Industrial Crops and Products 64 (2015) 135–151

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Analysis of phenolic compounds in leaves from endemic trees from Madeira Island. A contribution to the chemotaxonomy of Laurisilva forest species

Eulogio J. Llorent-Martínez[a,b], Sandra Gouveia[a], Paula C. Castilho[a,*]

[a] Centro de Química da Madeira (CQM), Centro de Ciências Exactas e da Engenharia da Universidade da Madeira, Campus Universitário da Penteada, 9000-390 Funchal, Portugal
[b] Department of Physical and Analytical Chemistry, University of Jaén, Campus Las Lagunillas S/N, E-23071 Jaén, Spain

ARTICLE INFO

Article history:
Received 24 July 2014
Received in revised form 29 October 2014
Accepted 30 October 2014
Available online 15 November 2014

Keywords:
Macaronesia
Tandem mass spectrometry
Flavonoid
Procyanidin
Endemic species

ABSTRACT

Phenolic compounds present high antioxidant activity and, therefore, health promoting effects, serving as a type of preventive medicine. Hence, research on the chemical composition of plants with potential antioxidant value is of high interest.

Forest cleaning, thinning, and pruning are beneficial activities that help maintaining healthy forests. In addition, they can provide vegetal material as source of valuable bioactive compounds that can have health promoting effects. In this work, the phenolic composition of several trees native to Madeira Archipelago (Portugal) was studied. Specifically, the leaves from *Olea europaea* ssp. *cerasiformis*, *Ilex perado* ssp. *perado*, *Clethra arborea*, and *Heberdenia excelsa* have been analyzed. The screening of the main phenolic compounds from their methanolic extracts has been carried out using high-performance liquid chromatography with electrospray ionization mass spectrometric detection (HPLC-ESI-MS^n). This is the first report on the phenolic composition of these Madeira native species, and more than 100 compounds have been detected and identified or tentatively characterized.

1. Introduction

Phenolic compounds, secondary metabolites synthesized by the plants, present high antioxidant activity and, therefore, important health benefits, such as protection against cancer, and cardiovascular and neurodegenerative diseases (Gouveia and Castilho, 2009; Ignat et al., 2011). Hence, many studies are being performed to characterize phenolic compounds from natural sources. For this purpose, liquid chromatography coupled to tandem mass spectrometry has proved to be a very powerful tool.

The Madeira 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 its chemistry remains unexplored, even though several species have been used for centuries in the preparation of folk remedies (Rivera, 1995). In this work, the most important non-lauraceae trees of the Laurisilva forest (*Olea europaea* ssp. *cerasiformis*, *Ilex perado* ssp. *perado*, *Clethra arborea*, and *Heberdenia excelsa*) have been selected, and their phenolic composition studied.

The olive tree (*Olea europaea* L., Oleaceae) is a fruit crop of high economic importance. Six subspecies of *O. europaea* have been described. Of them, ssp. *cerasiformis* Webb and Berth. ex Kunkel and Sunding (previously named *O. europaea* L. ssp. *maderensis* Lowe) is native to the Madeira Archipelago (Brito et al., 2008). It is locally known as "Oliveira brava" or "zambujeiro" and grows widely at altitudes between 0 and 200 m. Unlike the *O. europaea* cultivar developed for fruit and oil production, the fruits of the endemic wild species are inedible, but infusions of its leaves are used as an antihypertensive. Although previous studies have been carried out regarding the phenolic composition of *O. europaea* (Fu et al., 2010; Quirantes-Piné et al., 2013; Savarese et al., 2007), this is the first study for the Madeira native species.

The genus *Ilex* L. (Aquifoliaceae) includes over 400 species of trees and shrubs. *I. perado* Aiton, a complex of four subspecies distributed in different Macaronesian archipelagos, is represented in Madeira by *I. perado* ssp. *perado* Aiton (Sosa et al., 2013). In the *Ilex* genus, the phenolic composition of *I. paraguensis* has been previously reported (Bastos et al., 2007; Dartora et al., 2011; Peres et al.,

* Corresponding author. Tel.: +351 291705102.
E-mail address: castilho@uma.pt (P.C. Castilho).

http://dx.doi.org/10.1016/j.indcrop.2014.10.068
0926-6690/

2013), but no data are available regarding ssp. *perado*. It is known as Madeira Holy or "azevinho".

Clethra arborea Aiton, also known as the lily-of-the-valley-tree or "folhado", is a flowering plant in the genus *Clethra*. It is native to Madeira, extinct in the Canary Islands (Spain), and an invasive species in the Azores (Portugal). To our best knowledge, the phenolic composition of the *Clethra* genus has not been studied to date.

Heberdenia excelsa Aiton (*Ardisia excelsa* A.) is an uncommon species of Laurisilva, native to Madeira and to the Canary Islands, and known locally as "aderno". It is currently included in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN, 2013). The phenolic composition of plants in the *Heberdenia* genus has been scarcely studied to date (de Mejía et al., 2006), the report presented here being the first one for the ssp. *excelsa*.

Being protected as part of UNESCO natural patrimony since 2000, every year the Laurisilva forest must be cleaned and thinned to prevent fire spread and to improve the growth of healthy trees. Presently, the felled specimens and cut branches are discarded or treated as biomass residue without further valorization. The present work is part of a prospective project that aims at finding uses for discarded material for forest valorization.

2. Experimental

2.1. Chemicals and reagents

HPLC grade acetonitrile (CH_3CN) (99%; LabScan; Dublin, Ireland), ultrapure water (Milli-Q Waters purification system; Millipore; Milford, MA, USA), and formic acid (analytical reagent grade; Sigma–Aldrich; St. Louis, MO, USA), were used in the LC-MS analysis. The methanol used for the extraction procedures, of analytical reagent grade, was obtained from Fisher (Lisbon, Portugal). Eluents LC-MS analysis were also filtered through 0.45 μm Nylon membranes (Millipore; Merck; Darmstadt, Germany). Charcoal activated powder was purchased from Sigma–Aldrich.

Quercetin (>99%) was obtained from ExtraSynthese (Lyon, France). Kaempferol (>99%) and 5-O-caffeoylquinic acid (99%) were purchased from Acros Organics (Geel, Belgium). 3,4-O-dicaffeoylquinic acid, 3,5-O-dicaffeoylquinic acid and 4,5-O-dicaffeoylquinic acid were purchased from Chengdo biopurity phytochemicals (Ltd China, Sichuan, China). Rutin (>94%) was obtained from Sigma–Aldrich.

2.2. Sample preparation and extraction of phenolic compounds

Samples of *Clethra arborea*, *Heberdenia excelsa*, *Ilex perado*, and *Olea europaea* were collected in the wild in Madeira Island, in June 2012, with the help of Professor Miguel Menezes de Sequeira from the Biology Department of Madeira University. Vouchers were deposited in the Madeira Botanical Garden Herbarium collection. Specimen collection was performed at full maturity of leaves, in a protected forest area, of restricted human access and free of contamination of introduced species. The leaves were de-stemmed, lyophilized to dryness (Savant vapour trap RVT400; Thermo Scientific Inc.; Waltham, MA, USA), ground to powder, and stored at −20 °C until analysis.

The phenolic compounds were extracted by ultrasound-assisted extraction. Using a sonicator Bandelin Sonorex (Germany), 1 g of plant material was extracted with 25 mL of methanol (room temperature) at 35 Hz and 200 W for 60 min. Then, chlorophylls 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 extracts were stored at −20 °C until use. For HPLC analysis, the extracts were dissolved in the initial HPLC mobile phase, to obtain solutions of 5 mg mL^{-1} concentrations.

2.3. Chromatographic conditions

The HPLC analysis was carried out 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 achieved on 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).

For HPLC-ESI-MS^n analysis, a Bruker Esquire model 6000 ion trap mass spectrometer (Bremen, Germany) with an ESI source was used. MS^n analysis was performed in negative and positive mode and scan range was set at *m/z* 100–1000 with speed of 13,000 Da/s. The conditions of ESI 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 with the auto MS^n mode, selecting an isolation width of 4.0 *m/z*, and a fragmentation amplitude of 1.0 V (MS^n up to MS^4). Samples were filtered through 0.45 μm PTFE membrane filters, and 10 μL were injected.

3. Results and discussion

For the analysis of the phenolic composition by HPLC-ESI-MS^n, both the positive and negative ionization modes were used. Practically all the information was obtained using the negative mode, and the positive mode was mainly used for confirmation purposes and for the screening of anthocyanidins. The base peak chromatograms of the methanolic extracts of each plant are shown in Figs. 1 and 2.

In general, in the negative ionization mode (ESI^-) MS^1 spectrum, the most intense peak corresponded to the deprotonated molecular ion $[M-H]^-$, allowing MS^n analysis. Losses of sugar moieties like hexosyl, deoxyhexosyl, pentosyl, rutinosyl, and glucuronyl (−162, −146, −132, −308, and −176 Da, respectively) were observed in conjugated phenolic compounds.

Compounds were numbered by their order of elution, maintaining the same numeration in all the samples. The structures of the most relevant compounds identified are shown in Figs. 3 and 4.

3.1. Olea europaea ssp. cerasiformis

The results obtained in the analysis of leaves extracts from *Olea europaea* are shown in Table 1 (ESI^-). Most of the compounds, as previously reported in scientific literature for other *Olea* subspecies, were secoiridoids and flavonoids (Fu et al., 2010; Quirantes-Piné et al., 2013).

3.1.1. Secoiridoids

Olea europaea L. was rich in oleosides, which are oleaceae-specific secoiridoids usually esterified to a phenolic moiety (Quirantes-Piné et al., 2013). Oleuropein (compound **61**) was the most abundant compound, which is in agreement with scientific bibliography (Altiok et al., 2008; Benavente-García et al., 2000; Briante et al., 2002; De Nino et al., 1997; Fu et al., 2010; Mylonaki et al., 2008; Pereira et al., 2007; Quirantes-Piné et al., 2013). The identification of oleuropein was based on its $[M-H]^-$ at *m/z* 539, and its characteristic fragmentation pattern (Bianco et al., 2001; Fu et al., 2010), with fragment ions at *m/z* 377, 307, and 275.