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Phytochemical profiling of underexploited Fabaceae species: Insights on the ontogenic and phylogenetic effects over isoflavone levels

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ABSTRACT

There is an increasing trend towards finding alternative sources of valued phytochemicals due to their diverse potentialities in food industry and pharmaceutical applications. Phenolic compounds, in particular, have been the focus of several profiling reports, but isoflavones characterization has been studied in fewer cases and in a very limited group of plant species. Despite their acknowledged bioactivity, there's actually a strict number of plants validated for their isoflavones contents. In a previous report, we have identified nine Leguminosae species (from genera *Biserrula*, *Lotus*, *Ornithopus* and *Scorpiurus*) as potential alternative sources of these phenolic compounds. However, the isoflavone profiles are highly modulated by the ontogenic stage. Therefore, the present study was conducted in the same Leguminosae species, but harvested at three sequential vegetative development stages: vegetative elongation, late bud and late flowering, with the main purpose of assessing the evolution of isoflavones content throughout the plant development. In general, the plant species from *Biserrula* and *Lotus* genera showed the highest potential as new natural sources of isoflavones, especially owing their high levels of biochanin A. Independently of the plant species, it was possible to identify the phenologic stages where each of the quantified isoflavones is maximized. These findings are useful to predict isoflavone yields according to harvesting time, validating the potential use of the studied plants in innovative food formulations.

1. Introduction

Fabaceae (or Leguminosae) is the third largest flowering plant family (surpassed only by Orchidaceae and Asteraceae), comprising 19,325 species organized in 727 genera. From an economical point of view, Fabaceae is actually the second most important plant family, being mainly used as a source of food and phytochemical compounds (Boerma, John, & Molen, 2001). Curiously, fewer than 50 species provide 90% of the typical requirements, such as supplying human food, edible oils, animal fodder and forage, nitrogen fixation or providing a diversity of phytochemicals (Lewis, Schrire, Mackinder, & Lock, 2005). This situation creates a serious need for alternative Fabaceae species with the potential to be used for similar purposes. In the case of isoflavone production, the studied species are usually limited to soybean (*Glycine max* L.) and red clover (*Trifolium pratense* L.). Nevertheless, some alternative species are being studied to evaluate their potential use as isoflavone sources (Barreira et al., 2015; Jacobs, Wegewitz, Sommerfeld, Grossklaus, & Lampen, 2009; Spínola, Llorent-Martínez, Gouveia-Figueira, & Castilho, 2016; Visnevschi-Necrasov et al., 2014, 2015). The species studied in this work (*Biserrula pelecinus* L., *Lotus conimbricensis* Brot., *Lotus subbiflorus* Lag., *Ornithopus compressus* L., *Ornithopus pinnatus* (Mill.) Druce, *Ornithopus sativus* Brot., *Scorpiurus muricatus* L., *Scorpiurus vermiculata* L. and *Scorpiurus vermiculatus* L.) are good examples of alternative sources of isoflavones (Visnevschi-Necrasov et al., 2015). In general, these species are self-regenerating annual pasture legumes, nutritious and palatable, having deep radical system, good quality of hay and silage, compatibility with summer-growing perennial grasses and tolerance to high levels of soil aluminum (Hackney, Dear, & Crocker, 2007). In some cases, they are inclusively tolerant to acid, sandy and duplex soils, allowing their cultivation in highly deprived regions (Nandasena et al., 2004). In addition, they have high resistance against insects and might control weeds that are not destroyed by herbicides, having also important actions (especially

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the species belonging to *Lotus* genus) in bacteria-plant symbiosis, mycorrhizal interactions and nitrogen metabolism (Diaz, Borsani, & Monza, 2005).

From a phytochemical point of view, these species produce health-promoting secondary metabolites that can exert important effects on human health (*e.g.*, blood cholesterol-reduction, hypoglycemic action, prevention of certain types of cancer, and protective effects against atherogenesis, angiogenesis, nervous system diseases, menopausal symptoms and osteoporosis) (Gepts et al., 2005; Mortensen et al., 2009; Sacks et al., 2006). Isoflavones, due to their structural resemblance with estradiol, have been recognized for their ability to decrease the morbidity rates linked to age-related cardiovascular diseases and osteoporosis, breast and prostate cancers, and menopausal symptoms (Cano, García-Pérez, & Tarín, 2010; Mourouti & Panagiotakos, 2013; Park & Weaver, 2012). These properties might render important pharmaceutical applications to these plant species, but their phytochemical profiles must be accurately characterize before being included in any novel product (Food Safety Commission Novel Foods Expert Committee, 2006; U.S. Department of Agriculture, 2008), particularly when those natural compounds are indicated as exerting some deleterious effects, such as the previously reported genotoxicity of isoflavones (Azarova et al., 2010; Setchell & Cassidy, 1999; Ye et al., 2001).

Besides the genotypic factors, the ontogenic stage is an important modulator of isoflavones profile (Barreira et al., 2015; Bednarek et al., 2001; D'Agostina, Boschin, Resta, Anniccniarico, & Arnoldi, 2008; Tsao, Papadopoulos, Yang, Young, & McRae, 2006). To avoid biased effects induced by environmental factors, all plants were harvested in an experimental field. Isoflavones were extracted from the leaves of the nine selected species by matrix solid-phase dispersion (MSPD), and studied for the first time throughout different ontogenic stages: i) vegetative elongation, ii) late bud and iii) late flowering, allowing understanding profile changes along the plants' growth.

2. Materials and methods

2.1. Standards and reagents

Purity-corrected individual isoflavone stock solutions (1 g/L) were prepared in methanol:H_2O (75:25, v/v). A composite stock standard solution of multiple isoflavones was prepared in methanol containing 40 mg/L of each standard: biochanin A (≥97%), puerarin (≥99%), glycitein (≥97%), daidzein (≥98%), daidzin (≥95%), prunetin (≥98%), genistein (≥98%), genistin (≥95%) and formononetin (≥99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA); pratensein, pseudobaptigenin and irilone (all ≥98%) were from Chromadex Inc. (Barcelona, Spain). The internal standard (IS) 2-methoxyflavone was obtained from Sigma. A working IS solution was prepared in methanol at 1 g/L. All the solutions were stored at −20 °C in amber glass vials when not in use.

Methanol (HPLC grade) and formic acid (analytical grade) were from Merck (Darmstadt, Germany). Purified demineralized water was from a "Seradest LFM 20" system (Seral, Ransbach-Baumbach, Germany). The eluents were filtered through 0.45 μm filters and degassed under reduced pressure and ultrasonic bath. Disposable syringe filter PTFE 0.45 μm was from Macherey-Nagel (Düren, Germany). The C_{18}-bonded silica (particle size 55–105 μm) used as sorbent for MSPD was from Waters (Milford, MA, USA). The limits of quantification and detection as well as the recovery of the method were acceptable as described previously (Visnevschi-Necrasov et al., 2015).

2.2. Plant material and field experimental site

Nine species of four Fabaceae genera (*Biserrula*, *Lotus*, *Ornithopus* and *Scorpiurus*) were cultivated from February to June of 2010, in the experimental field of the University of Porto at the Agrarian Station of Vairão, in Portugal. No chemical fertilizers were used and plants were not inoculated with nitrogen-fixing bacteria. Samples were collected in three phenologic stages: 1 - vegetative elongation (stem length <30 cm, no visible buds or flowers); 2 - late bud (three or more nodes with visible buds, no flowers or seed pods); and 3 - late flowering (one or more nodes with 50% open flowers, no seed pods). The vegetal germplasm was obtained from the Portuguese collection of Leguminosae provided by the National Institute of Biological Resources (Instituto Nacional dos Recursos Biológicos, I.P.). Voucher specimens of each species were numbered and deposited in the local herbarium.

For each species, three independent samples were selected (in different locations within the limits of the indicated Experimental Field) consisting of fresh leaves from randomly selected plants (5 plants for each accession) belonging to 2 different accessions; samples were dried at 65 °C for 72 h and milled, at particle size of 0.1 mm, using an A11 analysis mill (IKA Werke, Staufen, Germany). Samples were stored in silicone tubes at room temperature.

2.3. Extraction procedure

MSPD extraction of isoflavones was applied with small modifications of a previous method (Visnevschi-Necrasov et al., 2015). An aliquot of 500 mg of the previously milled dried sample, 2 g of C_{18} and 40 mg/kg of 2-methoxyflavone (200 μL at 100 mg/L), used as internal standard, were placed in a glass mortar and blended with glass pestle for 2–3 min. This mixture was then transferred to an empty column connected to a vacuum system. The column was washed with 10 mL of distilled water (reddish-brown phase eluted from the column) and the isoflavones were eluted with 5 mL of methanol:H_2O (9:1, v/v). Before HPLC analysis, the extracts collected in amber vials were filtered through a 0.45 μm PTFE membrane. Different samples of two distinct accessions of all species were extracted.

2.4. HPLC determination of isoflavones

Chromatographic analyses were performed with a Jasco (Tokyo, Japan) high-performance liquid chromatograph equipped with a PU-2080 quaternary pump and a Jasco AS-950 automatic sampler with a 20 μL loop. Detection was performed with a Jasco model MD-2010 multi-wavelength diode-array detector (DAD). Chromatographic separation of the compounds was achieved with a Luna 5 U C_{18} column (5 μm, 150 × 4.60 mm; Teknokroma, Barcelona, Spain) operating at 40 °C. The eluent was a gradient of acetonitrile (A) and 0.1% formic acid (B), at a flow rate of 1 mL/min, with a linear gradient as follows: 0 min 33% B, 7 min 45% B, 15 min 50% B, 25 min 60% B, 30 min 70% B, 35 min 50% B, 37 min 33% B, maintaining these conditions for 10 min and returning to the initial ones after 3 min. Data were analyzed using the Borwin-PDA Controller Software (JMBS, Le Fontanil, France). Compounds were identified by chromatographic comparisons with authentic standards and UV spectra. Quantification was made by DAD at 254 nm based on the internal standard (2-methoxyflavone) method.

2.5. Statistical analysis

Two accessions of each species and phenologic stage were used. From each accession, three independent extractions were performed, and each of the extracts was injected twice, resulting in 36 values/species (2 accessions × 3 stages × 3 extractions × 2 injections). Data were expressed as means ± standard deviations. All statistical tests were performed at a 5% significance level using the SPSS software, version 22.0 (IBM Corp., Armonk, NY, USA).

Initially, the effects of "plant species" (PSp) and "phenologic stage" (PhS) in isoflavone profiles were evaluated through an analysis of variance (ANOVA) with type III sums of squares, performed using the Repeated Measures Analysis procedure of the General Linear Model. Since the independence of variables could not be assumed, there was a need to verify the sphericity criterion, which evaluates if the