESI source was used operating in the negative mode. 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 13,000 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 1×10^{-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.

2.4. HPLC-DAD quantification

The analysis were performed with the HPLC system described above using a modified gradient that allowed for the separation of all detected caffeoylquinic acid isomers. The mobile phase consisted of acetonitrile: formic acid (100:0.1, v/v) (A) and water:formic acid (100:0.1, v/v) (B). The gradient program was used as follows: 20% B (0–1 min), 78% B (8–10 min), 76% B (12–14 min), 75% B (16–18 min), 73% B (20 min), 50% B (40 min), 0% B (41–45 min), and 80% B (46–50 min). The flow rate was 0.4 mL/min and the injections volume 10 μL. UV detection was performed at 320 nm.

2.5. TPC, TFC and antioxidant capacities assays

2.5.1. Total phenolic content (TPC)

TPC was measured by the Folin-Ciocalteu method (Zheng & Wang, 2001) with some modifications. The extract was dissolved in methanol to yield a concentration (w/v) of 10 mg/mL. 50 μL aliquots were mixed with 1.25 mL of Folin-Ciocalteu reagent (diluted 1:10) and 1 mL of 7.5% sodium carbonate solution. After 30 min, at room temperature, the decrease in absorbance was measured at 765 nm. The final result was expressed as mg of gallic acid equivalents per 100 g of dried plant (mg GAE/100 g).

2.5.2. Total flavonoid content (TFC)

TFC was measured using a published method slightly modified (Akkol, Göger, Kosar, & Baser, 2008). A methanolic solution of the extract with a concentration of 2.5 mg/mL was prepared. To a 10 mL test tube, 0.5 mL of sample solution, 1.5 mL of methanol, 2.8 mL of water, 0.1 mL of potassium acetate (1 M) and 0.1 mL of aluminum chloride (10% in methanol) were added and mixed. After incubation at room temperature for 30 min, the decrease in absorbance was measured at 415 nm. The total flavonoid content was expressed as milligrams of rutin equivalent per 100 g of dried plant (mg RUE/100 g).

2.5.3. $ABTS^{\bullet+}$ radical scavenging activity

The $ABTS^{\bullet+}$ assay used was an adapted version of that first reported by Re et al. (1999). The $ABTS^{\bullet+}$ radical solution was prepared by reacting 50 mL of 2 mM $ABTS^{\bullet+}$ solution with 200 μL of 70 mM potassium persulfate solution. This mixture was stored in the dark for 16 h at room temperature, and it was stable in this form for two days (Re et al., 1999). For each analysis, the $ABTS^{\bullet+}$ solution was

diluted with pH 7.4 phosphate buffered saline (PBS) solution to an initial absorbance of 0.700 ± 0.021 at 734 nm. This solution was newly prepared for each set of analysis.

To determine the antiradical scavenging activity, an aliquot of 100 μL methanolic solution (10 mg/mL) was added to 1.8 mL of $ABTS^{\bullet+}$ solution and the absorbance decrease, at a 734 nm, was recorded during 6 min. Results were expressed as μmol Trolox equivalent per 100 g of dried plant (μmol eq. Trolox/100 g), based on the Trolox calibration curve.

2.5.4. DPPH radical scavenging activity

The DPPH assay followed a reported method (Gordon, Paiva-Martins, & Almeida, 2001) with some modifications. In a few words, 100 μL of the methanolic solution (10 mg/mL) was added to 3.5 mL of a 0.06 mM methanol DPPH radical solution. The decrease in absorbance at 516 nm was measured every minute during 30 min, in the dark. The DPPH radical scavenging effect of the sample was expressed, based on the Trolox calibration curve, as μmol Trolox equivalent per 100 g of dried plant (μmol eq. Trolox/100 g).

2.5.5. Ferric reducing activity (FRAP assay)

The ferric reducing capacity of the extracts was measured based on the ferric reducing activity assay (FRAP) (Benzie & Strain, 1996). FRAP reagent was prepared daily by mixing 2.5 mL of solution ferric trichloride hexahydrate (20 mM), 2.5 mL of solution TPTZ (10 mM in 40 mM of hydrochloric acid) and 25 mL of acetate buffer 0.3 M (pH 3.6) and incubating at 37 °C. The extract was dissolved in methanol to yield a final concentration (w/v) of 1 mg/mL. For each analysis, 30 μL of methanolic solution was added to 180 μL of distilled water and 1.8 mL of FRAP solution. The increase of absorbance was recorded at 593 nm in 15 s intervals, during 30 min at 37 °C. The FRAP results were expressed as mmol $FeSO_4 \cdot 7H_2O$ per mg of dried plant (mmol Fe (II)/mg).

2.5.6. β-Carotene bleaching assay

A reported method for the β-carotene bleaching assay (Siddhuraju & Becker, 2003) was applied with minor modifications. Briefly, 2 mL of β-carotene solution 0.2 mg/mL in chloroform was added to a round-bottom flask, containing 0.04 mL of linoleic acid and 200 mg of Tween 40. The chloroform was removed by evaporation using nitrogen and then 50 mL of oxygenated ultrapure water, obtained by bubbling air through the water for 15 min, was added. The mixture was strongly shaken. The resulting emulsion was freshly prepared before each experiment. Stock solutions of the extracts were prepared in ethanol to yield a final concentration of 1 mg/mL. An aliquot of 250 μL of the β-carotene–linoleic acid emulsion was distributed in each of the wells of the 96-well microtitre plates and 30 μL of the samples solutions were added. An equal amount of ethanol was used as control. The samples were then subjected to thermal autoxidation at 45 °C for 210 min. The decrease of the solution's absorbance was followed at λ = 490 nm, at 15 min intervals. The antioxidant activity (AA) of each sample was evaluated in terms of the bleaching of β-carotene using the following equation: $AA\ (\%) = (1 - A0 - At/A'0 - A't) * 100$, where A0 and A′0 are the absorbance values measured at zero incubation time for the test and control respectively and At and A′t are the corresponding absorbance values measured after incubation for 210 min.

2.6. Statistical analysis

All measurements were performed in triplicates and results are expressed as mean ± SD (standard deviation). The statistical probability was considered to be significantly different at the level of $p<0.05$.

3. Results and discussion

Preliminary phytochemical screening of raw methanolic extracts of *A. argentea* revealed the presence of flavonoids and saponins and the absence of alkaloids. Also, the essential oil was analyzed by GC–MS (data not shown) on two columns of different polarity and neither thujene, thujone nor artemisia ketone was detected. Alpha-phellandrene was the major component. Since the plant is used for alcoholic beverages preparations, it was important to confirm the absence of those potential toxic compounds.

3.1. HPLC-DAD-ESI/MS^n screening

The HPLC method employed for the separation of phenolic components in the alcoholic extract of *A. argentea* revealed a good separation the majority of the compounds.

Four compounds were unequivocally identified based on the analysis of standard compounds and comparing their HPLC retention time, UV spectra and MS^n fragmentation pattern. The remaining compounds were characterized and their structures proposed based mainly on the MS^n fragmentation data conjugated with the UV-DAD spectra. Most of the peaks showed similar UV absorptions maxima with two bands at λ_{max} 230–240 nm and 320–330 nm and a shoulder at 290–300 nm. These types of UV absorption bands are characteristic of hydroxycinnamic acids. Some peaks with characteristic UV absorptions bands for flavonoids were also detected (Mabry et al., 1970).

The base peak chromatogram (BPC) of the alcoholic extract of the leaves from *A. argentea* is presented in Fig. 1(a). The most relevant components were caffeoylquinic acids. The slight but noticeable foam formation during extraction and solvent evaporation was a previous clue to the presence of saponins that were indeed detected, albeit in very small amounts.

In general, in the MS^1 spectrum the most intense peak corresponds to the deprotonated molecular ion $[M–H]^-$. The main fragments observed in the MS^n experiments (n = 1–n4) are given in Table 1 and their chemical structures are shown in Fig. 2.

3.1.1. Hydroxycinnamic acid derivatives

The linkage position of acyl substituent groups on the quinic acid molecule can be determined based on the main fragment ions obtained from MS^n fragmentation of $[M–H]^-$ ions. Acyl groups connected to the 4-OH position displayed a [caffeic acid–H]$^-$ ion at *m/z* 173 as base peak. When the acyl group is attached 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 (Clifford, Knight, & Kuhnert, 2005).

The quinic acid derivatives found in *A. argentea* 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., 2005).

Compound **2** (t_R = 3.0 min) was identified as quinic acid. Its deprotonated molecular ion, at *m/z* 191, under MS^n fragmentation gave as main fragment ions peaks at *m/z* 127 ($[M–CO–2H_2O]^-$) and *m/z* 173 ($[M–H_2O]^-$) in good agreement with literature reports (Gouveia & Castilho, 2009).

3.1.2. Mono-, di- and tricaffeoylquinic acids

Two compounds with deprotonated molecular ions at *m/z* 353 were observed indicating the presence of monocaffeoylquinic acid isomers. However, their MS^n behavior was considerably different.

Compound **3** (t_R = 4.4 min) showed a MS^2 spectrum with a base peak at *m/z* 191 and an intense peak at *m/z* 179 (ca. 59% of base peak) indicating that the caffeoyl group is linked to the 3-OH position of quinic acid. Therefore, **3** was identified as 3-*O*-caffeoylquinic acid.

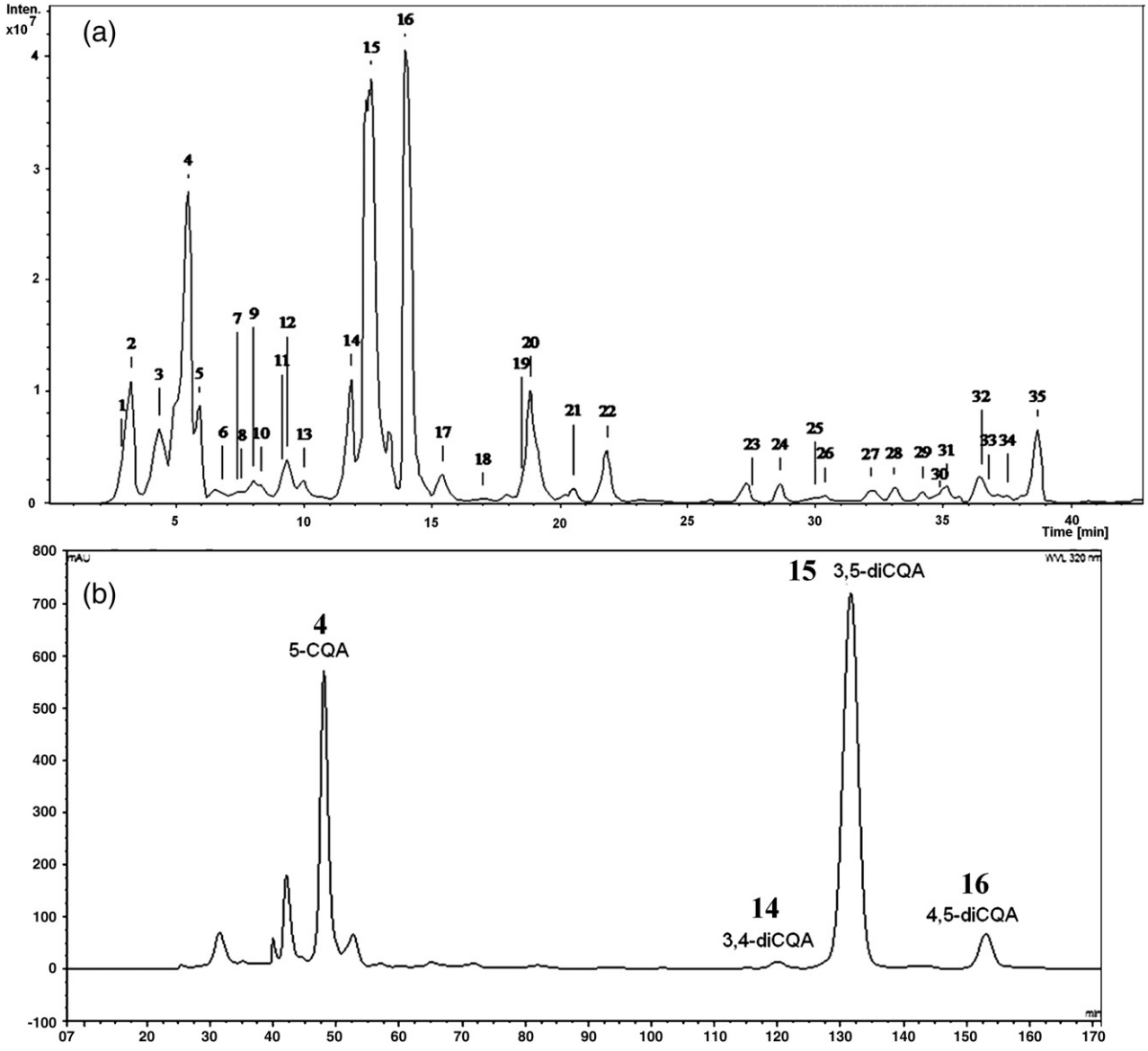


Fig. 1. HPLC-DAD-ESI/MS^n analysis of the methanolic extract of *A. argentea* L'Hér — (a) HPLC–MS negative ion ESI/MS^n base peak chromatogram (BPC) (gradient described in Section 2.3); (b) chromatografic profile of hydroxycinnamic acids at $\lambda = 320$ nm (gradient described in Section 2.4).

Compound **4** ($t_R = 5.0$ min) was identified as 5-*O*-caffeoylquinic acid by comparison of the HPLC retention time, UV and mass spectra with those found for a standard solution. The occurrence of this compound in *Asteraceae* family is well known (Carini, Aldini, Furlanetto, Stefani, & Facino, 2001) and in particular in *Artemisia* species (Han et al., 2008).

Compounds **14** ($t_R = 11.8$ min), **15** ($t_R = 12.9$ min) and **16** ($t_R = 14.2$ min) exhibited a $[M–H]^-$ ion at *m/z* 515 and were identified as 3,4-*O*-dicaffeoylquinic acid (**14**), 3,5-*O*-dicaffeoylquinic acid (**15**) and 4,5-*O*-dicaffeoylquinic acid (**16**). These identifications were made by comparison of HPLC retention times, UV and mass spectra of reference standards.

3.1.3. Coumaroylquinic acids

Compound **6** ($t_R = 6.8$ min) showed a $[M–H]^-$ ion at *m/z* 337. The MS^2 and MS^3 fragmentations gave fragment ions at *m/z* 191 and *m/z* 179, as base peak, respectively. The UV spectrum of this peak gave the characteristic absorptions bands of the *cis*-5-*O*-*p*-coumaroylquinic acid, previously described in our recent work on endemic *Asteraceae* species (*Helichrysum monizii* Lowe) from Madeira Archipelago (S.C. Gouveia & Castilho, 2011).

Compound **18** ($t_R = 17.1$ min) and compound **20** ($t_R = 19.6$ min) presented the same $[M–H]^-$ ion at *m/z* 499. Under MS^2 fragmentation, **18** gave a fragment ion at *m/z* 353 due to the lost of a 146 Da fragment, suggesting the presence of a coumaroyl group. The MS^3 spectrum gave a main fragment ion at *m/z* 191, due to the loss of 162 Da from a caffeoyl group and an intense ion at *m/z* 179 (ca. 55% of base peak). Based on these MS^n data and knowing that the first group lost is more easily expelled from a 5-OH position, **18** was identified as 3-*O*-caffeoyl-5-*O*-*p*-coumaroylquinic acid (Clifford, Marks, Knight, & Kuhnert, 2006).

Compound **20** showed a MS^2 ion at *m/z* 337, as base peak, suggesting the loss of a caffeoyl group in the first place. The sequential MS^n fragmentation resulted in a similar fragmentation pattern to that described for 3-*O*-caffeoyl-4-*O*-*p*-coumaroylquinic acid (Clifford et al., 2006).

Compound **19** was characterized as a coumaroylquinic acid derivative. It occurred at a retention time of 18.6 min and exhibited a

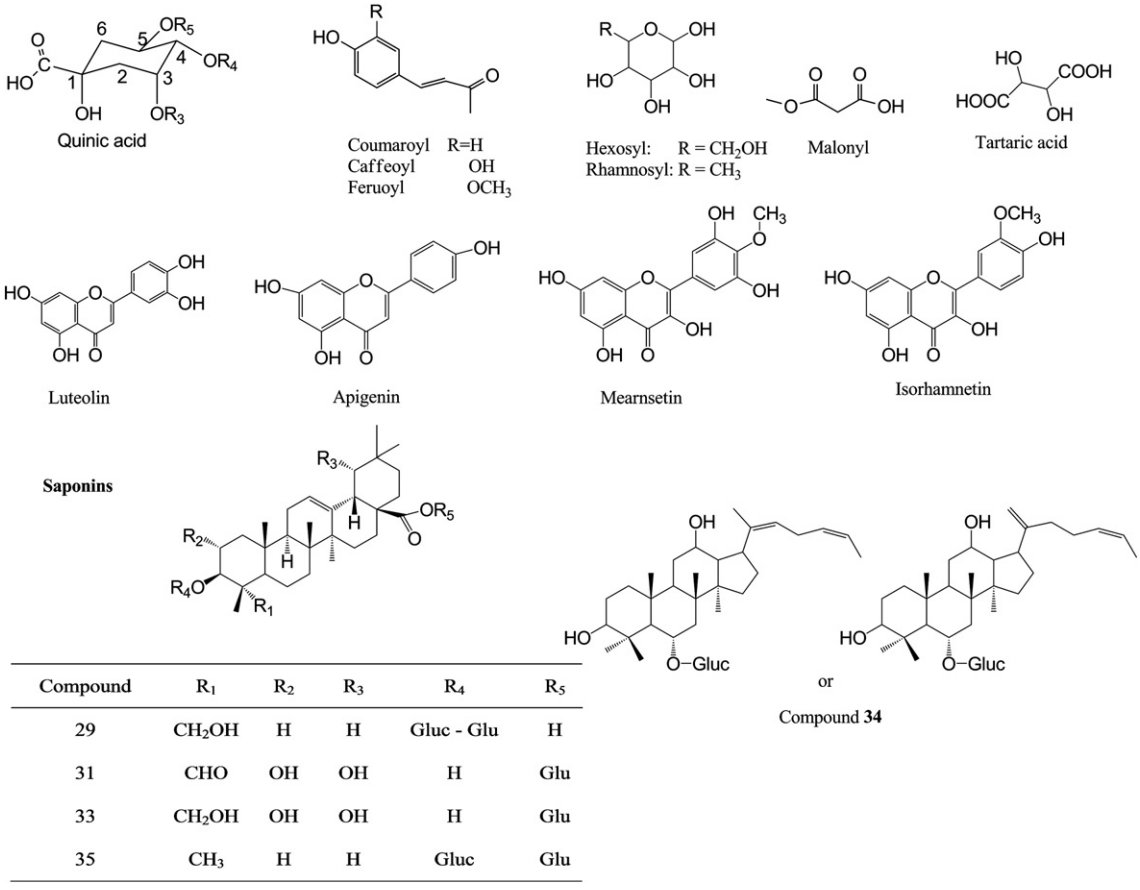
Table 1
Characterization of phenolic components of the alcoholic extract from *A. argenteae* L'Hér by HPLC-DAD-ESI/MS^n.

No	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	HPLC-DAD-ESI/MS^n *m/z* (% base peak)	Identification
1	2.8	275, 305	473	MS^2 [473]: 342 (16.8), 341 (100), 179 (17.5), 131 (10.2) MS^3 [473 → 341]: 179 (100), 161 (20.9), 143 (27.3), 119 (20.2) MS^4 [473 → 341 → 179]: 161 (100), 119 (85.3), 113 (54.0), 109 (35.3), 89 (67.9)	Caffeic acid hexoside derivative
2	3.0	269	191	MS^2 [191]: 173 (74.9), 127 (100), 111 (47.0), 109 (42.9), 85 (70.2) MS^3 [191 → 127]: 163 (30.8), 125 (14.6), 109 (69.7), 99 (100)	Quinic acid
3	4.4	241, 300, 324	353	MS^2 [353]: 191 (100), 179 (58.6), 135 (15.9) MS^3 [353 → 191]: 173 (22.7), 171 (21.9), 127 (100), 111 (52.4), 109 (28.1), 85 (47.8) MS^4 [353 → 191 → 127]: 109 (100), 99 (90.9), 85 (12.9)	3-*O*-Caffeoylquinic acid
4[a]	5.0	242, 300, 325	353	MS^2 [353]: 191 (100) MS^3 [353 → 191]: 173 (55.2), 127 (100), 109 (65.8), 85 (97.0) MS^4 [353 → 191 → 127]: 109 (100)	5-*O*-Caffeoylquinic acid
5	5.4	224, 272, 314	563	MS^2 [563]: 503 (49.8), 473 (68.6), 443 (100), 383 (63.0), 353 (65.0) MS^3 [563 → 443]: 383 (49.8), 354 (16.6), 353 (100) MS^4 [563 → 443 → 353]: 326 (35.1), 325 (100), 297 (56.2)	Apigenin-6-*C*-hexoside-8-*C*-pentoside.
6	6.8	304	337	MS^2 [337]: 191 (100), 163 (6.61) MS^3 [337 → 191]: 179 (100), 127 (44.8), 111 (47.2), 93 (85.3), 85 (85.7), 81 (26.4) MS^4 [337 → 191 → 179]: 93 (100)	*Cis*-5-*O*-*p*-coumaroylquinic acid
7	7.4	327	367	MS^2 [367]: 191 (100) MS^3 [367 → 191]: 179 (100), 134 (93.0), 111 (68.0), 127 (65.6), 109 (12.6), 85 (90.8)	5-*O*-Feruloylquinic acid
8		327	533	MS^2 [533]: 372 (18.6), 371 (100) MS^3 [533 → 371]: 353 (100), 191(32.4), 173 (98.7), 135 (28.8) MS^4 [533 → 371 → 353]: 191 (20.7), 179 (100), 173 (45.6)	Caffeic acid hexoside derivative
9	7.9	224, 272, 313	577	MS^2 [577]: 487 (41.2), 473 (2.7), 457 (100), 353 (36.9) MS^3 [577 → 457]: 383 (18.5), 354 (33.0), 353 (100) MS^4 [577 → 457 → 353]: 326 (55.5), 325 (100), 298 (22.0)	Apigenin-6-*C*-hexoside-8-*C*-rhamnoside
10	8.3	224, 272, 314	431	MS^2 [431]: 341 (23.0), 312(19.3), 311 (100) MS^3 [431 → 311]: 311 (11.5), 284 (12.2), 283 (100) MS^4[431 → 311 → 283]: 283 (31.1), 183 (100), 165 (64.6), 163 (37.6)	Apigenin-8-*C*-hexoside
11	9.3	257, 300, 342	493	MS^2 [493]: 331 (100), 330 (24.5), 316 (11.3) MS^3 [493 → 331]: 317 (15.6), 316 (100), 315 (10.6) MS^4 [493 → 331 → 316]: 287 (62.0), 271 (75.1), 215 (33.0), 166 (100)	Mearnsetin-*O*-hexoside
12		–	447	MS^2 [447]: 286 (14.1), 285 (100) MS^3 [447 → 285]: 258 (60.2), 241 (16.4), 176 (50.0), 175 (100), 149 (70.3)	Luteolin-7-*O*-hexoside
13	9.8	256, 270, 344	477	MS^2 [477]: 316 (17.1), 315 (100), 300 (29.3) MS^3 [477 → 315]: 301 (11.4), 300 (100) MS^4 [477 → 315 → 300]: 284 (60.2), 271 (57.5), 245 (67.8), 229 (109), 213 (64.4)	Isorhamentin-*O*-hexoside
14[a]	11.8	246, 299, 325	515	MS^2 [515]: 354 (15.3), 353 (100), 335 (14.7), 299 (12.2) MS^3 [515 → 353]: 191 (47.4), 179 (58.4), 173 (100), 135 (10.8) MS^4 [515 → 353 → 173]: 155 (16.5), 111 (100), 93 (79.5), 71 (10.6)	3,4-*O*-dicaffeoylquinic acid
15[a]	12.9	242, 300, 328	515	MS^2 [515]: 354 (14.0), 353 (100) MS^3 [515 → 353]: 191 (100), 179 (35.2) MS^4 [515 → 353 → 191]: 173 (100), 127 (28.2), 109 (17.6), 85 (49.9)	3,5-*O*-dicaffeoylquinic acid
16[a]	14.2	243, 300, 327	515	MS^2 [515]: 354 (12.4), 353 (100), 335 (1.80), 299 (12.6) MS^3 [515 → 353]: 191 (43.1), 179 (98.6), 174 (11.9), 173 (100), MS^4 [515 → 353 → 179]: 135 (100)	4,5-*O*-dicaffeoylquinic acid
17	15.3		491	MS^2 [491]: 371 (12.9), 330 (20.3), 329 (100), 314 (10.7) MS^3 [491 → 329]: 315 (18.2), 314 (100) MS^4 [491 → 329 → 314]: 300 (40.7), 299 (100)	Dihydroxy-dimethoxyl-O-hexoside flavone
18	17.1	–	499	MS^2 [499]: 354 (10.5), 353 (100), 337 (32.9) MS^3 [499 → 353]: 192 (15.2), 191 (100), 179 (55.1)	Coumaroyl-5-*O*-caffeoylquinic acid
19	18.6	–	819	MS^2 [819]: 787 (72.7), 518 (32.), 517 (100), 301 (17.6) MS^3 [819 → 517]: 489 (31.4), 338 (54.2), 337 (100), 314 (84.1), 305 (22.8) MS^4 [819 → 517 → 337]: 337 (55.5), 310 (100), 305 (36.6), 237 (37.6)	Coumaroylquinic acid derivative
20	19.6	225, 310	499	MS^2 [499]: 338 (14.8), 337 (100), 173 (19.7), 163 (20.5) MS^3 [499 → 337]: 173 (100), 163 (10.7) MS^4 [499 → 337 → 173]: 93 (100)	3-*O*-caffeoyl-4-*O*-*p*-coumaroylquinic acid
21	20.2	–	529	MS^2 [529]: 368 (12.8), 367 (100), 173 (16.6) MS^3 [529 → 367]: 193 (36.7), 175 (10.7), 174 (14.1), 173 (100) MS^4 [529 → 367 → 173]: 155 (43.5), 137 (34.8), 112 (44.8), 111 (100)	1 or 5-*O*-caffeoyl-4-*O*-feruoylquinic acid
22	21.6	-	457	MS^2 [457]: 412 (20.0), 411 (100), 179 (24.6), 161 (14.0) MS^3 [457 → 411]: 205 (16.4), 179 (100), 161 (62.3), 149 (15.2), 143 (36.5) MS^4 [457 → 411 → 179]: 143 (31.4), 131 (52.7), 101 (100), 85 (38.3)	Caffeic acid derivative
23	27.7	–	443	MS^2 [443]: 398 (20.5), 397 (100), 381 (18.6) MS^3 [443 → 397]: 179 (100), 119 (57.0) MS^4[443 → 397 → 179]: 89 (100)	Caffeic acid derivative
24	28.5	232, 300, 311	457	MS^2 [457]: 296 (12.4), 295 (100), 173 (18.3) MS^3 [457 → 295]: 163 (100), 121 (24.6) MS^4 [457 → 295 → 163]: 111 (100), 102 (16.2), 83 (14.0)	Caffeoylcoumaroyltartaric acid
25	29.8	299, 312	635	MS^2 [635]: 474 (15.5), 473 (100) MS^3 [635 → 473]: 312 (13.8), 311 (100), 179 (46.1), 173 (50.2) MS^4 [635 → 473 → 311]: 173 (100), 137 (12.0)	Dicaffeoyltartaric acid derivative
26	30.4	247, 329	449	MS^2 [449]:353 (10.8), 288 (20.5), 287 (100), 173 (31.6) MS^3 [449 → 287]: 173 (100), 113 (17.8) MS^4 [449 → 287 → 173]: 111 (33.6), 93 (100)	Eriodictyol-7-*O*-hexoside

Table 1 (*continued*)

No	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	HPLC-DAD-ESI/MS^n *m/z* (% base peak)	Identification
27	31.7	298, 325	657	MS^2 [657]: 625 (98.7), 355 (77.6), 301 (100), 257 (48.2) MS^3 [657→301]: 258 (11.7), 257 (100) MS^4 [657→301→257]: 242 (100)	Ellagic acid derivative
28	33.1	–	803	MS^2 [803]: 773 (12.9), 772 (44.7), 771 (100) MS^3 [803→771]: 753 (77.8), 744 (42.6), 743 (100), 725 (40.0) MS^4 [803→771→743]: 726 (48.9), 725 (100), 707 (10.8), 563 (16.4)	Unknown
29	34.2	–	809	MS^2 [809]: 48 (46.9), 647 (100), 471 (11.9) MS^3 [809→647]: 514 (20.1), 473 (30.7), 471 (100) MS^4 [809→647→471]: 405 (100), 406 (90), 393 (74.0)	Saponin 1
30	34.5	–	483	MS^2[483]: 322 (23.6), 321 (100), 173 (17.8) MS^3 [483→321]: 173 (100), 147 (26.3) MS^4 [483→321→173]: 111 (35.5), 109 (42.2), 93 (100)	Unknown
31	34.8	–	663	MS^2 [663]: 502 (21.7), 501 (100) MS^3 [663→501]: 328 (25.0), 327 (100), 173 (29.8) MS^4 [663→501→327]: 291 (62.7), 229 (93.4), 211 (100), 171 (29.9)	Saponin 2
32	36.4	–	563	MS^2 [563]: 402 (20.0), 401 (100) MS^3 [563→401]: 227 (100), 183 (20.7), 173 (65.4), 165 (18.8) MS^4[563→401→227]: 184 (14.7),183 (92.4), 145 (100)	Unknown
33	36.6	–	665	MS^2 [665]: 504 (26.5), 503 (100) MS^3 [665→503]: 330 (16.0), 329 (100), 173 (29.3) MS^4 [665→503→329]: 293 (12.4), 229 (100), 211 (32.3)	Saponin 3
34	37.5	–	619	MS^2 [619]: 458 (18.2), 457 (100) MS3 [619→457]: 335 (15.7), 296 (27.6), 295 (45.0), 174 (14.2), 173 (100) MS^4 [619→457→173]: 111 (35.8), 93 (100), 71 (11.7).	Saponin 4
35	38.6	–	793	MS^2 [793]: 632 (33.1), 631 (100) MS^3 [793→631]: 456 (40.3), 455 (100) MS^4 [793→631→455]: 453 (20.7), 409 (55.4), 407 (100), 191 (11.0)	Saponin 5

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



Compound	R_1	R_2	R_3	R_4	R_5
29	CH_2OH	H	H	Gluc - Glu	H
31	CHO	OH	OH	H	Glu
33	CH_2OH	OH	OH	H	Glu
35	CH_3	H	H	Gluc	Glu

Fig. 2. Chemical structures of the main class of phenolic compounds found in the methanolic extract of *A. argentea* L'Hér.

$[M–H]^-$ ion at *m/z* 819. The MS^n fragmentation showed main fragment ions MS^2 ion at *m/z* 517 (loss of 302 Da) and MS^3 ion at *m/z* 337 (loss of 180 Da). Only the ion at *m/z* 337 could be related to the coumaroylquinic acid group and no further identification was possible based with these data alone.

3.1.4. Feruloylcaffeoylquinic acids

Compound **7** (t_R = 7.4 min) displayed a $[M–H]^-$ ion at *m/z* 367 and its MS^2 fragmentation gave, as base peak, a fragment ion at *m/z* 191 [quinic acid–H]$^-$. MS^n data are consistent to those describe in literature for 5-*O*-feruloylquinic acid (Clifford et al., 2005).

Compound **21** (t_R = 20.4 min) showed a $[M–H]^-$ ion at *m/z* 529 suggesting the presence of a caffeoylferuloylquinic acid compound. In the MS^2 fragmentation a neutral fragment of 162 Da was expelled (caffeoyl moiety) resulting in a fragment ion at *m/z* 367. The base peak in the MS^3 spectrum was a fragment ion at *m/z* 173, pointing to a 4-*O*-feruloylquinic acid structure. The caffeoyl group can be linked to either of the following positions of the quinic acid structure: 1-OH, 3-OH and 5-OH.

Based on the MS^2 fragmentation data, which revealed a weak fragment ion at *m/z* 191 and no fragment ion at *m/z* 179, the 1-OH and 5-OH positions are preferable. Also, it is more difficult to caffeoyl groups located in 3-OH positions to be the first moiety to be lost in the MS^2 fragmentation.

Distinction between 1-OH and 5-OH positions could only be achieved based on their differences of retention time or using a solution of a standard compound, which was not available. So, compound **21** was tentatively characterized as 1 or 5-*O*-caffeoyl-4-*O*-feruloylquinic acid.

3.1.5. Caffeic acid derivatives

Compound **1** (t_R = 2.8 min) displayed a $[M–H]^-$ ion at *m/z* 473. The MS^2 spectrum showed a fragment ion at *m/z* 341, as base peak, indicating the loss of 132 Da (probably a pentose or tartaric acid unit). Further MS^n fragmentation of the ion at *m/z* 341 lead to the identification of a caffeic acid hexoside derivative, based on the loss of 162 Da (hexoside moiety) and the presence of a MS^3 ion at *m/z* 179, characteristics of caffeic acid. Therefore, compound **1** was identified as caffeic acid hexoside derivative.

At a retention time of 7.4 min, there were two co-eluted compounds: **7,** identified as 5-*O*-feruloylquinic acid and **8**.

Compound **8** showed a $[M–H]^-$ ion at *m/z* 533 and under MS^n fragmentation the characteristic fragment ions of caffeic acid were observed, such as *m/z* 179 [caffeic acid–H]$^-$ and *m/z* 135 [caffeic acid–CO_2–H]$^-$. Also, the MS^2 fragmentation of the deprotonated molecular ion gave a fragment ion at *m/z* 371 due to the neutral loss of 162 Da, suggesting a hexoside residue. In the absence of more specific data, **8** was tentatively characterized as another caffeic acid hexoside derivative.

Compound **22** (t_R = 21.6 min) was characterized as a caffeic acid derivative. The $[M–H]^-$ ion at *m/z* 457 gave a MS^2 fragment ion at *m/z* 411 (loss of 46 Da, probably a formic acid adduct). Fragmentation of this ion led to [caffeic acid–H]$^-$ ion at *m/z* 179. The nature of the 232 Da fragment was not possible to identify based on the MS^n data; the UV/visible spectrum was not useful for further identification.

3.1.6. Hydroxycinnamoyltartaric acids

Compound **24** (t_R = 28.5 min) showed a $[M–H]^-$ ion at *m/z* 457 and in the MS^2 fragmentation lost a neutral fragment of 162 Da, probably a caffeic acid. The base peak of the MS^2 spectrum is a fragment ion at *m/z* 295 corresponding to coumaroyltartaric (coutaric) acid. MS^3 fragmentation of this ion yielded a fragment ion at *m/z* 163 revealing a coumaroyl acid, and its decarboxylated ion at *m/z* 119. This fragmentation behavior was previously described (Schütz, Kammerer, Carle, & Schieber, 2005) and **24** was characterized as a caffeoylcoumaroyltartaric acid.

Compound **25** (t_R = 29.8 min) showed a $[M–H]^-$ ion at *m/z* 635 and a neutral loss of 162 Da to form a MS^2 base peak at *m/z* 473. This ion suffers a further loss of 162 Da leading to a MS^3 spectrum base peak of *m/z* 311 (caftaric acid), so 25 was characterized as a dicaffeoyltartaric acid derivative (one of the 162 Da neutral losses can be due either to a caffeoyl or hexoside moiety) (Maier et al., 2006).

3.1.7. Flavonoids

A number of flavonoids were also identified in the methanolic extract of *A. argentea*. Nearly all flavonoids were identified as glycosides containing one or more sugar moieties and, in some cases, esterified with acyl groups. Four of them were *O*-glycosylated flavonoids and three were *C*-glycosylated, reported here for *A. argentea* for the first time.

The distinction of these two groups of flavonoids is easily observed based on the MS^n fragmentation behavior. The carbon–carbon bond of *C*-glycosylated flavonoids is resistant to rupture, so the main cleavages are at the bonds of the sugar. For *O*-glycosylated flavonoids, the sugar moieties are easily lost by neutral losses (Jin et al., 2008).

3.1.8. O-glycosides

The tandem MS–MS experiments of the flavonoids gave the deprotonated molecular ion ($[M–H]^-$) and the deprotonated aglycone ion (Y_0^-) as a result of the loss of the sugar group. The flavonoid fragment ions were designated as proposed by Cuyckens and Claeys (2004).

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. Compound **11** (t_R = 9.3 min) exhibited a $[M–H]^-$ ion at *m/z* 493. The MS^2 fragmentation showed the loss of a hexoside moiety (162 Da) forming a fragment ion at *m/z* 331. This ion, under MS^n fragmentation, gave a radical fragment $[Y_0–CH_3]^{\bullet-}$ at *m/z* 316, consistent with literature data for mearnsetin (Han et al., 2008). Therefore, **11** was characterized as mearnsetin-*O*-hexoside.

Compound **12** (t_R = 9.4 min) gave a $[M–H]^-$ ion at *m/z* 447. When submitted to further MS^n fragmentation, this ion readily eliminated a hexoside residue (162 Da) to produce the deprotonated aglycone ion, Y_0^-, at *m/z* 285. The MS^3 spectrum of this ion formed fragments ions at *m/z* 199, 217 ($[M–H–C_3O_2]^-$), 175 ($[M–H–C_3O_2–C_2H_2O]^-$) and 241 ($[M–H–CO_2]^-$). These are characteristic ions of luteolin, as confirmed with a standard solution of the pure substance (data not shown) and literature data (Fabre, Rustan, Hoffmann, & Quetin-Leclercq, 2001). The preferred substitution position for flavones, such as luteolin, is the 7-OH position (Cuyckens & Claeys, 2004). Thus, **12** was identified as luteolin-7-*O*-hexoside.

Compound **13** (t_R = 10.0 min) displayed a $[M–H]^-$ ion at *m/z* 477. Its MS^2 spectrum showed a fragment ion Y_0^- at *m/z* 315 (loss of 162 Da), indicating the presence of a hexoside residue. Fragmentation of the MS^2 ion at *m/z* 315 was very similar to that of isorhamnetin reported in our previously studies (Gouveia & Castilho, 2009, 2010). So, **13** was identified as isorhamnetin-*O*-hexoside.

Compound **17** (t_R = 15.3 min) gave a $[M–H]^-$ ion at *m/z* 491. Its MS^2 fragmentation produced a fragment ion at *m/z* 329, by the loss of a hexoside residue (162 Da). The sequential MS^n fragmentation allowed for the identification of two losses of 15 Da each, due to two methoxyl groups. This fragmentation behavior is consistent with that described before for a dihydroxy-dimethoxy-*O*-hexoside flavones (Han et al., 2007). The same fragmentation pattern was found in other endemic *Asteraceae* species from Madeira (Gouveia & Castilho, 2010) albeit at higher retention time.

Compound **26** (t_R = 30.2 min) exhibited a $[M–H]^-$ ion at *m/z* 449 which easily expelled a hexoside residue (neutral loss of 162 Da) to form the deprotonated aglycone ion (Y_0^-) at *m/z* 287. Fragmentation of this ion gave MS^3 fragment ions at *m/z* 135 and *m/z* 151 which suggests the aglycone as being eriodictyol (Lin & Harnly, 2010).

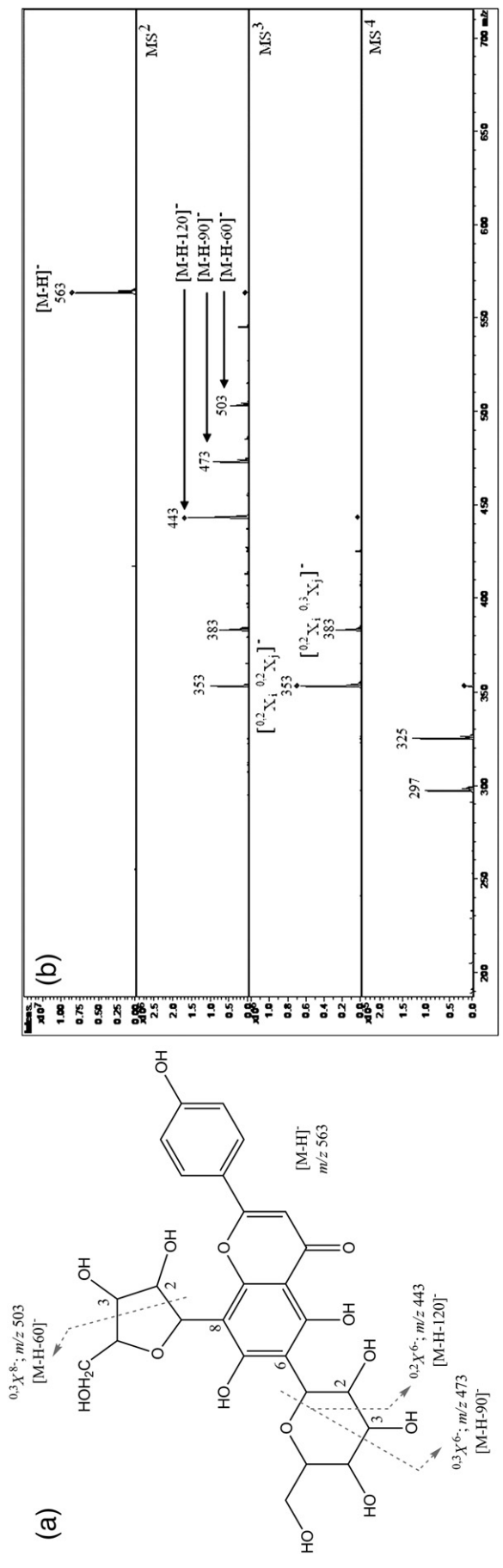


Fig. 3. (a) Fragmentation pathway proposed for apigenin-6-C-hexoside-8-C-pentoside (compound **5**). The nomenclature is according to Domon and Costello (Cuyckens & Claeys, 2004). (b) ESI/MS^n negative mode of compound **5**. Sequential fragmentation, MS^n (n: 1–4), of the ion at *m/z* 563.

Eriodictyol belongs to the flavonoid subgroup of flavanones and their most common glycosylation position is the 7-OH. Therefore, **26** was identified as eridictyol-7-*O*-hexoside.

3.1.9. C-glycosides

The UV spectra of compounds **5** ($t_R = 5.4$ min), **9** ($t_R = 7.9$ min) and **10** ($t_R = 8.3$ min) exhibited maximum absorption bands at $\lambda = 224$, 272 and 314 nm similar to the characteristic absorptions of flavones. However, the MS^n fragmentation experiments revealed a different fragmentation pathway from those of *O*-glycosides compounds.

Fragment ions at m/z $^{0,2}X^-[M–H–120]^-$, $^{0,3}X^-[M–H–90]^-$ and $X^-[M–H–60]^-$ related to the sugar ring were observed with different relative intensities, which are typical of *C*-glycosides (Cuyckens & Claeys, 2004). For this type of flavonoids, the absence of Y_0^- ions makes the characterization of the aglycone part rather difficult, since it is not possible to perform direct comparison with reference molecules (Vukics, Ringer, Kery, Bonn, & Guttman, 2008).

Compound **5** gave a $[M–H]^-$ ion at m/z 563 and a fragmentation pattern typical of the asymmetrical di-*C*-glycosides. In the MS^2 spectrum, the main fragments occurred at m/z 443 ($[M–H–120]^-$, base peak), 473 ($[M–H–90]^-$, ca. 69% of base peak) and 353 ($[M–H–210]^-$, ca. 65% of base peak), indicating the presence of a *C*-hexoside unit (Cuyckens & Claeys, 2004). A fragment ion at m/z 503 was observed ($[M–H–60]^-$, ca. 50% of base peak) which indicates the fragmentation of a *C*-pentose unit (Fig. 3).

It is known that the fragment ion $[M–H–60]^-$ has a higher intensity for *C*-6 glycosides than for *C*-8 glycosides (Han et al., 2008). For compound **5**, this MS^2 ion at m/z 503 had an intensity of ca. 50% of base peak, pointing to a *C*-6 glycoside compound. The MS^3 fragment ions at m/z 353 ($[^{0,2}X_i^{0,2}X_j]^-$, aglycone + 83) and m/z 383 ($[^{0,2}X_i^{0,3}X_j]^-$, aglycone + 113) suggest apigenin as the aglycone (Fig. 3) (Vukics et al., 2008). Thus, **5** was identified as apigenin-6-*C*-hexoside-8-*C*-pentoside.

Compound **9** ($t_R = 7.9$ min) displayed a $[M–H]^-$ ion at m/z 577. Its MS^2 fragmentation gave, as base peak, a $[M–H–120]^-$ ion at m/z 457 and also an intense fragment ion $[M–H–90]^-$ at m/z 487 (ca. 41% of base peak). These two ions indicate that **9** is a *C*-glycoside and, since the $[M–H–60]^-$ ion was not detected, the sugar unit should be a hexoside. Further fragmentation of the ion at m/z 457 led to the loss of 104 Da, forming a fragment ion at m/z 353. This fragment is indicative of a methylpentose group (Han et al., 2008). For the same reasons mentioned for compound **5,** the aglycone part was identified as apigenin and **9** identified as apigenin-6-*C*-hexoside-8-*C*-rhamnoside.

Compound **10** ($t_R = 8.3$ min) exhibited a $[M–H]^-$ ion at m/z 431 and its MS^2 spectrum showed typical fragment ions of *C*-glycosides at m/z 311 $[M–H–120]^-$and m/z 341 $[M–H–90]^-$. Based on the guidelines for the identification of isomeric mono-*C*-glycosides flavonoids presented by Waridel et al.(2001), this compound was identified as a *C*-8 flavonoid, since the MS^2 spectrum did not revealed the loss of water molecules representative of *C*-6 isomers. Taking into account the literature data (Han et al., 2008; Sánchez-Rabaneda et al., 2003) compound **10** was identified as apigenin-8-*C*-hexoside.

3.1.10. Saponins

Five saponins were identified, 4 of them with a triterpenic structure and one with a steroid structure. The basic proposed structures are presented in Fig. 2. With resource to MS^n and literature data only, since the information from UV/Vis spectra was not particularly useful, it was impossible to determine neither whether the glycosylation occurred at the α or the β position, nor the exact nature of the sugar moieties. These saponins correspond to minor components of the extract, so isolation was out of the question as a goal for the present work.

Compound **29** ($t_R = 34.2$ min) showed a $[M–H]^-$ ion at m/z 809. Its MS^2 fragmentation gave a fragment ion at m/z 647 due to the loss of 162 Da. The MS^2 ion at m/z 647 easily lost 176 Da (a glucuronide group) forming a fragment ion at m/z 471 as the deprotonated aglycone ion, $[aglycone–H]^-$, characteristic of hederagenin (Sun, He, Shi, Xiao, & Cheng, 2007). Thus, **29** was characterized as hederagenin esterified with a glucopyranosyl and a glucuronic acid groups and was identified as being saponin 1 (Fig. 2).

Compound **31** ($t_R = 34.8$ min) gave a $[M–H]^-$ ion at m/z 663 and, under MS^2 fragmentation, an ion at m/z 501 was observed as the main fragment, due to the loss of a neutral residue with 162 Da. A saponin compound with a similar fragmentation behavior has been reported (Weimin, Rensheng, Guowei, Vaisar, & Lee, 1996), assigning the 501 to the deprotonated aglycone ion of dihydroxy-olean-12-en-24,28-dioic acid. However, also in 1996, Terreaux et al. found two saponins in *Paradrymonia macrophilia* that eluted sequentially and showed $[M–H]^-$ ions at m/z 663 and m/z 665 respectively and gave aglycone m/z ions at 501 and m/z 503; the second was characterized as a trihydroxyl derivative of heredagenin and the first to its oxidized (aldehyde) form. Considering the data of compound **33**, which showed an $[M–H]^-$ ion at 665 and an aglicone ion at m/z 503, it can be seen that the same pattern of *P. macrophilia* is observed in *A. argentea*, so we proposed that compounds **31** and **33** correspond to saponins 2 and 3 (Fig. 2).

Compound **34** ($t_R = 37.5$ min) presented a $[M–H]^-$ ion at m/z 619 and its MS^2 spectrum showed the neutral loss of 162 Da to form a fragment at m/z 457. The MS^2 ion at m/z 457 corresponds to the $[aglycone–H]^-$ ion and, based on literature reports (Zhang & Cheng, 2006), it should be a steroid type saponin, here designated as saponin 4; the exact location of the double bond is not possible to derive from the available data.

Compound **35** ($t_R = 38.6$ min) displayed in the MS^2 spectrum a fragment ion at m/z 631 as base peak, due to the loss of a 162 Da (hexoside). In the MS^3 fragmentation, a neutral fragment of 176 Da (glucuronic acid) was eliminated originating the $[aglycone–H]^-$ ion at m/z 455. This pattern has been described for the saponin β-D-glucopyranosyl-(1 → 3)-O-β-D-glucuronopyranosyl-(1 → 3)]-3β-hydroxyolean-12-ene-28-oate (Magalhães et al., 2003). In this paper, we named it saponin 5.

Detection of saponins is difficult to perform by UV due to their poor absorption. However, for the application of HPLC-ESI/MS^n technique original standards are essential for their characterization (Han et al., 2007).

For this work no reference standards of saponins were available and under MS^n fragmentation it was difficult to produce MS^n fragmentation with exception of the $[M–H-162]^-$ fragment.

3.1.11. Other compounds

Compound **27** ($t_R = 31.7$ min) displayed a $[M–H]^-$ ion at m/z 657. Its MS^2 spectrum showed the loss of 356 Da resulting in a fragment ion at m/z 301 (base peak). Under MS^n fragmentations this ion gave as main fragment an ion at m/z 257 which is characteristics of ellagic acid. The 356 Da residue could not be identified based on the available data (Aaby, Ekeberg, & Skrede, 2007). Thus, **27** was identified as ellagic acid derivative.

Table 2
Contents of individual caffeoylquinic acids compounds in *A. argentea* L'Hér (mg/100 g of dried plant material).

Compounds	5-CQA	3,4-diCQA	3,5-diCQA	4,5-diCQA	Total amount
A. argenteae	283.72 ± 2.09	[a]	289.15 ± 0.62	28.39 ± 0.37	601.26 ± 3.08

[a] Compounds present in trace amounts (lower than the LOQ).

Three other signals, at high retention times, were observed and their MS^n fragmentation showed the loss of one or more 162 Da residues, indicating that probably these compounds are esterified with caffeic acid groups.

Their UV spectra did not provided any valuable information concerning to their chemical nature. The MS^n data were not conclusive and the structure of each of these compounds could not be established.

3.2. HPLC-DAD quantification of caffeoylquinic acid isomers

The main compounds detected by the HPLC-DAD-ESI/MS^n screening were caffeoylquinic acids. Recently, our group discovered that this type of compounds is present in high levels in some endemic species from Madeira (Gouveia & Castilho, 2009, 2010). The HPLC-DAD quantification method used in this study was validated and is being considered for publication elsewhere.

In *A. argentea,* five cafeoylquinic acids (mono and di-isomers) were detected (Table 1) and the quantification for four of them is shown in Table 2.

Fig. 1(b) represents the UV ($\lambda = 320$ nm) chromatogram obtained for *A. argentea* methanolic extract, using the optimized gradient for the separation of CQA isomers.

Despite that in the MS^n screening 4,5-*O*-dicaffeoylquinic acid signal appears with a higher intensity, using the UV-DAD detection the area of this compound is much lower than those found for 5-*O*-caffeoylquinic acid and 3,5-*O*-dicaffeoylquinic acid.

This can be explained by occasional saturation in the ion source of the mass spectrometer. The ion trap mass spectrometers are very useful in the identification/characterization of samples by tandem MS^n experiments. However, they do not always give linear and reproducible results for quantification purposes.

Therefore, we decided to consider the UV data to quantify these compounds in *A. argentea*.

The compounds present in higher amounts were 3,5-*O*-dicaffeoylquinic acid with 289.15 ± 0.62 mg/100 g and 5-*O*-caffeoylquinic acid with a value of 283.72 ± 2.09 mg/100 g (there is not a statistically difference at $p < 0.05$ level). 4,5-O-dicaffeoylquinic acid was present in low levels in the sample at 28.39 ± 0.37 mg/100 g (ca. 10 times lower when compared to main CQA isomers). Another dicaffeoylquinic acid, 3,4-*O*-diCQA, was observed but in amounts under the limit of quantification (LOQ), for this compound.

The phenolic composition described for *A. argentea* can be qualitatively compared to other subspecies of *Artemisia*, such *as Artemisia annua* (Han et al., 2008) and *Artemisia pectinata* (Ma, Hattori, Chen, Cai, & Daneshtalab, 2008) that presented a phenolic profile with a large variety of quinic acids derivatives such as mono-, di-, and tri-*O*-caffeoylquinic acids, caffeoylferuoylquinic acids and diferuoylquinic acids as major components. The therapeutic activity of the plants was associated with this class of compounds.

A. annua was also reported as a good source of flavonoids (apigenin, quercetin and mearnestin) *O*- and *C*-glycosylated aglycones (Han et al., 2008). These compounds were found in *A. argentea* in addition to other flavonoids aglycones such as luteolin and eriodictyol; conjugated to several substitution groups.

Kimura et al. (1985) studied the effects of extracts of leaves of several *Artemisia* species on lipid metabolic injury in rats fed peroxidized oil and on histamine release from rat peritoneal mast cells attributing those effects to caffeoylquinic acids.

A recent study (Carvalho, Cavaco, & Brodelius, 2011) compared six *Artemisia* species (*A. annua*, *arborescen*, *ludoviciana* Nutt, *oleandica* (Besser) Krasch, *princeps* Pamp., *stelleriana* Bess) in terms of its phenolic profile and antioxidant capacity. The main conclusion is that ferulic and caffeic acid conjugates are the main compounds in the six species as we here describe for *A. argentea*, but a note to the high levels of catechins reported and that were not even found at a trace level in our study.

In quantitative terms, the amount of hydroxycinnamic acids is lower of that found in *A. argentea*. For instance, the highest value reported for 5-CQA, was 4.119 ± 0.2001 mg/100 g of dry matter (*Artemisia stelleriana*) (Carvalho et al., 2011) and corresponds to ca. 2% of the value obtained for *A. argentea* (Table 2).

Table 3
Experimental determinations of total phenolic and flavonoid content and antioxidant capacity assays (ABTS, DPPH, FRAP and β-carotene) for *A. argentea*.

Artemisia Argenteae	
Alcoholic extract	
Yield (%)	13.05
Total phenolic content/(mg GAE/100 g dried plant)	152.8 ± 2.72
Total flavonoid content/(mg RUE/100 g dried plant)	109.201 ± 0.041
ABTS/(μmol eq. Trolox/100 g dried plant)	7773.35 ± 12.10
DPPH/(μmol eq. Trolox/100 g dried plant)	773.26 ± 0.362
FRAP/(mmol $FeSO_4 \cdot 7H_2O$/mg dried plant)	3090.0 ± 38.3
β-Carotene (%) (10 mg/mL)	67.50 ± 1.18

3.3. TPC, TFC and antioxidant capacities

Regardless that the compounds present in higher amounts in *A. argentea* were caffeoylquinic acids, the HPLC-DAD-ESI/MS^n analysis revealed a vast number of other minor phenolic compounds.

In a complex sample such as a plant extract, even a compound present in low levels can show an individual high contribution for a particular bioactivity and synergistic effects can occur. Most of the phenolic compounds detected such as caffeoylquinic acid isomers, luteolin, apigenin, quercetin and the saponins are known as antioxidants.

Table 3 shows the results obtained for the total phenolic (TPC) and flavonoid (TFC) content and the data for each antioxidant capacity assay of the alcoholic extract of *A. argentea*.

The TPC value was 152.80 ± 2.72 mg GAE/100 g and the TFC value obtained was 109.20 ± 0.041 mg RUE/100 g. The lower amount of total flavonoid compounds is in good agreement to the phenolic HPLC-MS/ESI profile characterization. The TPC value is comparable to those reported for other *Artemisia* species (Carvalho et al., 2011) with TPC values between 220 and 390 mg GAE/100 g.

For the ABTS assay the value obtained was of 7773.35 ± 12.10 μmol eq. Trolox/100 g, a value 10 times higher than the value found in the DPPH assay which as 773.26 ± 0.362 μmol eq. Trolox/100 g.

It is quite usual to obtain higher values in the ABTS test, due to differences in the sensitivity of these methods. ABTS assay measures the antioxidant activity of both hydrophilic and lipophilic antioxidants.

Many antioxidants that react quickly with transient radicals such as peroxyl radical may react slowly or maybe even inert to DPPH, which is a long-lived nitrogen radical.

A recent report on *Artemisia selengensis* (Shi, Jia, Zhao, & Chen, 2010) measured the antioxidant activity of extracts obtained with different solvents. In that work, the ABTS values are expressed in the same units as in our study; therefore a comparison is possible for the alcoholic extract, with *A. argentea* showing a higher antioxidant potential (more than 10 times higher). Corke and co-workers (Cai, Luo, Sun, & Corke, 2004) studied 112 traditional medicinal antioxidant plants (aqueous and alcoholic extracts) using the ABTS method. Comparing alcoholic extracts, *A. argentea* is one of the most active plants. However, comparison between studies of different authors or labs must be considered qualitative since small variations in experimental conditions can largely affect the results. Apart from *Helichrysum* endemic species, we have recently studied other Asteraceae plants (studies submitted or in preparation) such as *A. annua* and *Cynara cardunculus* var. *scolymus* (artichoke), using exactly the same methods as those applied to *A. argentea*, finding that this endemic plant was as active

(slightly more so) as artichoke (6943.9 ± 17.29 μmol eq. Trolox/100 g) and *A. annua* (2197.27 ± 19.94 μmol eq. Trolox/100 g) towards ABTS.

The chemistry of FRAP assay is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous colored form in the presence of antioxidants. *A. argentea* FRAP performance as 3090.0 ± 38.3 mmol $FeSO_4 \cdot 7H_2O$/mg indicates a moderate reducing power of this plant. However, it compared poorly with other *Asteraceae* endemic species from Madeira from our previous studies where values of 36,000 mmol $FeSO_4 \cdot 7H_2O$/mg were reached (*H. monizii*) (S.C. Gouveia & Castilho, 2011) but it proved to be at the same order of magnitude as *A. annua* or artichoke (1861.0 ± 76.2 and 6759.1 ± 58.3 mmol $FeSO_4 \cdot 7H_2O$/mg, respectively) or *A. selengensis* with about 2940 mmol $FeSO_4 \cdot 7H_2O$/mg (Shi et al., 2010).

The β-carotene method is based on the capacity of a sample to inhibit the formations of peroxyl radicals during the oxidation of linoleic acid by β-carotene.

Several concentrations in the range 1–10 mg/mL were tested. All of them inhibit bleaching of linoleic acid in some extension, the 10 mg/mL gave the highest value at 67.50 ± 1.18 (%), comparable to our studies in artichoke leaves (52.53 ± 1.76 (%)).

This assay is performed on an emulsion, which puts it closer to the real food properties, but the data obtained are dependent on the polarity of the compounds and consequently on their partition between the two phases (aqueous and lipidic).

A result of this magnitude indicates that *A. argentea* contains compounds with good capacity to prevent the oxidation of lipids.

4. Conclusion

The phenolic profile of *A. argentea* was established for the first time by an HPLC-DAD-ESI/MS^n method. Flavonoid conjugates and phenolic acid derivatives were the main classes of characterized compounds and 5 saponins, 4 triterpenic and 1 steroidal, were also identified in the polar extracts.

A large number of caffeoylquinic acid conjugates were detected and four of them were quantified by HPLC-DAD. 3,5-*O*-dicaffeoylquinic acid and 5-*O*-caffeoylquinic acid provided the highest amounts (ca. 300 mg/100 g dried plant).

Colorimetric methods were used to measure the total phenolic and flavonoid contents of the alcoholic extract of the plant. In addition, the antioxidant capacity was evaluated by four different methods. ABTS, DPPH and FRAP and β-carotene. The results showed that the plant extract presents a high antioxidant capacity to scavenge free radicals (ABTS and DPPH assays) and a moderate reducing capacity as measured by the FRAP assay.

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