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ORIGINAL ARTICLE

Winter wild fennel leaves as a source of anti-inflammatory and antioxidant polyphenols

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Abstract In the course of a screening program on the seasonal phenol composition of wild Mediterranean medicinal and aromatic plants, broadly used for culinary purposes, *Foeniculum vulgare* Mill. was the focus of the present study. Hydroalcoholic extracts from fennel freeze-dried leaves, collected in different seasons along 2012 and 2013 years, were quali-quantitatively analyzed through LC/MS/MS techniques. Winter extract contained, beyond several hydroxycinnamoyl quinic acids and flavonol glycosides, two chromone derivatives. Flavonol hexuronides were the main spring sample constituents. Phenol profile differences among the extracts influenced massively their bioactivity. When the antioxidant screening was performed, winter extract effectively scavenged DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ and reduced Fe^{3+}. Although all the extracts did not show cytotoxicity, they were differently able to exert cytoprotection in H_2O_2-oxidized cell systems and to affect COX-2 gene expression in THP-1 cells. The most active one was winter extract, which inhibited COX-2 expression by 40%, whereas spring sample showed a weak pro-inflammatory capability.

1. Introduction

Traditional medicine is an important part of human health care in many developing and developed countries, increasing their commercial value. Although the use of medicinal plants in therapy has been known for centuries in all parts of the world, the demand for herbal medicines has grown massively in recent years (Kartal, 2007). According to World Health Organization, a medicinal plant is a plant organism containing

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compounds that can be used for therapeutic purposes or which are precursors of semi-synthetic pharmaceutical species. The synergistic and/or additive action of plant chemicals finds its strength in the formulation of pharmacologically active phytocomplexes (Pacifico et al., 2013), whose preparation requires proper knowledge on the drug plant chemical constitution, as well as its variation over the plant life. This latter represents a key issue when wild medicinal plants are used. It is well known that the content of active ingredients in a medicinal plant is not constant; it undergoes significant variations, due to endogenous and/or exogenous factors (Galasso et al., 2014). In particular, environmental conditions heavily influence the development and the ability to synthesize secondary metabolites in spontaneous medicinal plants, which, even today, play a strategic role in the production of plant-based products (Ramakrishna and Ravishankar, 2011). Indeed, although the cultivation of medicinal species is in constant increase, it is still a marginal reality, and an amount between 75% and 90% of medicinal plants commercialized in the world still comes from wild harvest (Rapporto ISMEA – Osservatorio delle piante officinali, giugno, 2013).

In the Mediterranean area several medicinal and aromatic plants grow and develop, still today, spontaneously in rural areas. Among them, *Foeniculum vulgare* Mill., commonly called fennel, has been extensively used in traditional medicine since ancient times, not only in Europe but also in several parts of the world. In fact, it has been attested to have a number of therapeutic properties related to digestive, endocrine, reproductive and respiratory systems (Badgujar et al., 2014; Piras et al., 2014). The osteoprotective effects of a *F. vulgare* ethanol extract were also reported (Mahmoudi et al., 2013). Although the volatile chemical composition of wild fennel samples, harvested in different geographical areas, was broadly reported (Piccaglia and Marotti, 2001; Díaz-Maroto et al., 2006) different scientific evidences suggest that the various components of fennel (seeds, leaves and fruits) are rich in molecules with antioxidant activity (Oktaya et al., 2003; Parejo et al., 2004a,b; De Marino et al., 2007; Ghanem et al., 2012). The beneficial properties of fennel appeared to be due to its content of natural polyphenols, particularly abundant in the shoots (Barros et al., 2009). Plant polyphenols are molecules biosynthesized from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s) and chemically feature more than one phenolic ring and devoid of any nitrogen-based functional group in their most basic structural expression (Quideau et al., 2011). As the interest in dietary polyphenols has been evaluated in several epidemiological studies, which indicated an inverse association between the intake of foods rich in these compounds and the incidence of diseases such as cardiovascular diseases, diabetes mellitus, and cancer (Quideau et al., 2011 and references therein; Rodrigo et al., 2014), the definition of the environmental conditions that favor the concentration of bioactive polyphenols could represent a strategy for the formulation of fennel products with healthy actions. To this aim in the present study four hydroalcoholic extracts from wild *F. vulgare* leaves, collected in the four different seasons along 2012 and 2013 years, were prepared by ultrasound-assisted maceration. The phenol profile of the obtained extracts was achieved through LC–MS/MS techniques. An assessment of the bioactivity of the extracts in terms of antioxidant, cytotoxic and anti-inflammatory effectiveness was also carried out.

2. Materials and methods

2.1. Plant collection and fractionation

Leaves from *F. vulgare* were collected in the wild in Durazzano (Italy) on tenth day of July and October 2012 and of January and April 2013, and identified by Dr. Assunta Esposito of the Second University of Naples. A voucher specimen has been deposited at the Herbarium of the Department of Environmental, Biological and Pharmaceutical Sciences and Technologies of the Second University of Naples. Three replicate samples (10.0 g each) of *F. vulgare* leaves for each collection time were ground in a porcelain mortar and pestle chilled with liquid N_2, until particles of homogeneous size were obtained. Frozen powdered samples were lyophilized using a FTS-System Flex-DryTM instrument (SP Scientific, Stone Ridge, NY, USA). Aliquots of dried leaves (1.0 g) underwent ultrasound-assisted extraction (Dr. Hielscher UP 200S, Germany) using a hydro-alcoholic solution (H_2O:MeOH; 1:1, v:v) as a extracting solvent. Four sonication cycles were performed (30 min each) in order to achieve the maximum recovery of the leaf metabolic content. At the end of each sonication cycle, samples were centrifuged at 2044*g* for 10 min in a Beckman GS-15R centrifuge (Beckman Coulter, Milano, Italy) fitted with rotor S4180. Obtained supernatants were dried under vacuum by a rotary evaporator (Heidolph Hei-VAP Advantage, Germany) to yield crude extracts: FvSu = summer extract; FvAu = autumnal extract; FvWi = winter extract; and FvSp = spring extract). All the extracts were stored at −20 °C until use.

2.2. RP-HPLC-ESI-MS/MS analyses

Chromatographic analyses were carried out on a Dionex Ultimate 3000 HPLC system (ThermoScientific Vienna, Austria) equipped with Ultimate 3000 RS pump, Ultimate 3000 RS autosampler, Ultimate 3000 RS Column Compartment and Ultimate 3000 RS diode array detector (DAD). A Phenomenex® Synergy RP-80A column (4.0 μm particle size, 150 × 2 mm) was used for chromatographic separation. The mobile phase consisted of A: 0.1% formic acid in water and B: acetonitrile. Starting with 5% B, a linear gradient was followed to 15% B at 10 min, then increasing to 35% B at 30 min, to 80% B at 40 min, and to 100% B at 45 min, continuing for 5 min, before re-equilibration to starting conditions. The flow rate was 0.3 mL/min and the injection volume was 5.0 μL. The DAD acquisition range was 190–450 nm.

The LC system was coupled to a LTQ-XL mass spectrometer (Thermo Scientific Vienna, Austria) with an ESI ion source and controlled by Thermo Tune Plus 2.7.0 software. ESI ion source operated in negative mode with the following parameters: dry gas flow (N_2) 8.0 L/min with a capillary temperature set at 330 °C; source heater temperature set at 250 °C; sheath gas flow set at 50 arb; auxiliary gas flow set at 10 arb; source voltage set at 3.00 kV; source current set at 100 μA; capillary voltage set at −16 V. Mass spectra were recorded between m/z 50–2000. To obtain further structural information, these ions were trapped and fragmented. Collision-induced fragmentation experiments were performed in the ion trap using helium as a collision gas, with voltage cycles from 0.3 up to 2 V and collision energy set at 35 eV. The