Food Chemistry 135 (2012) 1253–1260

Contents lists available at SciVerse ScienceDirect

Food Chemistry

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

Thymus lotocephalus wild plants and *in vitro* cultures produce different profiles of phenolic compounds with antioxidant activity

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ARTICLE INFO

Article history:
Received 9 February 2012
Received in revised form 15 May 2012
Accepted 16 May 2012
Available online 24 May 2012

Keywords:
Thymus lotocephalus
Wild plants
In vitro cultures
Antioxidant activity
Phenolic compounds

ABSTRACT

We compared the phenolic metabolites and antioxidant activities of *Thymus lotocephalus* G. López & R. Morales wild plants and *in vitro* cultures using different extraction solvents. HPLC–DAD analysis allowed the identification and quantification of phenolic (caffeic and rosmarinic) acids and flavones (luteolin and apigenin) in extracts from both sources. The *in vitro* cultures accumulated large amounts of rosmarinic acid. However, extracts from both sources were able to neutralise free radicals in different test systems (TEAC and ORAC assays), to form complexes with Fe^{2+} and to protect mouse brains against Fe^{2+}-induced lipid peroxidation. The solvent significantly influenced the phenolic content and antioxidant activity of the extracts, water/ethanol being the most efficient for the extraction of antioxidant phytochemicals. We conclude that *in vitro* cultures of *T. lotocephalus* represent a promising alternative for the production of valuable natural antioxidants and an efficient tool for the *in vitro* biosynthesis of rosmarinic acid, therefore avoiding the need to exploit populations of wild plants.

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1. Introduction

Free radicals are an integral component of many redox processes in eukaryotic cells, which maintain homeostasis by synthesising antioxidant enzymes and metabolites. Excess free radicals are produced under stress and in response to pathogens, often resulting in oxidative damage to lipids, proteins and nucleic acids. In humans, this damage is implicated in ageing and in numerous diseases (Valko et al., 2007). Synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene have been used in food processing and preservation (Botterweck, Verhagen, Goldbohm, Kleinjans, & van den Brandt, 2000; Hinneburg, Dorman, & Hiltunen, 2006). However, these chemicals are toxic and their risk to health has increased the demand for natural antioxidants (Liu et al., 2011).

Phenolic compounds can prevent oxidative damage via a number of different mechanisms, such as free radical scavenging, transition metal chelation and interactions with lipid membranes, proteins and nucleic acids (Dai & Mumper, 2010). The spectrum of phenolic compounds isolated from plants depends on several factors, such as the nature of the tissue matrix, the extraction time and temperature, and the polarity of the solvent system (Dai & Mumper, 2010). Water, ethanol, methanol, acetone and aqueous/organic solvent mixtures are frequently used to extract phenolic compounds from plants (Chew et al., 2011; Sultana, Anwar, & Ashraf, 2009; Trabelsi et al., 2010).

Plants of the mint family (Lamiaceae) produce many metabolites with bioactive properties, but large-scale extraction is challenging because the family is genetically heterogeneous (Shetty, 1997). For this reason, there is great interest in the production of specific metabolites under controlled environmental conditions that maintain a given phytochemical profile (Makunga & van Staden, 2008; Pérez-Tortosa, López-Orenes, Martínez-Pérez, Ferrer, & Calderón, 2012; Shetty, 1997; Zuzarte, Dinis, Cavaleiro, Salgueiro, & Canhoto, 2010).

Thymus is a taxonomically complex genus of aromatic plants that is widely distributed in the Mediterranean region and

Abbreviations: AAPH, 2,2'-azobis (2-methylpropionamidine) dihydrochloride; $ABTS^{\cdot+}$, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation; BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; F–C reagent, Folin–Ciocalteu reagent; GAE, gallic acid equivalents; HAT, hydrogen atom transfer; HPLC–DAD, high-performance liquid chromatography–diode array detection; MDA, malondialdehyde; MS, Murashige and Skoog medium; ORAC, oxygen radical absorbance capacity; SET, single electron transfer; TBA, thiobarbituric acid; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

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0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2012.05.072

traditionally used as culinary herbs, ornamental plants and flavouring agents (Figueiredo, Miguel, Duarte, Barroso, & Pedro, 2001; Figueiredo et al., 2008). *Thymus* spp. also produce secondary metabolites that can be used as antioxidants, expectorants, antitussives, antiplatelet drugs, antimicrobials and drugs for the treatment of skin disorders (Dandlen et al., 2010; Faleiro et al., 2003; Figueiredo et al., 2008; Hazzit, Baaliouamer, Veríssimo, Faleiro, & Miguel, 2009; Oh et al., 2009; Okazaki, Kawazoe, & Takaishi, 2002). *Thymus lotocephalus* G. López & R. Morales is an aromatic species endemic to the Algarve region, which blossoms from April to June and is typically found in dry open areas and scrublands (Figueiredo et al., 2001, 2008). It produces phytochemicals with antimicrobial, antioxidant and anticholinesterase activities, although only for a limited time (Costa et al., 2012; Faleiro et al., 2003). The species is considered to be critically endangered and is legally protected by both the European Habitats Directive (92/43/CEE) and by Portuguese law (Reference 140/99, April 24 1999; ICN, 2006). These restrictions mean that natural populations cannot be exploited as a source of bioactive compounds and alternatives are required.

Biotechnology can be used to facilitate the large-scale production of plant material without a negative impact on natural habitats (Matkowski, 2008; Zuzarte et al., 2010). In particular, *in vitro* culture under controlled environmental conditions allows bioactive compounds to be extracted throughout the year, with no seasonal constraints (Dias, Barros, Sousa, & Ferreira, 2011; Shaik, Singh, & Nicholas, 2011; Zuzarte et al., 2010). We have therefore recently developed a micropropagation protocol for *T. lotocephalus* that could be used to replenish natural populations and also to generate *in vitro* cultures for the extraction of phytochemicals (Coelho, Gonçalves, González-Benito, & Romano, 2012). The suitability of the latter approach depends on the ability of micropropagated plants to synthesise useful phenolic profiles, but the capacity of both wild plants and *in vitro* cultures to produce phenolic molecules is not understood in detail.

We therefore set out to compare the phenolic profiles of wild plants and *in vitro* cultures, and to evaluate the effect of different solvents on the recovery of phenolic compounds with antioxidant activity by measuring the Trolox equivalent antioxidant capacity, oxygen radical absorbance capacity, Fe^{2+} chelation activity and the inhibition of Fe^{2+}-induced lipid peroxidation in mouse brains. The phenolic compounds present in *T. lotocephalus* extracts were identified and quantified by high-performance liquid chromatography with diode-array detection (HPLC–DAD).

2. Materials and methods

2.1. Standards and reagents

Apigenin, luteolin and rosmarinic acid were purchased from Extrasynthése (Genay, France). Caffeic acid, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) tablets, potassium persulphate, thiobarbituric acid (TBA) and Triton X-100 were purchased from Sigma–Aldrich (Steinheim, Germany). Formic acid, methanol, iron(II) sulphate ($FeSO_4$) were acquired to Merck (Darmstadt, Germany). Fluorescein, 1,10-phenanthroline and absolute ethanol were obtained from Panreac (Barcelona, Spain). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), butylated hydroxytoluene (BHT) and sodium dodecylsulphate (SDS) were purchased from Acros Organics (Geel, Germany). Qualitative filter paper, Folin–Ciocalteu (F–C) reagent, gallic acid and sodium carbonate were purchased from VWR (Leuven, Belgium). Ethylenediaminetetraacetic acid (EDTA) and sodium chloride (NaCl) were purchased from Fluka (Steinheim, Germany).

2.2. Plant material

Aerial parts from *T. lotocephalus* were collected from wild plants growing in a natural population in Algoz (Algarve region, Portugal) during the flowering period. The *T. lotocephalus* voucher was previously deposited in the Herbarium of the University of Algarve (number ALGU 8081).

In vitro cultures of *T. lotocephalus* were produced as previously described (Coelho et al., 2012). Briefly, plants were cultured in MS medium (Murashige & Skoog, 1962) supplemented with 2% (w/v) sucrose and 1% (w/v) agar. The cultures were incubated at 25 ± 2 °C with a 16-h photoperiod (cool white fluorescent lamps, 40 $\mu mol\ m^{-2}\ s^{-1}$) and were subcultured every 6 weeks.

2.3. Extraction procedure

Material from wild plants and *in vitro* cultures was dried at 40 °C and powdered in a blender to achieve a mean particle size less than 2 mm. We used water and ethanol separately and in a 1:1 mixture to generate extracts with different compositions. The plant material (10 g) was soaked overnight at room temperature in 200 ml of each solvent. After soaking, the mixture was filtered through a membrane with a retention size of 5–13 μm; the extract was concentrated to dryness (the water extract was lyophilised and the ethanol and water/ethanol extracts were dried in a rotary evaporator) and the residue stored at −20 °C.

2.4. Identification and quantification of phenolic compounds by HPLC–DAD

The extracts were analysed by HPLC (Gilson, Villiers le Bel, France) using a Spherisorb ODS2 column (4.6 × 250 mm, 5 μm particle size) and a solvent system comprising solvent A (19:1 water/formic acid) and solvent B (methanol). The gradient comprised 5% solvent B at 0 min increasing to 15% at 3 min, 25% at 13 min, 30% at 25 min, 35% at 35 min, 45% at 39 min and 42 min, 50% at 44 min, 55% at 47 min, 70% at 50 min, 75% at 56 min and 100% at 60 min. The injection volume was 20 μl and the flow rate was 0.9 ml/min. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were collected in the 200–400 nm range, and chromatograms were recorded at 320 and 350 nm for phenolic acids and flavones, respectively. The data were processed using the Unipoint Software platform (Gilson Medical Electronics). Peak purity was determined using the software contrast facilities. Phenolic compounds were quantified by comparison with standards.

2.5. Total phenolic content (F–C assay)

The Folin–Ciocalteu (F–C) colorimetric method was carried out as described by Ainsworth and Gillespie (2007), with slight modifications. Briefly, 10% (v/v) F–C reagent was added to each sample, standard or phosphate buffer (blank) in a microtube. A 700 mM sodium carbonate solution was added to each microtube and incubated for 2 h at room temperature, then 200 μl were transferred to a clear 96-well microplate (NUNC, Rochester, NY) and the absorbance at 765 nm was determined using an Infinite 200 microplate reader (Tekan, Grödig, Austria). The standard curve was calculated using gallic acid and the results were expressed as gallic acid equivalents (GAE) per gram of extract. All experiments were carried out in triplicate.

2.6. Trolox equivalent antioxidant capacity (TEAC) assay

Antioxidant activity was determined using the TEAC assay with the radical cation $ABTS^{\cdot+}$ as described by Re et al. (1999). The 7 mM

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