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# *Lotus conimbricensis* Brot. as an alternative source of isoflavones

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## ABSTRACT

Legumes (Fabaceae) are important crops, being used in several food applications and as raw materials for industry. Their ability to capture atmospheric nitrogen during symbiotic processes with soil bacteria is important to include these plants in biological agriculture, reducing the need for expensive chemical fertilizers, while improving soil and water quality. Several Fabaceae species are acknowledged for their high levels of secondary metabolites with important biological functions. Isoflavones are a typical example of these compounds, requiring detailed profiling studies due to their differential biological activity. Lotus conimbricensis Brot. represents a scarcely studied Fabaceae species, which might represent an alternative natural source of different bioactive compounds. Herein, L. conimbricensis samples were collected in different physiological stages along the vegetative cycle. Their isoflavones fractions were extracted using matrix solid-phase dispersion and analyzed by high performance liquid chromatography/diode-array detection. Eight different isoflavones were quantified: genistin, daidzein, glycitein, genistein, pratensein, irilone, prunetin and biochanin A. The levels in the latest isoflavone are particularly relevant because biochanin A is a O-methylated isoflavone, with diverse health effects, such as neuroprotective, cardioprotective, nephroprotective, antitumoral, antiviral and anti-inflammatory. Furthermore, it is usually found in relatively low amounts in most plant species, with few exceptions (such as it is observed for red clover). Furthermore, biochanin A might suffer demethylation by human hepatic enzymes, yielding genistein, which is also acknowledged for its biological activity. Accordingly, L. conimbricensis might represent a promising new source of isoflavones, with particular relevancy for biochanin A.

## 1. INTRODUCTION

There is an increasing trend towards finding alternative sources of valued phytochemicals due to their diverse potentialities in food industry and pharmaceutical applications. Legumes

(Fabaceae) are actually as the second most important plant family, being mainly used as a source of food and phytochemical compounds [1] Several Fabaceae species are acknowledged for their high levels of secondary metabolites with important biological functions (*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) [2]. Isoflavones, due to their structural resemblance with estradiol, have been recognized for their ability to decrease the morbidity rates related to age-related cardiovascular diseases and osteoporosis, breast and prostate cancers, and menopausal symptoms [3]. In the case of isoflavone production, the studied are usually limited to soybean (*Glycine max* L.) and red clover (*Trifolium pratense* L.). Nevertheless, Fabaceae species are being studied to evaluate their potential use as isoflavone sources with important pharmaceutical applications [4]. The *O*-methylated isoflavone biochanin A are particularly relevant because their diverse health effects, such as neuroprotective, cardioprotective, nephroprotective, antitumoral, antiviral and anti-inflammatory [5]. Therefore, the present study was conducted in leaves of *Lotus conimbricensis* Brot. a scarcely studied Fabaceae species, but harvested at three sequential vegetative development stages: i) vegetative elongation, ii) late bud and iii) late flowering, with the main purpose was assessing the evolution of isoflavones content throughout the plant development. *Lotus conimbricensis* Brot. phytochemical profiles must be accurately characterize before being included in any novel product, particularly when those natural compounds are indicated as exerting some deleterious effects, such as the previously reported genotoxicity of isoflavones [6].

## 2. MATERIALS AND METHODS

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). Three independent samples were selected (in different locations within the limits of the indicated Experimental Field) consisting in fresh leaves belonging to 2 different accessions; samples were dried at 65 ºC during 72 hours 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.

### 2.1 Extraction procedure

Matrix solid-phase dispersion (MSPD) extraction of isoflavones was applied with small modifications of a previous method [5]. 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 during 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.

## 2.2 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 5U $C_{18}$ column (5 μm, 150×4.60 mm; Teknokroma, Barcelona, Spain) operating at 40 ºC. 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.

## 3. RESULTS AND DISCUSSION

Besides being unequally distributed in the plant tissues and modulated by biotic and abiotic stress factors, isoflavones content depends on the ontogenic stage of a specific plant. However, the research on the isoflavones evolution during plant development is rather scarce [7]. Herein, eight different isoflavones were quantified in the different development stages of *L. conimbricensis*: six in the vegetative elongation and late bud stages and seven in late flowering (**Table 1**).

**Table 1.** Isoflavone contents (mg/kg of dry matter) in the leaves of the evaluated *L. conimbricensis*.

| Isoflavones | Vegetative elongation | Late bud | Late flowering |
|---|---|---|---|
| Genistin | 5.3±0.3 | 15±10 | 11.2±0.3 |
| Daidzein | nd | nd | 10.1±0.3 |
| Glycitein | nd | nd | 12.3±0.5 |
| Genistein | 33±1 | 87±15 | 97±1 |
| Pratensein | 80±3 | 149±22 | 119±2 |
| Irilone | 17±1 | 25±8 | 18±1 |
| Prunetin | 20±1 | 10±4 | nd |
| Biochanin A | 2750±81 | 3728±595 | 3092±91 |
| **Total** | 2907±84 | 4015±530 | 3360±92 |

nd: non detected

The levels in biochanin A are particularly remarkable, being inclusively higher than those showed by red clover, which is the most acknowledged source of this isoflavone. In fact, the high contents of biochanin A detected in *L. conimbricensis* represent a promising result considering that this *O*-methylated isoflavone is acknowledged for its neuroprotective, cardioprotective, nephroprotective, antitumoral, antiviral and anti-inflammatory activities [8]. Furthermore, biochanin A might suffer demethylation by human hepatic enzymes, yielding genistein, which is also acknowledged for its biological activity [9].

## 4. CONCLUSION

Biochanin A is usually found in relatively low amounts in most plant species, with few exceptions (such as it is observed for red clover). Accordingly to our results, *L. conimbricensis* might represent a promising new source of biochanin A. In addition to the health effects of this isoflavone, *e.g.*, neuroprotective, cardioprotective, nephroprotective, antitumoral, antiviral and anti-inflammatory, biochanin A might suffer demethylation by human hepatic enzymes, yielding genistein, which is also acknowledged for its biological activity. Despite the acknowledged variation of isoflavones throughout the plant development, the levels of bioachanin A were very similar among the studied growth stages. This might represent a useful advantage regarding the exploitation of *L. conimbricensis* as a natural source of this particular isoflavone.

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