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Characterization of four wild edible *Carduus* species from the Mediterranean region via phytochemical and biomolecular analyses

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ABSTRACT

Carduus species (Compositae) are widely distributed in the Mediterranean area, and traditionally used for both food and medicinal purposes.

The hydroalcoholic extracts of four wild edible *Carduus* species collected in Sardinia (*Carduus argyroa* Biv., *Carduus nutans* subsp. *macrocephalus* (Desf.) Nyman, *Carduus pycnocephalus* L., *Carduus cephalanthus* Viv.) were analyzed and characterized by HPLC-PDA-MS/MS and PCR-RFLP of the nrDNA internal transcribed spacer (ITS).

Flavonoids and caffeoylquinic acid derivatives were the predominant classes of secondary metabolites characterizing the extracts. The ITS region was sequenced in parallel, and a PCR-RFLP method was applied with three selective restriction enzymes. Statistical analyses, on both chemical and biomolecular results, revealed that individuals clustered according to their taxonomic classification.

The combination of the two techniques discriminates the four species within the genus, giving further information on these little-investigated plants, traditionally used in the Mediterranean area and in Sardinia.

1. Introduction

Wild edible species are traditionally consumed mainly for their taste, as well as for their healthy and nutritional properties. Many popular dishes prepared with wild plants are still consumed, nowadays increasingly so with the return to traditions, with the primary aim of finding healthy alternatives to commercial foods (Guarrera & Savo, 2016). This is part of a trend aiming at re-discovering local products, often offered as culinary specialities in fairs and markets. Further, several edible plants are traditionally used as depuratives or to treat trivial illnesses. These species may potentially play an important role as functional foods, thanks to the great variety of physiologically-active components providing health benefits (Guarrera & Savo, 2016; Lentini & Venza, 2007; Pardo-de-Santayana et al., 2007; Ranfa, Maurizi, Romano, & Bodesmo, 2014).

Species from the genus *Carduus* (Compositae family), known in English as thistles, are traditionally consumed for their taste and biological effects. They are annual or perennial plants, 0.5–2 m high, with lance-shaped, spiny-toothed leaves, spiny-winged stems and white-to-purple flowers. The genus includes approximately 100 species worldwide, which are widely distributed over the Mediterranean area (Al-Shammari, Hassan, & Al-Youssef, 2015; Dimitrova-dyulgerova, Zhelev, & Mihaylova, 2015; Lahaye et al., 2008; Thao et al., 2011; Tutin et al., 1968). They are consumed as raw or cooked, and are used as medicinal plants for the treatment of liver disorders or, more in general, for their diuretic and digestive properties (Al-Shammari et al., 2015; Atzei, 2003; Dimitrova-dyulgerova et al., 2015; Guarrera & Savo, 2016; Lentini & Venza, 2007; Rinchen & Pant, 2014; Signorini, Piredda, & Bruschi, 2009; Tardío, Pardo-de-santayana, & Morales, 2006). Several classes of secondary metabolites, chiefly flavonoids, phenolic acids, lignans, coumarins, alkaloids, sterols, and triterpenes, have been found in these species (Al-Shammari et al., 2015; Cardona, García, José, & Pérez, 1992; Dimitrova-dyulgerova et al., 2015; Fernández, Garcia, Pedro, & Varea, 1991; Jordon-Thaden & Louda, 2003). The presence of these compounds may be associated to the documented wide range of biological and nutraceutical properties that are associated to *Carduus* species: liver tonicity, anti-inflammatory, antioxidant, antispasmodic, anticancer, antiviral, and antibacterial activity (Al-Shammari et al., 2015; Jordon-Thaden & Louda, 2003; Koc et al., 2015; Slavov, Mihayloiva, & Dimitrova-dyulgerova, 2014).

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This study aims to verify similarities and dissimilarities in the chemical and biomolecular profiles of four wild edible *Carduus* species (*Carduus argyroa* Biv., *Carduus nutans* subsp. *macrocephalus* (Desf.) Nyman, *Carduus pycnocephalus* L., *Carduus cephalanthus* Viv) growing in the Mediterranean area and in particular in Sardinia, where these species are traditionally consumed. Little has been published on these species, in particular on those growing in Sardinia. Some data on the characteristic compounds (mainly flavonoids) of *C. pycnocephalus* and *C. nutans* L. (Al-Shammari et al., 2015; Bain & Desrochers, 1988; Jordon-Thaden & Louda, 2003; Marrelli, Loizzo, Nicoletti, Menichini, & Conforti, 2013) and on the polyacetylenes of *C. argyroa* extracts are available (Harborne, Baxter, & Moss, 1999; Jordon-Thaden & Louda, 2003). To the best of the authors' knowledge, no information is available on *C. cephalanthus* and *C. nutans* subsp. *macrocephalus*. At the same time, because of their relevance as traditional foods and remedies, it is of interest to learn more about these species, to verify the presence of compounds with nutraceutical properties, and to identify them in their extracts. The identification and discrimination of these closely-related species were approached by combining high performance liquid chromatography with diode array and mass spectrometry detectors (HPLC-PDA-MS/MS) and Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) sequences, together with unsupervised multivariate data analysis and cluster analysis (PCA, HCA, Neighbor Joining, UPGMA). ITS gene as a DNA barcode marker is a useful tool to authenticate raw herbal materials, and in particular when (dried or processed) closely related species show similar chemical compositions. DNA barcoding has successfully been applied to authenticate plant and animal samples, also in terms of food safety and quality control. However, a limit of this method is that reference sequences of uncommon plants are usually lacking in databases (Ali et al., 2014; Galimberti et al., 2013; Ha et al., 2015; Hebert, Cywinska, Ball, & Jeremy, 2003).

2. Materials and methods

2.1. Plant material

Aerial parts of the four wild species belonging to the genus *Carduus* were collected from different sites in Sardinia, in May and June 2015 (Table S1). They were identified at the Department of Life and Environmental Sciences, University of Cagliari, Italy, where a voucher specimen for each species was deposited. In total 10 specimens of *C. argyroa*, 6 of *C. cephalanthus*, 13 of *C. nutans* subsp. *macrocephalus* and 10 of *C. pycnocephalus* were collected. All plants growing at each site were separated by 1–50 m from one another, and were collected randomly. The fresh materials were dried at 40 °C to constant weight.

2.2. Chemicals

HPLC-grade acetonitrile (LC-MS grade), formic acid (> 98% purity), chlorogenic acid, rutin, apigenin 7-*O*-glucoside, apigenin, diosmin and kaempferol were from Sigma Aldrich (Bellefonte, USA). De-ionized water (18.2 MΩ cm) was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Luteolin, quercetin 3-*O*-glucoside, kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucoside were from Extrasynthese (Genay Cedex, France). Cryptochlorogenic acid, 1,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide, apigenin 7-*O*-glucuronide, kaempferol 3-*O*-rhamnoside, diosmetin and tricin were from Phytolab (Vestenbergsgreuth, Germany).

2.3. Sample preparation and HPLC-PDA-MS/MS analysis

500 mg of each dried and ground aerial part were submitted to ultrasonic extraction with 10 mL of methanol/water (70:30, v/v) three times for 10 min. The extracts were then combined and centrifuged at 4000 rpm for 10 min. The supernatant was brought to a volume of 30 mL and filtered with a 13 mm diameter, 0.22 μm pore diameter hydrophilic PTFE syringe filter, before the HPLC-PDA-MS/MS analysis.

2.4. HPLC-PDA-MS/MS analysis

Each extract (5 μL) was analyzed in duplicate with a Shimadzu Nexera × 2 system equipped with a photodiode detector SPD-M20A in series to a triple quadrupole Shimadzu LCMS-8040 system provided with electrospray ionization (ESI) source (Shimadzu, Dusseldorf Germany). Samples were analyzed on an Ascentis Express C18 column (15 cm × 2.1 mm, 2.7 μm, Supelco, Bellefonte, USA) using water/formic acid (999:1, v/v) and acetonitrile/formic acid (999:1, v/v) as mobile phases A and B, respectively. The flow rate was 0.4 mL/min and the column temperature was maintained at 30 °C. The gradient program was as follows: 5% B for 3 min, 5–15% B in 17 min, 15–25% B in 10 min, 25–75% B in 12 min, 75–100% B in 10 min, 100% B for 1 min. Total pre-running and post-running time was 60 min. UV spectra were acquired over the 220–450 nm wavelength range and the resulting chromatograms were integrated at 330 nm. MS operative conditions were as follows: heat block temperature: 200 °C; desolvation line (DL) temperature: 250 °C; nebulizer gas flow rate: 3 L/min drying gas flow rate: 15 L/min. Mass spectra were acquired both in positive and in negative full-scan mode over the range 100–1000 *m/z*, event time 0.5 s. Product Ion Scan mode (collision energy: - 35.0 V for ESI^+ and 35.0 V for ESI^-, event time: 0.2 s) was applied to compounds for which a correspondence between the pseudomolecular ions $[M+H]^+$ in ESI^+ and $[M-H]^-$ in ESI^- had been confirmed. Multiple Reaction Monitoring acquisition (collision energy: - 35.0 V for ESI^+ and 35.0 V for ESI^-, dwell time: 20) was carried out on specific product ions derived from precursor ions fragmentation (Table S2). Some of the main components were identified by comparing their retention times, UV and MS spectra to those of authentic standards (chlorogenic acid, cryptochlorogenic acid, dicaffeoylquinic acids, rutin, quercetin 3-*O*-glucoside, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide, kaempferol 3-*O*-rutinoside, kaempferol 3-*O*-glucoside, kaempferol 7-*O*-rhamnoside, apigenin 7-*O*-glucoside, apigenin 7-*O*-glucuronide, diosmin, apigenin, luteolin, kaempferol, tricin, diosmetin). The other components were tentatively identified on the basis of their UV spectra and mass spectral information, compared to those given in the literature. Data were processed using LabSolution software (Shimadzu, Dusseldorf Germany).

2.5. DNA extraction, PCR amplification and sequencing

Ten milligrams of the same material employed for chemical analyses were ground to a fine powder, with the addition of approximately 5 mg of polyvinylpolypyrrolidone (PVPP, Sigma Aldrich, Bellefonte, USA). Genomic DNA was extracted from the ground powder using the Eurogold Plant DNA Mini Kit (Euroclone, Pero, Italy) following the manufacturer's instructions. The quantitative and qualitative analyses of the isolated genomic DNA were assessed by spectrophotometry using the Nanophotometer (Implen GmbH, Munich, Germany) and by gel electrophoresis. Approximately 20 ng of genomic DNA were used as a template for PCR amplification with forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White, Bruns, Lee, & Taylor, 1990). The amplification was carried out in a 25 μL reaction mixture containing 2.5 μL of 10 × PCR buffer (Thermo-Scientific, Waltham, MA USA), 0.2 mM deoxynucleotide triphosphates (dNTPs), 20 pmol of forward and reverse primers, and 0.5 U of *Taq* DNA polymerase (Thermo-Scientific, Waltham, MA USA). PCR reactions were carried out in a T-Gradient Thermalcycler (Biometra GmbH, Göttingen, Germany). Cycling conditions consisted of an initial 4 min at 94 °C, followed by 30 s of denaturing at 94 °C, 45 s of annealing at 53 °C and 45 s of elongation at 72 °C, repeated for 35 cycles and with 10 min of final extension at