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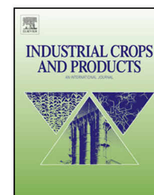

Screening of lipophilic and phenolic extractives from different morphological parts of *Halimione portulacoides*

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ABSTRACT

The chemical composition of the lipophilic and phenolic extractives of the leaves, stems and roots of the salt marsh plant *Halimione portulacoides* from the Aveiro Lagoon was thoroughly investigated by gas chromatography–mass spectrometry (GC–MS) and ultra-high-performance liquid chromatography–mass spectrometry (UHPLC–MS), respectively. The lipophilic fraction of leaves and stems is mainly composed of long chain aliphatic acids and alcohols (both in the C16–C30 range) and smaller amounts of sterols, such as schottenol, β-sitosterol and β-sitostanol. The major component of roots extract is a triterpenic ketone, hop-17(21)-en-3-one, accounting for 2.8 g kg^{-1} of dry material. Furthermore, thirteen phenolic compounds were firstly reported as constituents of this halophytic shrub. Among the studied plant tissues, leaves are the richest in phenolic compounds with 4.6 g kg^{-1} of dry material, most of which correspond to sulfated flavonoids (3.1 g kg^{-1} of dry material), particularly derivatives of isorhamnetin-sulfate.

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1. Introduction

Halimione portulacoides (L.) Aellen, a low perennial shrub of the Chenopodiaceae family and Caryophyllales order, is an abundant species in Mediterranean salt marshes (Waisel, 1972), particularly in those located nearby Aveiro, Portugal (Mendonça et al., 2004; Micaelo et al., 2003). The Aveiro Lagoon, a shallow coastal lagoon on the northwest Atlantic coast of Portugal characterized by narrow channels and significant intertidal zones, is a very important area due to its economic, social and environmental importance (Dias et al., 1999). Nevertheless, the salt marshes of this lagoon became the repositories of industrial effluents from a chlor-alkali plant between the 1950s and the 1990s, turning this coastal area into one of the most mercury-contaminated in Portugal (Pereira et al., 2009; Válega et al., 2009).

The presence of *H. portulacoides* in salt marshes has been the subject of several studies (Anjum et al., 2011; Dias et al., 1999; Mendonca et al., 2004; Micaelo et al., 2003; Pereira et al., 2009; Válega et al., 2009, 2008) some of which bear out the importance of this halophytic shrub at an environmental level given its potential as a bioindicator of metal contamination (Válega et al., 2009, 2008). Thus, the cultivation of *H. portulacoides* plants in sediments featuring extremely high concentrations of metals could help in bioremediation of soils contaminated with toxic metals. In the salt marshes of Aveiro lagoon, *H. portulacoides* has demonstrated its ability to strongly influence the concentration of mercury (Hg) in the sediments colonized by this species (Anjum et al., 2011; Válega et al., 2008). In fact, the Hg concentrations were found to be an order of magnitude higher than those found in non-colonized sediments (Micaelo et al., 2003).

In view of the importance of *H. portulacoides* at an environmental level and also the growing interest in plant-derived bioactive compounds, it is crucial to deepen the insight into the chemical composition of this halophytic shrub. Actually, to the best of our knowledge, only the presence of long chain chloroalkanes in leaf waxes (Grossi and Raphel, 2003), and volatile and semi-volatile organic compounds in the root exudates (Oliveira et al., 2012) have been reported. In this context, and following our interest in bioactive phytochemical compounds from vegetable biomass (Freire et al., 2006, 2005, 2004; Oliveira et al., 2005; Perestrelo et al., 2012; Santos et al., 2013a,b, 2012; Vilela et al., 2013), in the present paper we report the detailed characterization of the lipophilic and phenolic fractions of the leaves, stems and roots of *H. portulacoides* by gas chromatography–mass spectrometry (GC–MS) and

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ultra-high-performance liquid chromatography-mass spectrometry (UHPLC–MS) analysis, respectively.

2. Materials and methods

2.1. Chemicals

Dichloromethane (99% purity), *N,O*-bis(trimethylsilyl)-trifluoroacetamide (99% purity), trimethylchlorosilane (99% purity), tetracosane (99% purity), pyridine (99% purity) and Folin–Ciocalteu's phenol reagent were supplied by Sigma-Aldrich. (Madrid, Spain). Hexadecanoic acid (97% purity), β-sitosterol (99% purity), isorhamnetin (purity higher than 99%) and luteolin (purity higher than 97%), formic acid (purity higher than 98%) and methanol (purity higher than 99.8%) were purchased from Fluka Chemie (Madrid, Spain). HPLC-grade methanol, water, and acetonitrile were supplied by Fisher Scientific Chemicals (Loures, Portugal) and further filtered using a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, PA, USA).

2.2. Samples preparation

Samples of *Halimione portulacoides* (L.) Aellen were collected in the salt marshes nearby Aveiro, Portugal, in July 2012. Samples were air dried and handle separated into leaves, stems and roots; each fraction was then ground and sieved and the granulometric fraction of 40–60 mesh was used for analysis.

2.3. Extraction

Three powdered aliquots (20 g) of each fraction (leaves, stems and roots) of *H. portulacoides* were Soxhlet extracted during 10 h with dichloromethane, a fairly specific solvent for lipophilic extractives isolation for analytical purposes (Freire et al., 2004). The solvent was evaporated to dryness and the corresponding extracts weighed. Subsequently, the residues of each fraction were suspended (m/v 1:100) in a methanol/water (MeOH/H_2O, 50:50, v/v) mixture, at room temperature for 24 h under constant stirring (Santos et al., 2012, 2013a,b). The suspension was then filtered, MeOH removed by reduced pressure evaporation, and the extracts freeze-dried. The extraction yields were expressed in percent of dry vegetal material.

2.4. GC–MS analysis

Before GC–MS analysis of the dichloromethane extracts, approximately 20 mg of each dried extract were trimethylsilylated as follows: the residue was dissolved in 250 μL of pyridine and compounds containing carboxyl and hydroxyl groups were converted into trimethylsilyl (TMS) esters and ethers, respectively, by adding 250 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 50 μL of trimethylchlorosilane, standing the mixture at 70 °C for 30 min. The derivatized extracts were analyzed by GC–MS on a Trace Gas Chromatograph 2000 Series equipped with a Finnigan Trace MS Mass Spectrometer (Austin, USA), using helium as carrier gas (35 cm s^{-1}), and a DB-1 J&W (Folsom, USA) capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness). The chromatographic conditions were as follows: initial temperature: 80 °C for 5 min; temperature rate: 4 °C min^{-1}; final temperature: 285 °C for 15 min; injector temperature: 320 °C; transfer-line temperature: 290 °C; split ratio: 1:100.

For quantitative analysis, the GC–MS was calibrated with pure reference compounds representative of the major lipophilic components in the extracts relative to tetracosane as internal standard. The multiplication factors needed to obtain the correct quantification of the peak areas were calculated from an average of six GC–MS runs. All derivatized extracts were injected in triplicate and the compounds were identified as TMS derivatives.

In order to verify the presence of esterified structures, the extracts were also analyzed by GC–MS using a J&W Scientific short DB-1 capillary column (15 m × 0.32 mm i.d.; 0.25 μm film thickness). The chromatographic conditions were as follows: initial oven temperature 100 °C, held for 3 min, increased to 340 °C at a rate of 5 °C min^{-1}, and held for 12 min: the injector temperature was 320 °C, the transfer line temperature was 290 °C, and the split ratio was 1:100.

2.5. Isolation of hop-17(21)-en-3-one

The crude dichloromethane extract of *H. portulacoides* roots was fractionated by thin layer chromatography on silica gel 60 GF_{254} (1.5 mm thickness layers over 20 cm × 20 cm Glass-backed plates; Merck Darmstadt, Germany), eluting with a mixture of dichloromethane:light petroleum (50:50, v/v). Hop-17(21)-en-3-one was isolated at Rf ~0.6 and crystallized from methanol, yielding whitish crystals.

2.6. NMR analysis

^{1}H and ^{13}C NMR spectra of hop-17(21)-en-3-one was recorded in $CDCl_3$ using a Bruker Avance (Wissembourg, France) spectrometer operating at 300.13 and 75.47 MHz, respectively. Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as internal reference. ^{1}H and ^{13}C NMR assignments were made using two-dimensional COSY, NOESY, HSQC and HMBC (the delay for the evolution of long-range couplings was optimized at 7 Hz) experiments.

2.7. Total phenolic content (TPC)

The total phenolic content (TPC) of the methanol:water extracts was determined by the Folin–Ciocalteu method following a described procedure (Santos et al., 2012). 2.5 mL of Folin–Ciocalteu reagent, previously diluted with water (1:10, v/v), and 2 mL of aqueous sodium carbonate (75 g L^{-1}) were added to accurately weighed aliquots of the extracts dissolved in 0.5 mL of methanol, corresponding to concentration ranges of 0.5–1.0 mg of extract mL^{-1}. Each mixture was kept for 5 min at 50 °C and, after cooling, the absorbance was measured at 760 nm, using a UV–Vis V-530 spectrophotometer (Jasco, Tokyo, Japan). TPC was calculated as gallic acid equivalent from the calibration curve of gallic acid standard solutions (30–125 μg mL^{-1}) and expressed as g of gallic acid equivalent (GAE) g^{-1} of extract. Triplicate measurements were carried out.

2.8. UHPLC procedure

The UHPLC system consisted of a variable loop Accela autosampler (200 vial capacity set at 15 °C), an Accela 600 LC pump and an Accela 80 Hz PDA detector (Thermo Fisher Scientific, San Jose, CA, USA). The separation of the compounds was carried out at 25 °C, with a gradient elution program at a flow rate of 0.6 mL min^{-1}, by using a Hypersil™ Gold C_{18} (50 mm x 2.1 mm × 1.9 μm) column supplied by ThermoFisher (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase consisted in water:acetonitrile (99:1, v/v) (A) and acetonitrile (B), both with 0.1% of formic acid. The following linear gradient was applied: 0–3 min: 0%B, 3–30 min: 0–30%B, 30–32 min: 30–100%B, 32–34.5 min: 100–0%B, followed by re-equilibration of the column for 2.5 min before the next run. Single online detection was carried out in the diode array detector, at 340 nm, and UV spectra in a range of 200–600 nm were also recorded. Before the injection, each extract was dissolved

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