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Phenolic profiles of Lauraceae plant species endemic to Laurisilva forest: A chemotaxonomic survey

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ABSTRACT

In this work, the phenolic composition of several trees endemic to Madeira archipelago (Portugal) was studied. Specifically, the leaves of the most relevant species of the Lauraceae family (*Laurus novocanariensis*, *Apollonias barbujana*, *Ocotea foetens*, and *Persea indica*) have been analyzed. The screening of the main phenolic compounds in their methanol extracts has been performed by high-performance liquid chromatography with electrospray ionization mass spectrometric detection (HPLC-ESI–MS^n), identifying or tentatively characterizing almost 100 compounds, including a high number of proanthocyanidins (A- and B-type), which have been reported to present remarkable health benefits. Thirty-four compounds have been quantified, observing total individual phenolic contents (TIPCs) between 18.43 and 88.99 mg g^{-1} dry extract, with the lowest TIPC in *O. foetens* and the highest in *A. barbujana*.

1. Introduction

Madeira (Madeira archipelago, Portugal) laurel forest, Laurisilva, is a subtropical forest with a very rich bryophyte and vascular flora. It is well characterized from the botanical point of view, but little attention has been paid to the composition of most plant species, even though several species have been used for centuries in folk medicine (Rivera and Obón, 1995). Among the different compounds that are present in plants, phenolic compounds are of special interest to scientists, as they exhibit important biological activities, such as anti-oxidant, anti-microbial, anti-cancer and anti-mutagenic. Therefore, the characterization of these compounds, mainly uninvestigated in wild plants, is an important research field nowadays. The chemical and biological characterization of these plants may lead to promising sources of biologically active compounds.

In a previous study (Llorent-Martínez et al., 2015a), our research group established the phytochemical composition of the most important non-lauraceae trees of the Laurisilva forest (*Olea europaea* ssp. cerasiformis, *Ilex perado* ssp. perado, *Clethra arborea*, and *Heberdenia excelsa*). In this sense, the aim of the present work was to investigate the phenolic profile of selected plants from the Laurisilva belonging to the Lauraceae family: *Laurus novocanariensis*, *Apollonias barbujana*, *Ocotea foetens*, and *Persea indica*. Other species from the Lauraceae family have been reported to present health benefits, due to their phytochemical profile. For instance, cinnamon (*Cinnamomum zeylanicum* and *C. cassia* barks) has been the most studied species, which extracts are recognized by their high levels of procyanidins (Rao and Gan, 2014). The study of other non-edible *Lauraceae* species may lead to new sources of proanthocyanidins.

Laurus is a genus of evergreen trees belonging to the Lauraceae family, and three autochthonous species [*Laurus nobilis* L., *Laurus azorica* (Seub.) Franco and *Laurus novocanariensis* Rivas-Mart., Lousã, Fern. Prieto, E. Díaz, J.C. Costa & C. Aguiar] are described in Portugal (Vinha et al., 2015). *L. novocanariensis* is the most abundant endemic laurel from the Madeira archipelago and can grow from 3 to 20 m tall, presenting aromatic, shiny dark-green foliage. It presents male and female flowers on separate plants (Press and Short, 1994); the latter produce ovoid berries (1–1.5 cm) black when ripe. These berries derive a fatty oil that has been used in traditional medicine to treat skin ailments (Viciolle et al., 2012). Additionally, leaves (non-edible) are used in traditional cuisine to flavor dishes (Vinha et al., 2015), and to prepare infusions to relieve common cold and as sudorific (Rivera and Obón, 1995). A previous investigation on *L. novocanariensis* leaves documented monomeric and oligomeric flavan-3-ols as major phenolics (Vinha et al., 2015).

Apollonias barbujana (Cav.) Bornm. is an evergreen tree of 3–30 m tall. The simple leaves are alternate, elliptic, entire and petiolate, 6–8 cm length and 3–4 cm width. This tree produces panicles of white six-stellate flowers from June to September. Its berries are ovoid, approximately 15 mm long and brownish-grey color when ripe (Press

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and Short, 1994). This species has been used in folk medicine as diuretic, analgesic, antiulcerogenic, cytostatic, cardiotonic, expectorant, stomachic, sedative or carminative effects, and against rheumatic pain (Rivera and Obón, 1995). The total phenolics content and antioxidant activity of *A. barbujana* have been determined before (Tavares et al., 2010), but not the individual composition of phenolics.

Ocotea foetens (Aiton) Baill is an evergreen tree up to 30 m height. It usually grows with multiple trunks branched from its base. Leaves are 9–12 cm long and 3–5 cm wide, oblong lanceolate. Flowering season is from June to August. It produces hard and fleshy berries, dark-green, approximately 3-cm long. Its leaves have been traditionally used to prepare infusions, used as antihypertensive. It has also been used to treat malignant diseases with poultices made of tender leaves and branchlets (Rivera and Obón, 1995). Its total phenolics content and antioxidant activity have been previously reported (Tavares et al., 2010), but its phytochemical profile remains unknown.

The genus *Persea*, belonging to the family Lauraceae, comprises about 190 species including its main species *P. americana* Miller (avocado) (Alvárez et al., 2016). *Persea indica* (L) Spreng is an evergreen tree up to 15–20 m tall, with a broad, rounded crown. It presents leaves without glands, 10–20 × 3–8 cm, elliptic. It grows berries of about 2 cm, ellipsoid, bluish-black when ripe (Press and Short, 1994). The phytochemical composition of *P. indica* has been scarcely studied to date; only the presence of diterpenes has been reported (Alvárez et al., 2016).

The Laurisilva is part of UNESCO natural patrimony since 2000, and the forest is cleaned and thinned every year to prevent fire spread, and to improve the growth of healthy trees. Considering that the felled specimens and cut branches are discarded, the present work is part of a project aiming to validate traditional claims in order to promote applications of the discarded material from the forest.

2. Experimental

2.1. Chemicals and reagents

All reagents and standards were of analytical reagent (AR) grade unless stated otherwise. Caffeic acid (≥98%), diosmin (≥90%), kaempferol (≥97%), protocatechuic acid (98%) and rutin (≥95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). (+)-catechin hydrated (> 99%), apigenin (≥99%) and hesperidin (≥98.5%) were obtained from Extrasynthese (Genay, France); and quercetin dihydrate (> 99%) from Riedel-de Haen. Stock solutions of 200 mg/L were prepared in ethanol (HPLC grade; Sigma). LC–MS grade acetonitrile (CH_3CN, 99%) (LabScan; Dublin, Ireland) and ultrapure water (Milli-Q Waters purification system; Millipore; Milford, MA, USA) were used for the HPLC–MS analyses.

2.2. Sample preparation and extraction of phenolic compounds

Plant material was collected in different locations of Madeira Island (Portugal) as described in Table 1. Branches were cut from healthy plants in the mentioned locations.

Leaves were lyophilized to dryness (Alpha 1–2 LD Plus freeze dryer, CHRIST), ground to powder, and stored at −20 °C. Phenolic extraction followed a previous procedure (Spínola et al., 2014): 1 g of dry material was extracted with 25 mL of methanol in an ultrasonic bath (Bandelin Sonorex, Germany) at 35 kHz and 200 W for 60 min (room temperature). Chlorophylls (which can interfere in the analyses) were removed by adsorption on activated charcoal and extracts were filtered and concentrated to dryness in a rotary evaporator (Buchi Rotavapor R-114; USA) at 40 °C. The resulting extracts were stored at 4 °C until further analysis.

2.3. Chromatographic conditions

The HPLC-DAD analysis was performed on a Dionex ultimate 3000 series instrument (Thermo Scientific Inc.) coupled to a binary pump, an autosampler and a column compartment (kept at 20 °C). Separation was carried out in a Phenomenex Gemini C_{18} column (5 µm, 250 × 3.0 mm i.d.) using a mobile phase composed by CH_3CN (A) and water/formic acid (0.1%, v/v) at a flow rate of 0.4 mL min^{-1}. The following gradient program was used: 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). Sample solutions (5 mg mL^{-1}) were prepared by dissolving the dried extract in the initial HPLC mobile phase; after filtration through 0.45 µm PTFE membrane filters, 5 µL was injected.

For HPLC-ESI–MS^n analysis, a Bruker Esquire model 6000 ion trap mass spectrometer (Bremen, Germany) with an ESI source was used in hyphenation with the former described Dionex HPLC system. MS^n analysis was performed in negative and positive modes and scan range was set at *m/z* 100–1000 with a speed of 13,000 Da/s. The ESI conditions were as follows: drying and nebulizer gas (N_2) flow rate and pressure, 10 mL min^{-1} and 50 psi; capillary temperature, 325 °C; capillary voltage, 4.5 keV; collision gas (He) pressure and energy, 1×10^{-5} mbar and 40 eV. The acquisition of MS^n data was made in auto MS^n mode, with an isolation width of 4.0 *m/z*, and a fragmentation amplitude of 1.0 V (MS^n up to MS^4). Esquire control software was used for the data acquisition and Data Analysis for processing.

2.4. Quantification of polyphenols

For this experiment, one polyphenol was selected as the standard for each group, and it was used to calculate individual concentrations by HPLC-DAD (Spínola et al., 2014). Caffeic and protocatechuic acids were used, respectively, for hydroxycinnamic and hydroxybenzoic acids determinations. Quercetin, (+)-catechin, hesperidin and apigenin were the standards used for flavonols, flavanols, flavanones and flavones, respectively. Stock standard solutions (1000 mg L^{-1}) were prepared in methanol, and calibration curves were built by diluting the stock solutions with the initial mobile phase. Six concentrations (5–100 mg L^{-1}) were used for the calibration, plotting peak area versus concentration ($R^2 \geq 0.967$ in all cases). Total individual phenolic contents (TIPC) were defined as the sum of the quantified phenolic compounds.

2.5. Statistical analysis

All samples were assayed in triplicate and results are given as means ± standard deviations. Data analysis was carried out by means

Table 1
List of analyzed species and nomenclature used.

Species	Common name		Collection Area	Collection data	Voucher number
L. novocanariensis	Loureiro	L1	Ribeiro Frio	March 2013	MADJ 9677
		L2	Chão dos Louros	March 2013	MADJ 11285
		L3	Ponta do Pargo	July 2013	MADJ 14155
A. barbujana	Barbusano	AJ	Ribeiro Frio	March 2013	MADJ 4765
O. foetens	Til	OF	Ribeiro Frio	March 2013	MADJ 13159
P. indica	Vinhático	PI	Ribeiro Frio	March 2013	MADJ 13157

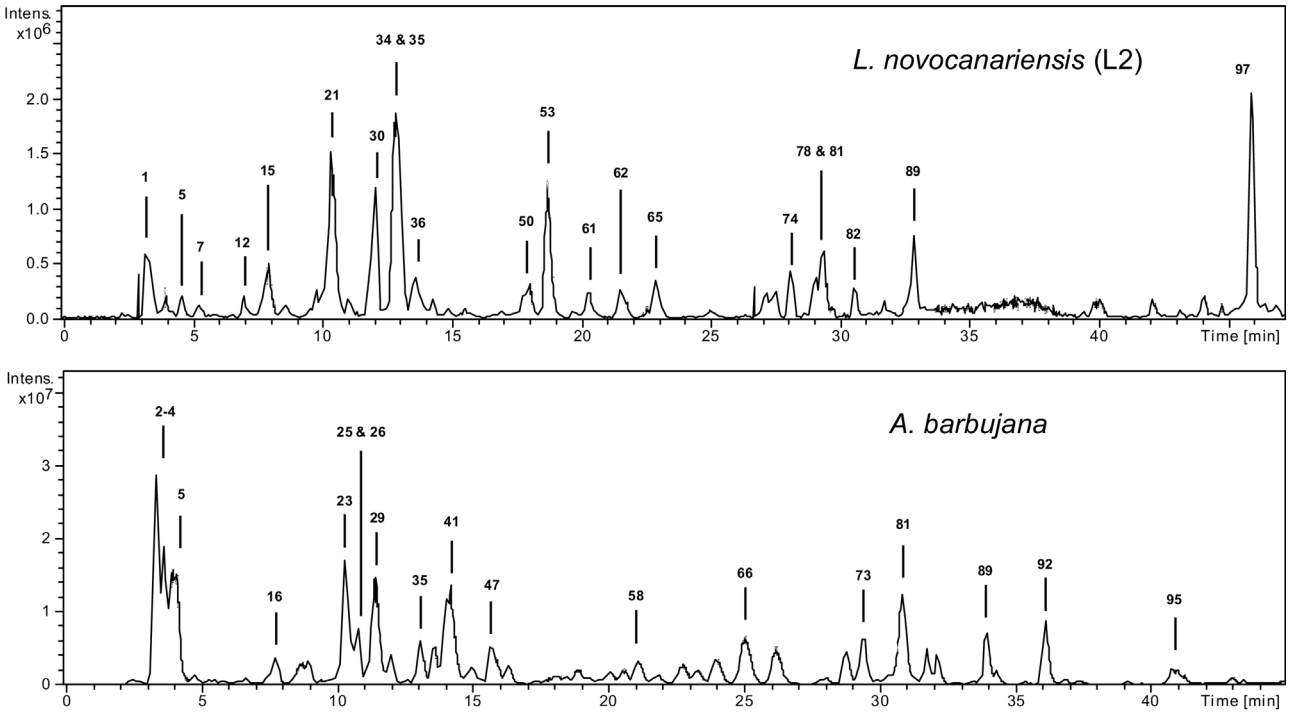

Fig. 1. HPLC-ESI/MS^n base peak chromatograms (BPCs) of *L. novocanariensis* and *A. barbujana* methanol extracts (negative mode).

of a one-way ANOVA with Tukeys post-hoc test using SPSS for Windows, IBM SPSS Statistics 20 (SPSS, Inc., USA). A value of $p < 0.05$ was considered statistically significant. Principal component analysis (PCA) was applied to the concentrations of polyphenols determined in different Lauraceae plants.

3. Results and discussion

3.1. HPLC-ESI–MS^n

The analysis of the phenolic composition in the extracts of the leaves from the different plants was carried out by HPLC-ESI–MS^n using negative and positive ionization modes. Three independent assays were carried out for each sample, obtaining similar data concerning the nature and relative intensities of the fragments. The base peak chromatograms of the methanol extracts are shown in Figs. 1 and 2.

The initial step for the characterization of the compounds consisted in the determination of the molecular weights. With this purpose, the negative ionization mode was used. The base peak usually corresponded to the deprotonated molecular ion $[M-H]^-$, although several formate adducts ($[M-H+HCOOH]^-$) were also detected. For the identification of the flavonoid glycosides, the mass spectra of the aglycones were compared with analytical standards when available (apigenin, kaempferol and quercetin) and the chemical nature of the sugars was determined by the neutral losses observed. Rutin and diosmin were identified by comparison with analytical standards. When reference compounds were not available, the tentative characterization was carried out by comparison of the experimental mass spectra with data from scientific literature.

The positive ionization mode was used for confirmation of the identifications. Compounds were numbered in all the chromatograms by their order of elution, keeping the same numbering in the different plants. A total number of 97 compounds were characterized, distributed among the different species analyzed. The tentative characterization of the detected compounds is shown in Tables 2 and 3, and the discussion of the characterization is explained in the following sub-sections.

3.1.1. Phenolic acids

Compound **10**, $[M-H]^-$ at *m/z* 361, exhibited its MS^2 base peak at *m/z* 163, which was identified as coumaric acid due to the 163 → 119 transition (Gruz et al., 2008). Hence, it was characterized as a coumaric acid derivative.

Compound **14** displayed an $[M-H]^-$ ion at *m/z* 353, MS^2 base peak ion at *m/z* 191 and a relatively intense fragment ion at *m/z* 179. According to Clifford et al. (2003), it was identified as 3-*O*-caffeoylquinic acid.

Compounds **19** and **35** presented $[M-H]^-$ ions at *m/z* 289 and identical fragmentation patterns. Considering that catechin elutes before epicatechin in reversed-phase HPLC, **19** and **35** were identified as catechin and epicatechin, respectively (Stöggl et al., 2004).

Compound **25** exhibited the deprotonated molecular ion at *m/z* 337, and its MS^2 base peak at *m/z* 163. Considering bibliographic data (Clifford et al., 2003), it was characterized as 3-*p*-coumaroylquinic acid.

Compound **35** was identified as epicatechin as described before. Compound **42**, with deprotonated molecular ion at *m/z* 647, presented an MS^2 base peak ion at *m/z* 289, and MS^3 [647 → 289] fragment ions at *m/z* 245, 205 and 203, so it was tentatively characterized as an (epi) catechin derivative.

Compound **79**, with $[M-H]^-$ at *m/z* 551, displayed an MS^2 fragment ion at *m/z* 153, which had its main fragment ion at *m/z* 109. Hence, it was characterized as a dihydroxybenzoic acid derivative.

3.1.2. Proanthocyanidins (PAs)

Ten PAs were detected in extracts of *L. novocanariensis*. Compounds **15**, **33**, **36**, **65** and **75** exhibited $[M-H]^-$ at *m/z* 575, and their mass fragmentation profiles (Tables 2 and 3) were similar to those reported for PA dimers (A-type), probably (epi)catechin-(epi)catechin (Soong and Barlow, 2005; Tomás-Barberán et al., 2001). Compounds **21** and **62** exhibited the deprotonated molecular ion at *m/z* 577, and fragment ions at *m/z* 451, 425, 407, and 289 (Table 2), typical from B-type PA dimers (Kajdžanoska et al., 2010; Ruiz et al., 2005). The fragment ion at *m/z* 289 proceeds from the loss of an (epi)catechin unit, so they were characterized as (epi)catechin-(epi)catechin dimers (Fig. 3). Compounds **30** and **34**, with $[M-H]^-$ at *m/z* 863, were characterized as A-type trimers (epi)catechin-(epi)catechin-(epi)catechin, comparing

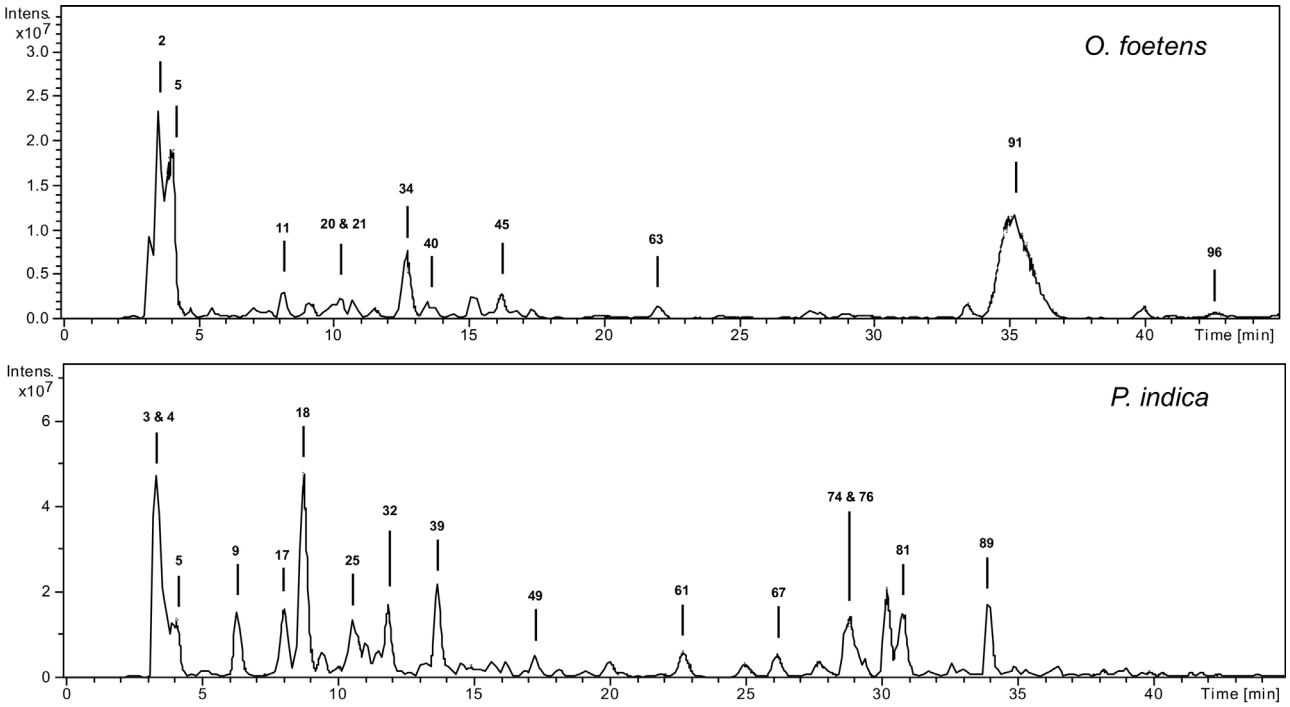

Fig. 2. HPLC-ESI/MS^n base peak chromatograms (BPCs) of *O. foetens* and *P. indica* methanol extracts (negative mode).

the mass fragmentation (positive and negative ion modes) with data from bibliography (Määttä-Riihinen et al., 2005). **30** and **34** are PA trimers with one A-linkage, whereas compounds **83** was characterized as a trimer with two A-linkages (Appeldoorn et al., 2009).

Five proanthocynidins were detected in the analyzed extracts of *A. barbujana*. Compounds **43** and **65** exhibited $[M-H]^-$ at *m/z* 575 and were characterized as PA dimers, probably (epi)catechin-(epi)catechin (A-type). Compounds **29** and **62**, with $[M-H]^-$ at *m/z* 577, were characterized as (epi)catechin-(epi)catechin dimers of the B-type (Kajdžanoska et al., 2010; Ruiz et al., 2005), as previously explained. Finally, compound **41** was characterized as an (epi)catechin-(epi)catechin-(epi)catechin-(epi)catechin trimer with one A-linkage.

Four PAs were detected in extracts of *O. foetens*, all of them consisting of (epi)catechin units. Compounds **21** and **29** were characterized as dimers (*m/z* 577), whereas compounds **34** and **83** as trimers, with deprotonated molecular ions at *m/z* 863 and 861, respectively.

Six PAs were characterized in *P. indica*. Compounds **17**, **21**, **29**, and **46** exhibited the deprotonated molecular ion at *m/z* 577, and fragment ions at *m/z* 451, 425, 407, and 289, typical from B-type PA dimers (Kajdžanoska et al., 2010; Ruiz et al., 2005). Compound **22**, with $[M-H]^-$ at *m/z* 865, was characterized as an (epi)catechin-(epi)catechin-(epi)catechin-(epi)catechin trimer (B-type) (Kajdžanoska et al., 2010; Ruiz et al., 2005), whereas compound **34** (*m/z* at 863) was identified as an A-type trimer (Määttä-Riihinen et al., 2005).

3.1.3. Flavonoids

High percentages – approximately 40% – of flavonoids were observed in all the analyzed extracts. The discussions in the following sub-sections are carried out grouping the compounds by their aglycones.

3.1.3.1 L. novocanariensis. Three apigenin derivatives were characterized. Compounds **50** and **53** were characterized as *C*-glycosil flavones, *O*-glycosylated on the sugar moiety, based on the characteristic peaks [(M−H)−164] and [aglycone + 41 − 18] at *m/z* 413 and 293. They were identified as rhamnosylvitexin and 2“-*O*-rhamnosylvitexin, respectively, considering their elution order (Dou et al., 2007; Ferreres et al., 2007). Compound **54** was identified as apigenin-8-*C*-hexoside (vitexin), based on its fragment ions at *m/z* 341, 311 and 283 and the absence of a fragment ion at *m/z* 413, which would indicate isovitexin (Gouveia and Castilho, 2013; Waridel et al., 2001).

Compounds **64** and **81** were quercetin-*O*-glycosides, exhibiting the aglycone at *m/z* 301. **64** and **81** suffered neutral losses of hexoside (162 Da) and deoxyhexoside (146 Da), respectively.

Five kaempferol-*O*-glycosides were characterized, showing the aglycone at *m/z* 285. Compounds **72**, **74**, **82** and **89** suffered neutral losses of 308, 162, 132 and 146 Da, respectively, and were characterized as kaempferol *O*-rutinoside, *O*-hexoside, *O*-pentoside, and *O*-coumaroyl, respectively. Compound **97** could not be fully identified and was characterized as a kaempferol derivative.

Finally, compounds **78** and **85** corresponded to isorhamnetin derivatives, with the aglycone at *m/z* 315 and typical fragment ion at *m/z* 300. Compound **78** was characterized as isorhamnetin-*O*-hexoside, whereas compound **85** corresponded to isorhamnetin-*O*-rutinoside or isorhamnetin-*O*-neohesperidoside (Bakr and El Bishbishy, 2016).

3.1.3.2 Apollonias barbujana. Nine quercetin derivatives were characterized (aglycone at *m/z* 301). Compound **47** suffered two neutral lossses of 162 Da (625 → 463 and 463 → 301), and was assigned to quercetin-*O*-dihexoside. Compound **58** presented the loss of hexoside + pentoside units. Compounds **64** and **66** corresponded to quercetin-*O*-hexoside isomers. Compounds **73** and **81** suffered neutral losses of 132 and 146 Da, respectively, and were characterized as quercetin-*O*-pentoside and quercetin-*O*-deoxyhexoside. Compound **68** was characterized as quercetin-*O*-*xylo*-pentoside (Sánchez-Rabaneda et al., 2004). Two compounds were identified as quercetin diglycosides, presenting the same $[M-H]^-$, together with the common neutral loss of 308 Da (609 → 301). Compound **61** was unambiguously identified as rutin after comparison with an analytical standard, whereas compound **71** was tentatively identified as quercetin-*O*-neohesperidoside, since rutinosides elute earlier than their corresponding neohesperidoside analogues (Abad-García et al., 2009).

Compound **55** presented the $[M-H]^-$ at *m/z* 521. After the neutral

Table 2
Characterization of the methanol extract of leaves from *L. novocanariensis*.

No.	t_R (min)	$[M-H]^-$ *m/z*	*m/z* (% base peak)	Assigned identification	L1	L2	L3
1	3.4	275	MS^2 [275]: 239 (100), 221 (11), 179 (9), 161 (7), 149 (33), 131 (11), 119 (5) MS^3 [275 → 239]: 179 (100), 161 (24), 149 (93), 131 (52), 119 (36), 89 (23)	Saccharide derivative	✓	✓	✓
5	4.0	191	MS^2 [191]: 173 (20), 111 (100)	Quinic acid	✓	✓	✓
7	5.5	315	MS^2 [315]: 153 (100), 123 (9) MS^3 [315 → 153]: 123 (100)	Hydroxytyrosol hexoside		✓	
8	5.8	319	MS^2 [319]: 97 (100)	Unknown			✓
10	6.6	361	MS^2 [361]: 163 (100) MS^3 [361 → 163]: 119 (100)	Coumaric acid derivative		✓	
12	7.2	315	MS^2 [315]: 153 (100), 152 (29) MS^3 [315 → 153]: 109 (100), 108 (39)	Dihydroxybenzoic acid-*O*-hexoside	✓	✓	✓
15	7.8	575	MS^2 [575]: 499 (41), 451 (54), 289 (35), 245 (100), 205 (17)	Proanthocyanidin dimer (A-type)	✓	✓	✓
19	9.5	289	MS^2 [289]: 245 (100), 205 (27), 203(7), 179 (26) MS^3 [289 → 245]: 227 (8), 203 (100)	Catechin		✓	
21	10.0	577	MS^2 [577]: 451 (12), 425 (94), 407 (100), 289 (14), 287 (10), 245 (14)	(Epi)catechin-(epi)catechin (B-type)	✓	✓	✓
28	10.9	431	MS^2 [431]: 385 (35), 179 (100) MS^3 [431 → 179]: 161 (6), 149 (57), 143 (65), 125 (53), 119 (6), 113 (56), 89 (100)	Saccharide derivative (formate adduct)	✓	✓	✓
30	11.5	863 865 (+)	MS^2 [863]: 712 (35), 711 (100), 695 (10), 693 (46), 559 (33), 451 (17), 411 (19), 289 (10) MS^3 [863 → 711]: 694 (64), 693 (100),559 (77), 407 (37) MS^2 [865]: 713 (72), 695 (54), 533 (100), 287 (29) MS^3 [865 → 533]: 515 (15), 407 (42), 287 (100)	Proanthocyanidin trimer (A-type)	✓	✓	
33	12.4	575	MS^2 [575]: 499 (55), 490 (55), 423 (37), 407 (16), 289 (100) MS^3 [575 → 289]: 271 (100), 245 (54), 203 (24)	Proanthocyanidin dimer (A-type)		✓	
34	12.6	863 865 (+)	MS^2 [863]: 712 (40), 711 (100), 693 (45), 559 (30), 573 (18), 451 (20), 411 (31), 289 (9) MS^3 [863 → 711]: 693 (100), 559 (36), 541 (21), 425 (6), 407 (23) MS^2 [865]: 713 (67), 695 (49), 533 (100), 287 (19) MS^3 [865 → 533]: 515 (10), 407 (41), 287 (100)	Proanthocyanidin trimer (A-type)	✓	✓	✓
35	13.0	289	MS^2 [289]: 245 (100), 205 (31), 203 (9), 179 (11)	Epicatechin	✓	✓	✓
36	13.3	575	MS^2 [575]: 499 (100), 490 (95), 451 (83), 423 (43), 289 (80)	Proanthocyanidin dimer (A-type)	✓	✓	
50	17.6	577	MS^2 [577]: 413 (100), 293 (76) MS^3 [577 → 413]: 293 (100)	Rhamnosylvitexin	✓	✓	✓
53	18.2	577	MS^2 [577]: 457 (37), 431 (34), 413 (28), 293 (100)	2"-*O*-rhamnosylvitexin	✓	✓	✓
54	18.6	431	MS^2 [431]: 341 (10), 312 (20), 311 (100) MS^3 [431 → 311]: 284 (99), 283 (100), 268 (39),	Apigenin-8-*C*-hexoside			✓
61	20.9	609	MS^2 [609]: 301 (100) MS^3 [609 → 301]: 271 (21), 179 (92), 151 (100)	Rutin	✓	✓	
62	21.5	577	MS^2 [577]: 559 (23), 451 (9), 425 (100), 407 (54), 289 (28), 287 (14)	(Epi)catechin-(epi)catechin (B-type)	✓	✓	✓
64	23.0	463	MS^2 [463]: 301 (100), 300 (18) MS^3 [463 → 301]: 179 (100), 151 (84)	Quercetin-*O*-hexoside	✓	✓	
65	23.1	575	MS^2 [575]: 449 (45), 423 (100), 407 (7), 289 (6), 287 (10)	Proanthocyanidin dimer (A-type)	✓	✓	✓
72	28.3	593	MS^2 [593]: 285 (100), 163 (56), 151 (20)	Kaempferol-*O*-rutinoside	✓		
74	28.7	447	MS^2 [447]: 285 (43), 284 (100), 257 (11), 255 (39) MS^3 [447 → 285]: 255 (100), 241 (12), 151 (35)	Kaempferol-*O*-hexoside	✓	✓	✓
75	28.7	575	MS^2 [575]: 449 (49), 423 (100), 327 (44), 289 (39), 287 (50)	Proanthocyanidin dimer (A-type)		✓	
78	29.0	477	MS^2 [477]: 315 (100), 314 (91), 285 (22), 271 (16) MS^3 [477 → 315]: 301 (96), 300 (100), 285 (82), 271 (58)	Isorhamnetin-*O*-hexoside		✓	
81	30.2	447	MS^2 [447]: 301 (100) 1 MS^3 [447 → 301]: 271 (24), 179 (100), 151 (94)	Quercetin-*O*-deoxyhesoxide	✓	✓	
82	30.2	417	MS^2 [417]: 327 (22), 285 (42), 284 (100), 255 (12), 227 (7) MS^3 [417 → 285]: 255 (100), 229 (12)	Kaempferol-*O*-pentoside	✓	✓	
83	30.6	861	MS^2 [861]: 735 (100), 693 (28), 575 (61), 571 (98), 539 (45), 449 (29), 447 (18) MS^3 [861 → 735]: 717 (46), 613 (22), 575 (70), 573 (16), 553 (50), 487 (37), 445 (100), 409 (33)	(Epi)catechin-(epi)catechin-(epi)catechin (A-type)			✓
85	31.8	623	MS^2 [623]: 315 (100), 300 (35) MS^3 [623 → 315]: 300 (100), 271 (8) MS^4 [623 → 315 → 300]: 271 (76), 255 (100), 151 (66)	Isorhamnetin-*O*-rutinoside or isorhamnetin-*O*-neohesperidoside	✓	✓	
89	33.1	431	MS^2 [431]: 285 (100), 284 (40), 255 (5), 151 (5) MS^3 [431 → 285]: 285 (100), 257 (50), 255 (89), 241 (15), 229 (46), 151 (11)	Kaempferol-*O*-coumaroyl	✓	✓	✓
95	40.5	327	MS^2 [327]: 291 (53), 229 (78), 211 (30), 171 (100), 165 (26)	Oxo-dihydroxy-octadecenoic acid	✓	✓	✓
96	43.0	329	MS^2 [329]: 311 (29), 293 (45), 229 (28), 211 (55), 171 (100)	Trihydroxyoctadecenoic acid	✓	✓	✓
97	45.7	723	MS^2 [723]: 577 (59), 559 (20), 437 (76), 285 (100) MS^3 [723 → 285]: 285 (100), 257 (13), 255 (16), 241 (38), 151 (72)	Kaempferol derivative		✓	

loss of 132 Da (pentoside), the aglycone (*m/z* 389) suffered several neutral losses of 15 Da (methyl), so it was tentatively characterized as a polymethoxylated flavonoid-*O*-pentoside.

Four kaempferol derivatives were identified, based on the aglycone at *m/z* 285. Compounds **72**, **74**, **84** and **89** suffered neutral losses of 308, 162, 162 + 162, and 146 Da, respectively, and were characterized as kaempferol-*O*-rutinoside, kaempferol-*O*-hexoside, kaempferol-*O*-coumaroylhexoside, and kaempferol-*O*-coumaroyl, respectively.

Table 3
Characterization of the methanol extract of leaves from *A. barbujana* (AB), *O. foetens* (OF) and *P. indica* (PI).

No.	t_R (min)	$[M-H]^-$ *m/z*	*m/z* (% base peak)	Assigned identification	AB	OF	PI
2	3.4	487	MS^2 [487]: 341 (100) MS^3 [487 → 341]: 179 (100), 161 (16), 143 (18), 131 (7), 119 (12), 113 (14), 101 (6) MS^4 [487 → 341 → 179]: 161 (36), 131 (37), 119 (58), 113 (42), 101 (36), 89 (75)	Saccharide	✓	✓	
3	3.5	683	MS^2 [683]: 341 (100) MS^3 [683 → 341]: 179 (100), 161 (20), 131 (5), 119 (35), 113 (17) MS^4 [683 → 341 → 179]: 161 (100), 149 (35), 143 (26), 119 (36), 113 (21), 89 (40)	Saccharide	✓		✓
4	3.6	533	MS^2 [533]: 191 (100), 173 (2) MS^3 [533 → 191]: 173 (14), 171 (23), 127 (100), 125 (12), 93 (68), 85 (53) MS^4 [533 → 191 → 127]: 109 (100)	Quinic acid derivative	✓		✓
5	4.0	191	MS^2 [191]: 173 (54), 127 (100), 111 (76), 93 (35)	Quinic acid	✓	✓	✓
6	4.8	465	MS^2 [465]: 375 (9), 345 (100) MS^3 [465 → 345]: 327 (100), 317 (25), 167 (53) MS^4 [465 → 345 → 327]: 309 (30), 283 (39), 151 (100)	Unknown			✓
9	6.3	465	MS^2 [465]: 375 (8), 345 (100), 327 (12) MS^3 [465 → 345]: 327 (100), 317 (21), 167 (53), 151 (8) MS^4 [465 → 345 → 327]: 283 (30), 201 (19), 175 (61), 165 (28), 151 (100)	Unknown			✓
11	7.1	451	MS^2 [451]: 417 (15), 405 (100) MS^3 [451 → 405]: 241 (100), 224 (60), 179 (39), 161 (9)	Saccharide		✓	
13	7.3	315	MS^2 [315]: 153 (100) MS^3 [315 → 153]: 109 (100)	Dihydroxybenzoic acid-*O*-hexoside	✓		
14	7.7	353	MS^2 [353]: 191 (100), 179 (36), 135 (17) MS^3 [353 → 191]: 173 (96), 127 (100)	3-*O*-caffeoylquinic acid	✓		
16	7.8	451	MS^2 [451]: 405 (100) MS^3 [451 → 405]: 179 (100), 161 (30), 149 (8), 143 (32), 131 (13), 119 (17)	Saccharide (formate adduct)	✓	✓	
17	8.1	577	MS^2 [577]: 451 (32), 425 (100), 407 (89), 289 (25), 287 (11)	(Epi)catechin-(epi)catechin (B-type)			✓
18	8.8	449	MS^2 [449]: 359 (22), 329 (100), 301 (27) MS^3 [449 → 329]: 311 (14), 301 (100), 285 (12), 167 (13) MS^4 [449 → 329 → 301]: 167 (100)	Unknown			✓
19	9.5	289	MS^2 [289]: 245 (100), 205 (48), 203 (16), 179 (21)	Catechin			✓
20	9.8	431	MS^2 [431]: 391 (17), 179 (100), 161 (15), 131 (15) MS^3 [431 → 179]: 161 (100), 149 (57), 143 (16), 113 (35), 107 (82), 89 (44)	Saccharide		✓	
21	9.9	577	MS^2 [577]: 451 (20), 425 (100), 407 (70), 289 (25), 287 (12)	(Epi)catechin-(epi)catechin (B-type)		✓	✓
22	10.1	865	MS^2 [865]: 739 (27), 713 (30), 695 (100), 543 (25), 407 (37), 287 (24) MS^3 [865 → 695]: 677 (33), 543 (100), 525 (50), 451 (53), 391 (28), 289 (14), 243 (51) MS^4 [865 → 695 → 543]: 525 (100), 457 (11), 373 (10)	(Epi)catechin-(epi)catechin-(epi)catechin (B-type)			✓
23	10.3	567	MS^2 [567]: 522 (28), 521 (100) MS^3 [567 → 521]: 389 (100), 293 (12), 233 (17), 227 (12), 161 (57) MS^4 [567 → 521 → 389]: 227 (8), 161 (100), 143 (7)	Saccharide	✓		
24	10.3	557	MS^2 [557]: 522 (21), 521 (100) MS^3 [557 → 521]: 389 (100), 293 (22), 179 (10), 161 (39) MS^4 [557 → 521 → 389]: 161 (99), 131 (73), 129 (21), 115 (100)	Saccharide	✓		
25	10.6	337	MS^2 [337]: 163 (100), 119 (8) MS^3 [337 → 163]: 119 (100)	3-*p*-coumaroylquinic acid	✓		✓
26	10.6	435	MS^2 [435]: 389 (100), 345 (29) MS^3 [435 → 389]: 273 (14), 227 (69), 161 (100), 131 (32) MS^4 [435 → 389 → 161]: 143 (100)	Saccharide	✓		
27	10.7	593	MS^2 [593]: 503 (43), 473 (100), 383 (36), 353 (61) MS^3 [593 → 473]: 353 (100) MS^4 [593 → 473 → 353]: 325 (100), 297 (31)	Vicenin-2 (apigenin-6,8-di-*C*-glucoside)		✓	
28	10.9	431	MS^2 [431]: 225 (31), 179 (100), 161 (14), 143 (22) MS^3 [431 → 179]: 161 (65), 143 (99), 131 (50), 119 (64), 113 (29), 89 (100)	Saccharide	✓	✓	✓
29	11.3	577	MS^2 [577]: 559 (12), 451 (22), 425 (92), 407 (100), 289 (21), 287 (12)	(Epi)catechin-(epi)catechin (B-type)	✓	✓	✓
31	11.6	433	MS^2 [433]: 387 (100), 385 (18) MS^3 [433 → 387]: 223 (100), 205 (72), 161 (43), 153 (71), 143 (10) MS^4 [433 → 387 → 223]: 205 (44), 153 (100)	Dihydro-roseoside (formate adduct)			✓
32	11.9	431	MS^2 [431]: 387 (13), 385 (100), 223 (16) MS^3 [431 → 385]: 223 (41), 205 (78), 161 (25), 153 (100)	Roseoside (formate adduct)			✓
34	12.6	863	MS^2 [863]: 712 (32), 711 (100), 693 (20), 559 (27), 411 (28) MS^3 [863 → 711]: 693 (100), 559 (77), 541 (30), 407 (13) MS^4 [863 → 711 → 693]: 657 (32), 567 (100), 525 (22), 407 (31)	(Epi)catechin-(epi)catechin-(epi)catechin (A-type)		✓	✓
35	13.0	289	MS^2 [289]: 245 (100), 205 (39), 203 (19)	Epicatechin	✓		✓
37	13.5	435	MS^2 [435]: 390 (24), 389 (100) MS^3 [435 → 389]: 161 (100), 159 (10), 143 (6)	Saccharide	✓		

(continued on next page)

Table 3 *(continued)*

No.	t_R (min)	$[M-H]^-$ *m/z*	*m/z* (% base peak)	Assigned identification	AB	OF	PI
			MS^4 [435 → 389 → 161]: 129 (29), 143 (13), 113 (16), 101 (100)				
38	13.5	415	MS^2 [415]: 269 (100), 161 (49) MS^3 [415 → 269]: 161 (100), 143 (21), 115 (46), 113 (59), 101 (69)	Unknown		✓	
39	13.7	559	MS^2 [559]: 514 (32), 513 (100) MS^3 [559 → 513]: 496 (31), 495 (100), 477 (20) MS^4 [559 → 513 → 495]: 478 (12), 477 (100)	Unknown			✓
40	13.8	563	MS^2 [563]: 503 (53), 473 (77), 443 (72), 383 (77), 353 (100) MS^3 [563 → 353]: 353 (100), 325 (72), 299 (11), 297 (37), 267 (37), 163 (20)	Apigenin-*C*-hexoside-*C*-pentoside		✓	
41	14.0	863	MS^2 [863]: 711 (100), 693 (32), 573 (15), 559 (25), 451 (19), 411 (29) MS^3 [863 → 711]: 693 (100), 567 (18), 559 (82), 541 (31), 407 (24) MS^4 [863 → 711 → 693]: 657 (28), 567 (100), 525 (19), 407 (30)	(Epi)catechin-(epi)catechin-(epi)catechin (A-type)	✓		
		865 (+)	MS^2 [865]: 713 (55), 695 (49), 533 (100), 287 (17)				
42	14.7	647	MS^2 [647]: 603 (49), 523 (35), 495 (34), 449 (83), 343 (60), 289 (100) MS^3 [647 → 289]: 245 (100), 205 (94), 203 (28), 179 (50), 125 (79)	(Epi)catechin derivative	✓		
43	15.1	575	MS^2 [575]: 499 (100), 423 (37), 289 (44) MS^3 [577 → 499]: 490 (100), 451 (50), 423 (64), 407 (72), 377 (44), 289 (87) MS^4 [577 → 499 → 490]: 429 (100), 420 (86), 415 (51) MS^4 [577 → 499 → 289]: 287 (58), 245 (100), 205 (29), 203 (15), 125 (28)	Proanthocyanidin dimer	✓		
44	15.1	565	MS^2 [565]: 520 (25), 519 (100) MS^3 [565 → 519]: 505 (27), 504 (100), 487 (37), 387 (70), 233 (11) MS^4 [565 → 519 → 504]: 203 (57), 161 (100)	Unknown		✓	
45	15.3	433	MS^2 [433]: 387 (70), 179 (100), 161 (12), 143 (15) MS^3 [433 → 179]: 161 (28), 149 (35), 143 (100), 119 (23), 113 (43), 89 (46)	Saccharide		✓	
46	15.6	577	MS^2 [577]: 451 (22), 425 (100), 407 (65), 289 (14), 287 (21)	(Epi)catechin-(epi)catechin (B-type)			✓
47	15.8	625	MS^2 [625]: 505 (15), 463 (10), 445 (31), 301 (87), 300 (100), 271 (28) MS^3 [625 → 301]: 300 (100), 271 (58), 255 (52), 179 (59), 151 (53)	Quercetin-*O*-dihexoside	✓		
48	16.4	435	MS^2 [435]: 390 (25), 389 (100) MS^3 [435 → 389]: 225 (18), 161 (100), 159 (12), 143 (11) MS^4 [435 → 389 → 161]: 143 (17), 113 (34), 101 (100), 97 (21)	Saccharide	✓		
49	17.2	433	MS^2 [433]: 343 (21), 313 (100)	Naringenin-*C*-hexoside			✓
51	17.8	551	MS^2 [551]: 419 (100), 401 (46), 389 (25), 233 (22), 203 (17) MS^3 [551 → 419]: 405 (20), 404 (100), 373 (21)	Unknown	✓		
52	18.0	449	MS^2 [449]: 269 (100) MS^3 [449 → 269]: 269 (100), 225 (20), 197 (11), 151 (5)	Apigenin derivative			✓
55	19.0	521	MS^2 [521]: 390 (22), 389 (100), 359 (15) MS^3 [521 → 389]: 375 (18), 374 (100), 359 (12) MS^4 [521 → 389 → 374]: 359 (100), 343 (70), 203 (14), 189 (28)	Polymethoxylated flavonoid-*O*-pentoside	✓		
56	19.3	521	MS^2 [521]: 359 (100) MS^3 [521 → 359]: 344 (100) MS^4 [521 → 359 → 344]: 329 (55), 314 (38), 313 (59), 189 (50), 159 (100)	Polymethoxylated flavonoide-*O*-hexoside			✓
57	19.5	551	MS^2 [551]: 506 (22), 505 (100), 419 (7) MS^3 [551 → 505]: 373 (100), 161 (30) MS^4 [551 → 505 → 373]: 161 (100), 143 (25), 129 (8), 113 (3)	Saccharide	✓		
58	19.8	595	MS^2 [595]: 415 (14), 301 (69), 300 (100), 271 (21), 255 (13) MS^3 [595 → 300]: 271 (100), 255 (60), 179 (43), 151 (47)	Quercetin-hexoside-pentoside	✓		
59	19.9	557	MS^2 [557]: 511 (100) MS^3 [557 → 511]: 493 (100), 475 (33), 295 (10) MS^4 [557 → 511 → 493]: 475 (75), 295 (100), 249 (13)	Unknown			✓
60	20.4	435	MS^2 [435]: 390 (17), 389 (100) MS^3 [435 → 389]: 299 (100), 161 (34), 159 (41) MS^4 [435 → 389 → 299]: 253 (100), 227 (20), 173 (34)	Unknown	✓		
61	20.9	609	MS^2 [609]: 301 (100), 300 (25) MS^3 [609 → 301]: 271 (9), 257 (14), 179 (100), 151 (81)	Rutin	✓		✓
62	21.5	577	MS^2 [577]: 451 (23), 425 (100), 407 (81), 289 (24), 287 (12)	(Epi)catechin-(epi)catechin (B-type)	✓		
63	21.9	553	MS^2 [553]: 508 (30), 507 (100) MS^3 [553 → 507]: 375 (100), 161 (21) MS^4 [553 → 507 → 375]: 161 (100), 159 (13), 143 (3), 113 (4)	Saccharide	✓	✓	
64	23.0	463	MS^2 [463]: 301 (100), 300 (33) MS^3 [463 → 301]: 271 (16), 179 (100), 151 (69)	Quercetin-*O*-hexoside	✓		
65	23.1	575	MS^2 [575]: 449 (25), 423 (100), 289 (13), 285 (19)	Proanthocyanidin dimer	✓		
66	24.5	463	MS^2 [463]: 301 (100), 300 (17) MS^3 [463 → 301]: 179 (94), 151 (100)	Quercetin-*O*-hexoside	✓		✓
67	26.1	463	MS^2 [463]: 301 (100) MS^3 [463 → 301]: 179 (100), 151 (91)	Quercetin-*O*-hexoside	✓		✓
68	26.8	579	MS^2 [579]: 301 (39), 300 (100), 271 (14) MS^3 [579 → 301]: 271 (100), 255 (44), 179 (17), 151 (20)	Quercetin–*xylo*-pentoside	✓		
69	27.2	519	MS^2 [519]: 357 (100)	Pinoresinol-*O*-hexoside			✓

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Table 3 *(continued)*

No.	t_R (min)	$[M-H]^-$ *m/z*	*m/z* (% base peak)	Assigned identification	AB	OF	PI
			MS^3 [519 → 357]: 342 (8), 311 (2), 151 (100), 136 (35)				
70	27.5	473	MS^2 [473]: 428 (27), 427 (100), 293 (28) MS^3 [473 → 427]: 293 (100), 233 (13), 125 (41) MS^4 [473 → 427 → 293]: 233 (100), 161 (10), 149 (15), 131 (6), 113 (23), 99 (28), 89 (20)	Saccharide		✓	
71	27.7	609	MS^2 [609]: 301 (34), 300 (100), 271 (25) MS^3 [609 → 300]: 271 (100), 255 (79), 179 (20), 151 (21)	Quercetin-O-neohesperidoside	✓		
72	28.3	593	MS^2 [593]: 285 (100) MS^3 [593 → 285]: 257 (100), 229 (99), 107 (66)	Kaempferol-*O*-rutinoside	✓		✓
73	28.5	433	MS^2 [433]: 301 (100), 300 (75) MS^3 [433 → 301]: 271 (81), 255 (62), 179 (84), 151 (100)	Quercetin-*O*-pentoside	✓		
74	28.7	447	MS^2 [447]: 327 (14), 285 (92), 284 (100), 255 (22) MS^3 [447 → 284]: 257 (30), 255 (100), 213 (12)	Kaempferol-*O*-hexoside	✓		✓
76	28.8	417	MS^2 [417]: 371 (100) MS^3 [417 → 371]: 209 (10), 161 (100), 113 (15)	Saccharide (formate adduct)			✓
77	28.8	503	MS^2 [503]: 457 (100), 293 (42) MS^3 [503 → 457]: 293 (100), 233 (8) MS^4 [503 → 457 → 293]: 233 (65), 191 (75), 149 (84), 131 (100), 89 (50)	Unknown		✓	
79	29.6	551	MS^2 [551]: 444 (17), 443 (100) MS^3 [551 → 443]: 153 (100), 135 (12) MS^4 [551 → 443 → 153]: 109 (100)	Dihydroxybenzoic acid derivative	✓		
80	30.1	543	MS^2 [543]: 457 (100), 383 (19), 363 (61), 345 (42), 251 (39), 237 (44) MS^3 [543 → 457]: 413 (100), 277 (20), 233 (25) MS^4 [543 → 457 → 413]: 251 (29), 233 (100), 205 (6), 189 (12)	Unknown			✓
81	30.2	447	MS^2 [447]: 301 (100), 300 (18) MS^3 [447 → 301]: 271 (11), 179 (100), 151 (98)	Quercetin-*O*-deoxyhexoside	✓		✓
83	30.6	861	MS^2 [861]: 825 (15), 735 100), 575 (96), 571 (51), 449 (37) MS^3 [861 → 575]: 449 (100), 423 (15), 289 (33)	(Epi)catechin-(epi)catechin-(epi)catechin (A-type)		✓	
84	31.3	593	MS^2 [593]: 431 (12), 413 (66), 285 (100), 255 (30) MS^3 [593 → 285]: 257 (30), 255 (100), 229 (9), 227 (9), 151 (7)	Kaempferol-*O*-coumaroylhexoside	✓		
85	31.8	623	MS^2 [623]: 315 (100), 300 (87), 271 (28) MS^3 [623 → 315]: 300 (100) MS^4 [623 → 315 → 300]: 271 (100), 255 (34), 151 (5)	Isorhamnetin-*O*-rutinoside or isorhamnetin-*O*-neohesperidoside	✓		
86	32.4	475	MS^2 [475]: 460 (75), 313 (100) MS^3 [475 → 313]: 298 (100), 283 (81) MS^4 [475 → 313 → 298]: 283 (100)	Polymethoxylated flavonoid-*O*-hexoside		✓	
87	32.9	375	MS^2 [375]: 241 (100) MS^3 [375 → 241]: 223 (71), 151 (23), 139 (56), 97 (100)	Unknown	✓		
88	33.1	441	MS^2 [441]: 307 (100), 295 (23), 235 (22), 159 (22), 133 (31) MS^3 [441 → 307]: 163 (100)	Unknown		✓	
89	33.1	431	MS^2 [431]: 285 (100), 284 (25) MS^3 [431 → 285]: 257 (47), 255 (100), 229 (29)	Kaempferol-*O*-coumaroyl	✓		✓
90	33.8	461	MS^2 [461]: 315 (67), 314 (100), 300 (30), 299 (84) MS^3 [461 → 315]: 300 (61), 299 (100) MS^4 [461 → 315 → 299]: 271 (100), 255 (20), 151 (10)	Isorhamnetin-*O*-coumaroyl	✓		
91	35.0	375	MS^2 [375]: 241 (100), 139 (20) MS^3 [375 → 241]: 223 (49), 151 (15), 139 (48), 97 (100) MS^4 [375 → 241 → 223]: 205 (36), 193 (77), 141 (36), 125 (68), 113 (100)	Unknown		✓	
92	35.5	607	MS^2 [607]: 299 (100), 284 (44), 255 (22) MS^3 [607 → 299]: 284 (100) MS^4 [607 → 299 → 284]: 255 (100), 227 (6), 151 (2)	Diosmin	✓		
93	38.4	445	MS^2 [445]: 299 (45), 298 (100), 283 (47) MS^3 [445 → 298]: 284 (25), 283 (100), 255 (83) MS^4 [445 → 298 → 283]: 255 (100)	Kaempferide-*O*-coumaroyl	✓		
94	39.8	601	MS^2 [601]: 555 (100), 393 (64) MS^3 [601 → 555]: 393 (100) MS^4 [601 → 555 → 393]: 209 (18), 183 (100), 139 (10)	Unknown		✓	
95	40.5	327	MS^2 [327]: 291 (18), 229 (11), 211 (6), 171 (100)	Oxo-dihydroxy-octadecenoic acid	✓	✓	✓
96	43.0	329	MS^2 [329]: 327 (16), 311 (15), 309 (19), 229 (26), 211 (20), 171 (100)	Trihydroxyoctadecenoic acid	✓	✓	✓

Compound **90** suffered a neutral losses of 146 Da; therefore it was characterized as isorhamnetin-*O*-coumaroyl.

Compound **93** exhibited the deprotonated molecular ion at *m/z* 445. It suffered the neutral loss of 146 Da to yield the aglycone at *m/z* 299. The aglycone was characterized as kaempferide (Engels et al., 2012). Considering the high retention time, it was assigned to kaempferide-*O*-coumaroyl.

3.1.3.3 O. foetens. Only three flavonoid glycosides were characterized in these extracts. Compound **27**, with $[M-H]^-$ at *m/z* 593, was identified as vicenin-2 (Llorent-Martínez et al., 2015a). Compound **40** exhibited the deprotonated molecular ion at *m/z* 563, and presented an MS^2 fragmentation pattern typical of asymmetrical di-C-glycosides, with fragment ions at $[M-H-210]^-$, $[M-H-90]^-$ and $[M-H-60]^-$; it was characterized as apigenin-*C*-pentoside-*C*-hexoside (Llorent-Martínez et al., 2015b). Finally, compound **86** presented $[M-H]^-$ at *m/z* 475 and, after the loss of 162 Da, displayed the aglycone at *m/z* 313, which suffered several losses of

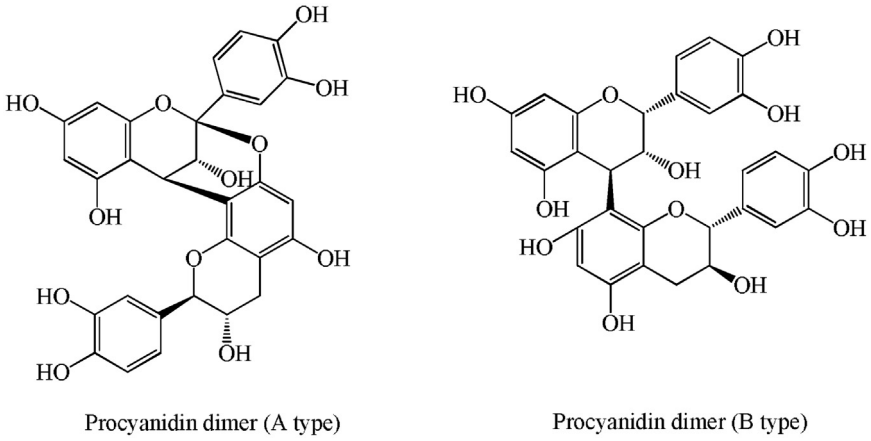

Fig. 3. Procyanidin dimers: A-type and B-type.

Table 4
Quantification of phenolic compounds in leaves of the analyzed plant species (mg g^{-1} DE).

N°	Assigned identification	*L. novocanariensis*			*A. barbujana*	*O. foentes*	*P. indica*
		L1	L2	L3			
Phenolic acids							
12	Dihydroxybenzoic acid-*O*-hexoside	0.52 ± 0.02	0.56 ± 0.04				
25	3-*p*-Coumaroylquinic acid						5.60 ± 0.09
Total		**0.52 ± 0.02^{a}**	**0.56 ± 0.04^{a}**				**5.60 ± 0.09^{b}**
Flavonols							
47	Quercetin-*O*-dihexoside				0.52 ± 0.13		
58	Quercetin-hexoside-pentoside				0.45 ± 0.03		
61	Rutin						0.76 ± 0.01
64	Quercetin-*O*-hexoside				1.83 ± 0.01		
66	Quercetin-*O*-hexoside				1.97 ± 0.07^{b}		0.40 ± 0.01^{a}
67	Quercetin-*O*-hexoside				1.81 ± 0.01^{b}		0.75 ± 0.01^{a}
73	Quercetin-*O*-pentoside				1.82 ± 0.12		
74	Kaempferol-*O*-hexoside	1.19 ± 0.10					
81	Quercetin-*O*-deoxyhesoxide	1.50 ± 0.06^{b}	0.87 ± 0.01^{a}		3.69 ± 0.21^{d}		2.14 ± 0.01^{c}
82	Kaempferol-*O*-pentoside		0.40 ± 0.02				
84	Kaempferol-*O*-coumaroylhexoside				0.61 ± 0.03		
85	Isorhamnetin-*O*-rutinoside				0.30 ± 0.01		
89	Kaempferol-*O*-coumaroyl		0.83 ± 0.04^{a}		1.27 ± 0.01^{b}		3.31 ± 0.04^{c}
97	Kaempferol derivative		10.33 ± 0.50				
Total		**2.69 ± 0.16^{a}**	**12.43 ± 0.58^{c}**		**14.29 ± 0.62^{d}**		**7.36 ± 0.01^{b}**
Flavanols							
15	Proanthocyanidin dimer	4.31 ± 0.21^{b}	4.43 ± 0.21^{b}	0.62 ± 0.01^{a}			
17	(Epi)catechin-(epi)catechin (B-type)						8.91 ± 0.38
21	(Epi)catechin-(epi)catechin (B-type)	4.04 ± 0.05^{c}	3.91 ± 0.10^{c}	1.23 ± 0.07^{b}			0.76 ± 0.04^{a}
29	(Epi)catechin-(epi)catechin (B-type)				4.66 ± 0.06		
34	A-type proanthocyanidin trimer	48.80 ± 0.85^{d}	34.13 ± 1.38^{b}	40.11 ± 0.30^{c}		17.45 ± 0.47^{a}	
35	Epicatechin	5.23 ± 0.49^{b}	7.00 ± 0.34^{c}	0.10 ± 0.06^{a}	5.20 ± 0.18^{b}		
36	Proanthocyanidin dimer		1.08 ± 0.06				
41	(Epi)catechin-(epi)catechin-(epi)catechin (A-type)				62.66 ± 1.81		
43	Proanthocyanidin dimer				1.56 ± 0.05		
65	Proanthocyanidin dimer	2.21 ± 0.09^{c}	1.25 ± 0.01^{a}	2.02 ± 0.09^{b}			
83	(Epi)catechin-(epi)catechin-(epi)catechin (A-type)			3.45 ± 0.01^{b}		0.90 ± 0.05^{a}	
89	Kaempferol-*O*-coumaroyl		0.83 ± 0.04^{a}		1.27 ± 0.01^{b}		3.31 ± 0.04^{c}
Total		**64.59 ± 1.70^{d}**	**51.80 ± 2.01^{c}**	**48.45 ± 0.54^{c}**	**74.09 ± 2.10^{e}**	**18.35 ± 0.52^{b}**	**9.67 ± 0.33^{a}**
Flavones							
27	Vicenin-2					0.09 ± 0.01	
50	Rhamnosylvitexin		0.13 ± 0.01				
52	Apigenin derivative						0.15 ± 0.01
57	2“-*O*-rhamnosylvitexin	0.23 ± 0.02^{a}	0.48 ± 0.01^{b}	0.22 ± 0.01^{a}			
92	Diosmin				0.60 ± 0.04		
Total		**0.23 ± 0.02^{b}**	**0.58 ± 0.02^{c}**	**0.22 ± 0.01^{b}**	**0.60 ± 0.04^{c}**	**0.09 ± 0.01^{a}**	**0.15 ± 0.01^{a}**
Flavanone							
49	Naringenin-*C*-hexoside						2.28 ± 0.06
Total							**2.28 ± 0.06**
TIPC		**68.02 ± 1.90^{d}**	**65.37 ± 2.73^{d}**	**48.67 ± 0.55^{c}**	**88.99 ± 2.76^{e}**	**18.44 ± 0.52^{a}**	**25.06 ± 0.57^{b}**
Yield (%)		**4.38**	**7.96**	**4.53**	**4.62**	**4.48**	**9.84**

Means in the same line not sharing the same letter are significantly different at $p < 0.05$ probability level.

15 Da, so it was tentatively characterized as the hexoside of a polymethoxylated flavonoid.

3.1.3.4 P. indica. Compounds **31** and **32** were characterized as the formic adducts of dihydro-roseoside and roseoside (drovomifoliol-*O*-glucoside), respectively, based on bibliographic information (Spínola and Castilho, 2016).

Compound **49**, with $[M-H]^-$ at *m/z* 433, and fragment ions at $[M-H-90]^-$ and $[M-H-120]^-$, was tentatively characterized as naringenin-*C*-hexoside (Vallverdú-Queralt et al., 2011).

Compound **52** exhibited MS^3 fragment ions at *m/z* 269 and 225, typical of apigenin, so it was tentatively characterized as a derivative.

Compounds **66**, **67** and **81** were quercetin glycosides (aglycone at *m/z* 301). **66** and **67** were quercetin-*O*-hexoside isomers, whereas **81** corresponded to quercetin-*O*-deoxyhexoside.

Compound **69** displayed an $[M-H]^-$ ion at *m/z* 519. It suffered the neutral loss of 162 Da. The aglycone, at *m/z* 357, presented fragment ions at *m/z* 342, 311, 151 and 136, typical from pinoresinol, so it was characterized as its *O*-hexoside (Ye et al., 2005).

3.1.4. Other compounds

A high number of compounds (Tables 2 and 3) presented MS^n fragment ions at *m/z* 131, 119, 113 and 89, typical from saccharides (Brudzynski and Miotto, 2011; Verardo et al., 2009), so they were tentatively characterized as saccharide derivatives. These compounds were observed in all the analyzed plants.

Compound **5**, $[M-H]^-$ at *m/z* 191, exhibited MS^2 fragment ions at *m/z* 173 and 111, typical from quinic acid (Llorent-Martínez et al., 2015a).

Compound **7** displayed $[M-H]^-$ at *m/z* 315 and two fragment ions at *m/z* 153 and 123, consistent with hydroxytyrosol hexoside (Savarese et al., 2007)

Compound **12** exhibited $[M-H]^-$ at *m/z* 315, and MS^n fragment ions at *m/z* 153 and 109, which has been previously mentioned as typical from a dihydroxybenzoic acid-O-hexoside (Han et al., 2008).

Compound **13**, detected in *A. barbujana*, exhibited $[M-H]^-$ at *m/z* 315, and MS^n fragment ions at *m/z* 153 and 109, previously mentioned as typical of dihydroxybenzoic acid-*O*-hexoside (Han et al., 2008).

Compound **25**, in *P. indica*, exhibited the deprotonated molecular ion at *m/z* 337, and MS^2 base peak ion at *m/z* 163, so it was characterized as 3-*p*-coumaroylquinic acid (Clifford et al., 2003).

Compounds **95** and **96**, detected in all plants, were tentatively characterized as oxo-dihydroxy-octadecenoic and trihydroxy-octadecenoic acids, respectively, considering bibliographic data (Van Hoyweghen et al., 2014).

3.2. Quantification of individual polyphenols

In the present study, 34 polyphenols were quantified by HPLC-DAD (Table 4) using the corresponding standards for calibration for each group. Only the most abundant compounds were identified, since the overlapping of some compounds or their low levels did not allow to carry out an accurate quantification.

The most relevant arboreal species in Laurisilva forest are *L. novocanariensis*, *O. foetens*, *A. barbujana* and *P. indica* from the Lauraceae family, which gives name to the forest, and *Olea europaea*, *Ilex perado*, *Clethra arborea*, *Heberdenia excelsa*, *Juniperus cedrus*, *Ilex canariensis* and *Myrica faya*, from other families. In previous works, our research group characterized the phenolic fractions of several of these non-lauraceae species (Llorent-Martínez et al., 2015a; Spínola et al., 2014). Hence, comparison between several species of the Laurisilva forest will be performed.

In this work, intra and inter-species differences were observed for TIPC ($p < 0.05$). *A. barbujana* presented the higher phenolic contents and *O. foetens* the lowest (88.99 and 18.43 mg g^{-1} DE, respectively) (Table 4). This trend is in agreement with a previous study (Tavares et al., 2010), but no individual quantitative data was shown before. Significant differences ($p < 0.05$) were found in TIPC of *Laurus* collected in different locations of Madeira Island (48.67–68.02 mg g^{-1} DE). Additionally, variations were also observed between Laurus samples for HCAs, flavonols, flavanols and flavones contents (Table 4). The lower phenolic amounts found in Ponta do Pargo samples (L3) may be related to different soil and climatic conditions, since these plants grown in the southside of the Island.

In non-lauraceae species, *O. europaea* profile was dominated by secoiridoids, mainly oleuropein derivatives; *I. perado* showed HCA esters and saponins as main components; *H. excelsa* was rich in flavonols and isoflavonols; and *C. arborea* contained several B-type procyanidin dimers and trimers, and flavonol derivatives as well (Llorent-Martínez et al., 2015a). In addition, *M. faya* presented mainly flavanols and flavonols (Spínola et al., 2014). Quantitative data in the present work indicated that flavanols (79.24–99.55%) were the most representative class of polyphenols on *Laurus* samples, followed by flavonols (0–19.01%), flavones and HCAs. Flavonoids were dominant in *A. barbujana*: flavanols (83.26%) > flavonols (16.05%) > flavones (0.68%). Flavanols (99.51%) and flavones (0.48%) composed the polyphenolic profile of *O. foetens*. *P. indica* showed the most diverse phytochemical composition among analyzed plants: flavanols (38.58%) > flavonols (29.38%) > HCAs (22.34%) > flavanones (2.28%) > flavones (0.58%).

A study conducted on *P. americana* leaves (Uysal et al., 2016) showed that chlorogenic acid was the main component (15.78–18.93 mg g^{-1} DE), which was absent in the analyzed *P. indica* samples. Regarding other analyzed *Lauraceae* species, no data in literature were found about their phenolic compositions. *M. faya* leaves yielded a total of 178 mg/100 g dry weight (DW) of flavanols, mainly gallocatechin derivatives, but the main components were flavonols (1204 mg/100 g DW) (Spínola et al., 2014).

The most important discovery in all the analyzed extracts was the presence of PAs. In fact, PAs represented at least 80% of the phenolic profile in *L. novocanariensis* and *A. barbujana*. An A-type PA trimer (compound **34**) was the most abundant phenolic in *L. novocanariensis* and *O. foetens* (17.45–48.80 mg g^{-1} DE). (Epi)catechin-(epi)catechin-(epi)catechin (A-type) (compound **41**) was major in *A. barbujana* and (epi)catechin-(epi)catechin (B-type) (compound **17**) in *P. indica* (62.66 and 8.91 mg g^{-1} DE, respectively).

Similarly to the present work, A-type PAs were also found as major phenolics in three *Laurus* species (including *L. novocanariensis*) (Vinha et al., 2015). By contrast, the same authors reported that PA dimers were more abundant than trimers. In general, the analyzed *Laurus* samples presented superior individual and total phenolic amounts than those studied by Vinha and co-workers (7.58–33.24 mg g^{-1} DE) (Vinha et al., 2015). These differences may be related to differences in the collection area, sample preparation, time of the year, and even the sexual dimorphism of *Laurus*.

PAs are compounds of high interest due to their anti-infectious, anti-inflammatory, cardioprotective and anticarcinogenic properties proved in clinical and laboratory studies (Nandakumar et al., 2008). These properties depend on the distribution of oligomers and polymers and on the type of proanthocyanidin: A or B depending on the number of bonds between catechin units and two and one linkage, respectively (Tsao, 2010).

PAs are found in plant material in a wide range of amounts (Kardel et al., 2013; Ropiak et al., 2016). However, only a few − such as plums, avocados, peanuts, cinnamon, and cranberries − contain A-type procyanidins. The major sources are some berries (blueberries, cranberries, and black currant) and plums (prunes), with a content of about 200 mg/100 g (fresh weight); cinnamon bark, which can reach > 2000 mg/100 g (dry weight) (Gu et al., 2004) and maritime pine (*Pinus pinaster*) bark (Maimoona et al., 2011). Cinnamon (another Lauraceae) bark has been widely recognized for its different health benefits, mainly associated to its PAs (A-type) content (Mateos-Martín et al., 2012; Peng

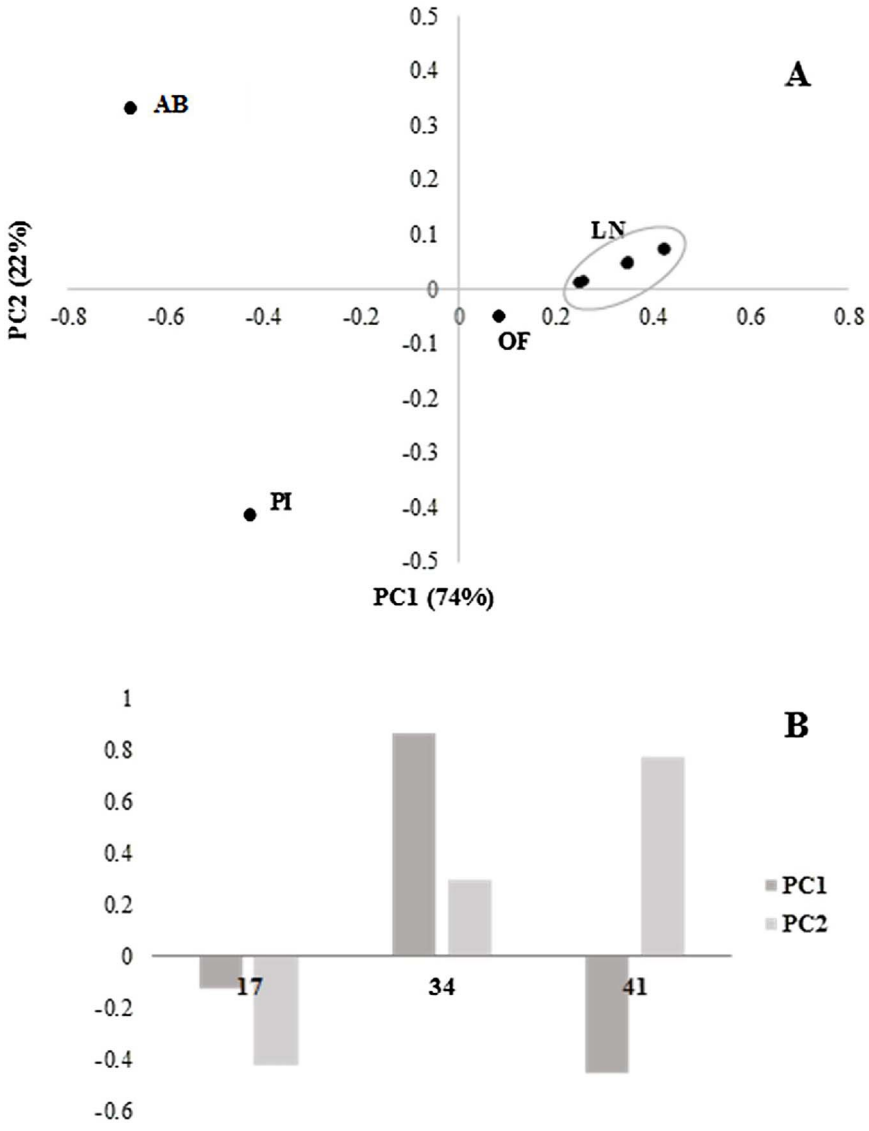

Fig. 4. (A) PC1 × PC2 of scores scatter plot between different Lauraceae plant species; (B) PC1 × PC2 of loading plot of the main source of variability between different Lauraceae plant species. AB: *A. barbujana*; PI: *P. indica*; LN: *L. novocanarienses*; OF: *O. Foetens*.

et al., 2010; Rao and Gan, 2014; Sun et al., 2016), although the coumarin contained in cinnamon is known to be hepatotoxic (Iwata et al., 2016), hence the importance of finding new sources of PAs.

The A-type variety is more active tan B-type in reduction of cell proliferation, increase of apoptosis, cell cycle arrest and modulation of the expression and activity of NF-kB and NF-kB target genes. For instance, A-type procyanidins exhibit, *in vitro*, a capacity of inhibition of P-fimbriated *Escherichia coli* adhesion to uroepithelial cells greater than B-type procyanidins. In fact, different extracts containing only B-type procyanidins had no anti-adhesion activity (Howell et al., 2005).

3.3. Principal component analysis

PCA statistical tool was applied to the concentrations of 34 polyphenols determined by HPLC-DAD to establish a relationship between targeted plant species. The PCA score scatter plot of the two first principal components (which explains 96% of the total variability) is shown in Fig. 4.

The loadings of each compound (variable) that contribute to explain the differentiation between plant species is shown in Fig. 4B. PC1, that explained 74% of the total variability, shows *Lauraceae* species discrimination based on phenolic profile, where *L. novocanariensis* (LN) and *O. foetens* (OF) samples are projected in PC1 positive, *A. barbujana* (AB) is above the positive PC2 axis and *P. indica* (PI) in PC2 negative. Taking into account the loading plot (Fig. 4B), the compounds responsible for these results were proanthocyanidins: (Epi)catechin-(epi)catechin (B-type) (**17**), A-type proanthocyanidin trimer (**34**) and (Epi)catechin-(epi)catechin-(epi)catechin (A-type) (**41**).

4. Conclusions

A report on the phenolic composition of leaves of the most important Lauraceae plants of the Laurisilva forest, part of UNESCO patrimony, is presented; the most important compounds detected in the analyzed extracts were characterized and quantified.

It was observed that flavonoids, glycosides and proanthocyanidins (A- and B- type), were the most abundant compounds. In particular, the highest amounts of these compounds were observed in *L. novocanariensis* and *A. barbujana*, which present amounts of A-type PA trimers similar to those found in commercial sources. It has been reported that PA trimers are mainly responsible for the activity of cinnamon extracts (Sun et al., 2016). Therefore, an interesting direction for upcoming research will be the isolation and NMR identification of these trimers, and the study of their individual biological properties as well. Con-

sidering the high number of health benefits that the identified compounds present, these species could be considered as potential novel sources of these compounds in the pharmaceutical industry. Hence, further research regarding antioxidant and toxicity assays will be performed in our group to improve the knowledge of these species.

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