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Antioxidant activity and verbascoside content in extracts from two uninvestigated endemic *Plantago* spp.

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ABSTRACT

This work describes for the first time the antioxidant activity of extracts from wild plants and *in vitro* cultures of two previously uninvestigated *Plantago* spp. endemic to Portugal, *Plantago algarbiensis* Samp. and *Plantago almogravensis* Franco. The well-known and widely distributed species *Plantago lagopus* L. was also studied for comparison. Although the extracts from the three species exhibited radical scavenging and iron-reducing properties, the extracts from the two endemic species showed greater activity irrespectively of plant material source. Both *P. algarbiensis* extracts showed similar antioxidant activity whereas, in the case of *P. almogravensis* higher activity was observed for extracts from wild plants compared to extracts from *in vitro* cultures. Liquid chromatography–diode array detection analyses indicate verbascoside as a major constituent of all extracts and the highest content was obtained from *in vitro* cultures of *P. algarbiensis*. In addition, a high correlation between the antioxidant activity measured with three methods and the total phenolic content was found, but no correlation was observed between antioxidant activity and verbascoside content. The results suggest that *P. algarbiensis and P. almogravensis* are sources of health-beneficial phytochemicals and indicate that *in vitro* cultures represent a promising alternative for the biosynthesis of verbascoside.

1. Introduction

Plantago (Plantaginaceae) is a diverse genus of about 275 annual and perennial herbs (rarely shrubs) with world-wide distribution. Several species are used in certain countries as part of the diet, the leaves are used as ingredients for salads, soups or for childrens' mush, and the seeds can be cooked and used as starch, or can be ground into a powder and added to flour when making bread and cakes (Pieroni et al., 2002; Gálvez et al., 2005; Heimler et al., 2007). Some species are also important for animal feeding, improving their physiological conditions and diminishing the need for antibiotic growth promoters (Tamura and Nishibe, 2002). In addition, the aerial parts of some species are used in folk medicine and phytotherapy for a large range of diseases, namely those related with digestive and respiratory organs, skin and infectious diseases, pain relief, and cancer (Wichtl, 1994; Samuelsen, 2000).

Some widely distributed *Plantago* species are the subject of many investigations that confirm their benefits (Gálvez et al., 2003, 2005; Velasco-Lezama et al., 2006; Fleer and Verspohl, 2007; Ozaslan et al., 2007; Stanisavljević et al., 2008; Harput et al., 2012; Hu et al., 2013). Phytochemical investigations showed that phenylethanoid glycosides and iridoid glycosides are the most important groups of bioactive metabolites in these species (Rønsted et al., 2003; Harput et al., 2012; Gonda et al., 2013). Verbascoside is a phenylethanoid glycoside present in several *Plantago* species that possesses beneficial activities for human health, namely antioxidant, anti-inflammatory and antimicrobial in addition to wound-healing and neuroprotective properties (Alipieva et al., 2014).

Recent surveys demonstrated that certain endemic and less studied *Plantago* species also possess considerable bioactivity and contain valuable phytochemicals (Beara et al., 2012a,b; Zhou et al., 2013). Nevertheless, there are species that are still uninvestigated as is the case of *Plantago algarbiensis* Samp. and *Plantago almogravensis* Franco that have not been examined with respect to any biological activity. *P. algarbiensis* and *P. almogravensis* are endemic species to Portugal, from the west/central Algarve region and the southwest coast, respectively. Both species are in risk of global extinction and are protected under the European Habitats Directive and by Portuguese law (ICN, 2006). These restrictions mean

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; F–C reagent, Folin–Ciocalteu reagent; GAE, gallic acid equivalents; LC–DAD, liquid chromatography–diode array detection; TCA, trichloroacetic acid; TE, trolox equivalents; TEAC, trolox equivalent absorbance capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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that natural populations cannot be exploited as source of bioactive compounds and alternatives are required. The use of *in vitro* culture allows the rapid and mass production of plant material without a negative impact on natural habitats. Moreover, culture under controlled environmental conditions allows bioactive compounds to be extracted throughout the year, with no seasonal constraints (Costa et al., 2012, 2013; Aremu et al., 2013). The investigation of the secondary metabolites on *in vitro* produced tissues is described for few *Plantago* species (Saker and Kawashity, 1998; Budzianowska et al., 2004). A micropropagation protocol for *P. algarbiensis and P. almogravensis* was described by our group (Gonçalves et al., 2009) that could be used to replenish natural populations and also to generate *in vitro* cultures for the extraction of phytochemicals. However, *in vitro* culture conditions have been found to affect the production of secondary metabolites (Coste et al., 2011, 2012; Aremu et al., 2012, 2013) and therefore the suitability of this approach depends on the ability of *in vitro* cultures to synthesise bioactive compounds. In this work, the antioxidant activity of extracts from wild plants and *in vitro* cultures of the endemic species *P. algarbiensis* and *P. almogravensis* and their verbascoside content was compared for the first time. Extracts from the widely distributed and well studied species *Plantago lagopus* L. were also investigated for comparison.

2. Materials and methods

2.1. Chemicals and reagents

2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) tablets, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, verbascoside, trichloroacetic acid (TCA) and $K_2S_2O_8$ were purchased from Sigma–Aldrich (Steinheim, Germany). Folin–Ciocalteu's phenol reagent (F–C reagent), gallic acid, Na_2CO_3 and $FeCl_3$ were acquired from VWR (Leuven, Belgium). $K_3[Fe(CN)_6]$ and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Acros Organics (Geel, Germany). Formic acid was obtained from Merck (Darmstadt, Germany).

2.2. Plant material and extraction procedure

Randomly selected leaf samples of wild growing plants were collected during the spring of 2012. Samples of *P. algarbiensis* and *P. lagopus* were collected in the Algarve region and samples of *P. almogravensis* in Vila Nova de Milfontes (southwest coast). Voucher specimens were deposited at the herbarium of the University of Algarve (number ALGU 8079, ALGU 10815 and ALGU 14263 for *P. algarbiensis*, *P. almogravensis*, and *P. lagopus*, respectively). *In vitro* cultures were grown in MS medium (Murashige and Skoog, 1962) supplemented with 0.2 mg/l 6-benzyladenine (BA) as described by Gonçalves et al. (2009). The cultures were maintained at 25 ± 2 °C with a 16-h photoperiod (cool white fluorescent lamps, 50 μmol/m/s) and were subcultured every 6 weeks.

Plant material was dried at 40 °C and powdered in a blender to achieve a mean particle size less than 2 mm. Dried plant material was extracted twice by maceration with 80% methanol (1:10, w/v) during 24 h at room temperature. After filtration, solvent was evaporated in vacuum and crude residues were dissolved in hot distilled water (100 mg/ml). With the aim of removing non-polar compounds, the extracts were washed exhaustively with *n*-hexane. Finally, the extracts were concentrated to dryness in a rotary evaporator and stored at −20 °C.

2.3. $ABTS^{\bullet+}$ radical cation decoloration assay

The ABTS free radical-scavenging activity of each sample was determined as described by Re et al. (1999) and the results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC). The 7 mM $ABTS^{\bullet+}$ stock solution was prepared using potassium persulfate as the oxidizing agent. The absorbance was determined at 734 nm, 1 min after mixing. The sample dilution that achieved 20–80% inhibition of the blank absorbance was used to calculate the TEAC values and the results were expressed as Trolox equivalents (TEs) per gram of extract.

2.4. DPPH free radical scavenging assay

The ability of the extracts to scavenge DPPH radicals was determined according to the procedure described by Soler-Rivas et al. (2000) with slight modifications. One hundred microliters of 90 μM DPPH methanolic solution was added to 10 μl of sample solution at different concentrations, and the mixture was diluted with 190 μl of methanol in a clear 96-well microplate (NUNC, Rochester, New York, USA). The extract was replaced with solvent for the control. After 30 min, the reduction of DPPH radicals was measured at an absorbance of 515 nm. The radical scavenger activity was expressed in terms of the amount of sample necessary to decrease the initial absorbance by 50% (IC_{50}).

2.5. Ferric reducing antioxidant power (FRAP)

The reducing properties of the extracts were determined using $FeCl_3$ as described by Pulido et al. (2000) with some modifications. Briefly, 100 μl of each extract was mixed with 250 μl sodium phosphate buffer (200 mM, pH 6.6) and 250 μl 1% $K_3[Fe(CN)_6]$ and was incubated at 50 °C for 20 min. After the addition of 250 μl 10% TCA the mixture was centrifuged at 650 rpm for 10 min. Then 100 μl of the supernatant were mixed with 100 μl of water and 20 μl 0.1% $FeCl_3$ in a 96-well microplate. Reducing activity was measured by determining the absorbance at 700 nm.

2.6. Determination of the total phenolic content

The total phenolic content of each extract was measured using an adapted F–C colorimetric method (Ainsworth and Gillespie, 2007). Briefly, 200 μl of 10% (v/v) F–C reagent was mixed with 100 μl of each extract in phosphate buffer (75 mM, pH 7.0). Then 800 μl of 700 mM Na_2CO_3 were added and the reaction was incubated at room temperature for 2 h. Instead of the plant extracts, gallic acid was used as a positive control or phosphate buffer as a negative control. Two hundred microliters were transferred from each reaction to a clear 96-well microplate and the absorbance was measured at 765 nm. A standard curve was calculated using gallic acid concentrations ranging from 0.004 to 0.5 mM and the results were expressed as gallic acid equivalents (GAE) per gram of extract.

2.7. Liquid chromatography–diode array detection (LC–DAD) analysis

The extracts were analysed on an Agilent 1100 series LC system (Agilent Technologies, Waldbronn, Germany). Separation was achieved using a Phenomenex Kinetex C18 column (4.6 × 150 mm, 2.6 μm particle size) (Tecnocroma, Caldas da Rainha, Portugal) and a binary solvent consisting of methanol (A) and formic acid aqueous solution (0.1%) with the following gradient: 25% A at 0 min, 35% A at 10 min, 45% A at 12 min, 45% A at 17 min, 50% A at 19 min, 55% A at 21 min and hold at 100% A for 5 min. The flow rate was 0.5 ml/min and the injection volume 20 μl.

Spectral data from all peaks were accumulated in the range 200–400 nm, and chromatograms were recorded at 325 nm. The LC3DChemStation software (Agilent Technologies) was used for instrumental control and data processing. Verbascoside (HWI Analytik GmbH, Ruelzheim, Germany) quantification was achieved by