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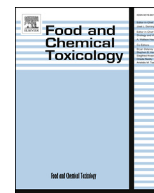

Influence of harvest season on chemical composition and bioactivity of wild rue plant hydroalcoholic extracts

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ABSTRACT

The rue (*Ruta graveolens*) copiousness in rural areas of the Campania Region based a thorough chemical and biological investigation aimed at exploring the seasonal variability of phenol constituents in rue leaves and its influence on their antioxidant, cytotoxic and anti-inflammatory capabilities. To this purpose, hydroalcoholic extracts were prepared from plant samples seasonally collected. LC-ESI-MS/MS techniques were employed to analyze qualitatively and quantitatively the seasonal rue phenol content, whereas different chemical antioxidant assays (by DPPH, ABTS, Fe^{3+} RP, ORAC, and FCR methods) and XTT redox metabolic activity assay were performed to screen the seasonal phenol complex-related antioxidant and cytotoxic power. The ability of the rue leaf extracts to counteract cyclooxygenase-2 (COX-2) expression was also evaluated. Data obtained highlighted that the adopted extraction procedure markedly pauperized the furanocoumarin content in all the prepared rue extracts. Flavonol glycosides, along with the flavone acacetin and two sinapic acid derivatives were the main constituents of the spring harvest-derived extract, which exerted the highest antioxidant capability in cell-free systems and was capable to inhibit COX-2 synthesis by 44% comparably to dexamethasone, used as positive control. Data provide new insights for developing a proper management of rue plants for new safe industrial purposes in herbal medicine field.

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1. Introduction

Healing plants have a long traditional history for their medicinal use (Petrovska, 2012; Ncube et al., 2012; Galasso et al., 2014; Pacifico et al., 2015a) and provide an unlimited source for drug discovery because of the unmatched availability of chemical diversity (Sasidharan et al., 2011). In fact, plants contain a broad array of bioactive compounds, mainly secondary metabolites, able to employ beneficial effects to humans, especially in the prevention of chronic degenerative diseases such as cancer, cardiovascular and neurodegenerative disorders. Indeed, the content in active ingredients of a medicinal plant is continuously affected by different endogenous, exogenous and/or biotic factors (Pacifico et al., 2015a, 2015b). Abiotic stresses deeply influence the secondary metabolites biosynthesis in spontaneous medicinal plants, which, even today, play a strategic role in the production of plant-based products. In fact, it is known that 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 (ISMEA, 2013). Peculiarly, harsh abiotic stresses could reduce plant growth with huge impact on agriculture (Atkinson and Urwin, 2012). Drought, salinity, heavy metals, extreme temperatures, nutrient poor soils and other abiotic stresses account for major crops lost worldwide (Rodriguez et al., 2005). However, not all the effects are detrimental, as plants exhibit various defense mechanisms (Boscaiu et al., 2008), among which the synthesis of phenol compounds (Agati et al., 2012, 2013), whose accumulation seems to counteract the overproduction of

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reactive oxygen species that triggers in plant. Thus, it is easy to image that depending on the habitat in which a plant grows and develops, it otherwise can modulate its ability to biosynthesize phenols, whose presence/absence makes the same plant an anti-oxidatively active/inactive source for human purposes (Nascimento and Fett-Neto, 2010; Ramakrishna and Ravishankar, 2011). Therefore, the variability of phenols biosynthesis as response to environmental and stress constraints overcoming (Hüsnü Can Baser, 2002; Edreva et al., 2008) cannot be neglected in the definition of pharmacological goodness of wild medicinal plants. It is feasible that the opportune knowledge of phenols stress-induced expression in wild medicinal plants could address the maintenance of their biodiversity safeguard and/or optimize their domestication in order to realize a production chain that exploits environmental conditions favorable to the production of plant characteristic phenols. Furthermore, the full knowledge of the phenolic constitution of medicinal and aromatic plant species, together with the determination of the health properties of their phenol plant complexes, could lead to their exhaustive and safe use in herbal medicine.

In this context, *R. graveolens* L. wild plants, seasonally collected, were of interest.

This herbaceous perennial plant, native to the Mediterranean region, commonly known as rue, has been used for a long time as contraceptive, anti-inflammatory, antipyretic, anti-helminthic, to relieve symptoms of hangover, and applied externally as a poultice against rheumatic pain (Raghav et al., 2006; Saieed et al., 2006; Ratheesh and Helen, 2007; Asgarpanah and Khoshkam, 2012; Malik et al., 2013). The rue copiousness in rural areas of the Campania Region based a thorough chemical and biological investigation aimed at exploring the seasonal variability of phenol constituents in rue leaves and its influence on their antioxidant, cytotoxic and anti-inflammatory capabilities.

2. Materials and methods

2.1. Plant collection and fractionation

R. graveolens leaves were collected in the wild in Durazzano (Italy) on the tenth day of July and October 2012 and of January and April 2013. 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. Durazzano is a small center in the Southern Italy (altitude 286 m above sea level) characterized by a mild Mediterranean climate, identified as Csa climate on the basis of Köppen and Geiger classification. Meteorological parameters, acquired from the Centro Agrometeorologico Regionale (C.A.R.) of the Campania Region, for the meteorological station located in Airola, are listed in Table 1.

Table 1
Agrometeorological mean parameters for harvest months, acquired from Campania Region C.A.R.

	Jul 2012[a]	Oct 2012[b]	Jan 2013[c]	Apr 2013[d]
Maximum temperature 24 h °C	32.6	23.3	12.1	21.2
Minimum temperature 24 h °C	16.7	10.5	3.8	8.84
Average temperature 24 h °C	24.8	16.7	7.65	15.0
Maximum humidity 24 h %Sat	86.4	92.2	89.2	87.1
Minimum humidity 24 h %Sat	30.4	49.0	58.2	39.2
Average humidity 24 h %Sat	59.2	76.2	77.3	65.7
Wind speed 24 h m/s	2.06	1.80	2.53	2.52
Precipitation 24 h mm	1.80	5.48	6.43	2.20

[a] RgSu sampling time.
[b] RgAu sampling time.
[c] RgWi sampling time.
[d] RgSp sampling time.

Three replicate samples (10.0 g each) of *R. graveolens* leaves for each collection time were ground in a porcelain mortar and pestle chilled with liquid N_2. Frozen powdered samples were lyophilized using an FTS-System Flex-DryTM instrument (SP Scientific, Stone Ridge, NY, USA). Aliquots of dried leaves (1.0 g) underwent ultrasound-assisted extraction using an ultrasonic bath (Branson M3800, Carouge, Switzerland) at 40 kHz frequency. A hydroalcoholic solution (H_2O:MeOH; 1:1, v:v) was used as extracting solvent with a drug/solvent ratio equal to 1:5. 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 four crude extracts, RgSu (*R. graveolens* summer extract), RgAu (*R. graveolens* autumn extract), RgWi (*R. graveolens* winter extract) and RgSp (*R. graveolens* spring extract), which 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, 80% B at 40 min, and 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 (Diode-Array Detector) acquisition range was 190–450 nm.

The liquid chromatography (LC) system was coupled to a LTQ-XL mass spectrometer (Thermo Scientific Vienna, Austria) with an ESI (Electrospray Ionization) 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; capillary voltage set at −16 V. Mass spectra were recorded between *m/z* 50–2000. 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. To obtain further structural information, these ions were trapped and fragmented to yield the product ions patterns of the analytes. The constituents were identified by comparison of their specific chromatographic data, including UV spectra and MS/MS fragmentation patterns to literature data and reference compounds. Metabolites content was expressed relative to quercetin used as external standard. For this purpose quercetin calibration curves were prepared using ten different concentrations of the flavonol (3.06, 6.125, 12.5, 25.0, 50.0, 100.0, 400.0, 600.0, 800.0, and 1000.0 μg/mL) injected in the same conditions of the samples.

2.3. Determination of total phenols

Total phenol amount of investigated crude extracts was determined according to the Folin–Ciocalteau procedure, as reported by Pacifico et al. (2012). Analyzed samples (1.0 mg/mL in DMSO) were mixed with 0.500 mL of Folin–Ciocalteau reagent (FCR) and 4.0 mL of Na_2CO_3 (7.5% w/v). After stirring reaction mixture at room

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