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Iridoid and caffeoyl phenylethanoid glycosides of the endangered carnivorous plant *Pinguicula lusitanica* L. (Lentibulariaceae)

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ABSTRACT

This work reports for the first time the identification of the major compounds of *Pinguicula lusitanica*, an endangered carnivorous plant species, using minimal amounts of plant material. A methanol extract was prepared from in vitro cultured plantlets and analyzed by HPLC–SPE–NMR/HPLC–MS. Three iridoid and five caffeoyl phenylethanoid glycosides were identified. These groups of natural compounds were previously reported in the Lentibulariaceae family and have been used as chemotaxonomic markers in related families.

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1. Introduction

Pinguicula lusitanica L., commonly known as the Pale butterwort, is a small rare carnivorous perennial plant with pharmacological value and limited reproductive capacity. The plant grows wild in areas along coastal western Europe from Scotland to Iberia and Morocco in north-western Africa (Heslop-Harrison, 2004). The butterworts constitute the second most diverse genus of the carnivorous Lentibulariaceae, with 85 currently accepted species (Cieslak et al., 2005).

Wild populations of this species are becoming increasingly scarce, probably due to the fact that reproduction of *P. lusitanica* relies entirely on seed production. Moreover, seedling establishment in a suitable environment is crucial because seedlings do not withstand competition (Heslop-Harrison, 2004). An efficient micropropagation protocol for *P. lusitanica* has been published recently (Gonçalves et al., 2008), which enabled this chemical study, as the natural populations cannot provide sufficient material for analysis due to their reduced number of individuals.

There are no reports on the biochemical or physiological data of *P. lusitanica*. In fact, only two of the some 85 species of the *Pinguicula* genus have been subject of a biochemical study, namely *Pinguicula vulgaris* (Damtoft et al., 1985; Marco, 1985) and *Pinguicula moranensis* (Damtoft et al., 1994). Several iridoid glucosides were identified in these two species, and a caffeoyl phenylethanoid glycoside, namely verbascoside, was found in *P. moranensis* as well. These groups of compounds are characteristic for the Lentibulariaceae and related families (Order Lamiales), and have proven to be good chemotaxonomic markers in several related families (Rønsted et al., 2003; Jensen et al., 2005; Taskova et al., 2005; Pedersen et al., 2007).

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The function of phenylethanoid glycosides in plants can be resistance to, or protection from, fungal or viral attacks (Jiménez & Riguera, 1994). The benefit of iridoid glucosides for the plant is not clear, although it has been demonstrated that the iridoid aucubin is a strong protein denaturant when hydrolyzed by the enzymes in the plant. This observation indicates that iridoid glucosides may play an important role in the defense mechanism against herbivores (Konno et al., 1999; Jensen et al., 2002). To better understand the biological function of these compounds, further chemical investigation on other *Pinguicula* species is needed.

The interfacing of liquid chromatography with NMR spectroscopy together with the introduction of on-line solid-phase extraction (SPE) provides structural information directly from crude extracts, allowing to analyze small biological samples (Albert, 2002; Clarkson et al., 2005; Exarchou et al., 2005; Jaroszewski, 2005), important in the study of endangered plant species such as *P. lusitanica*.

This is the first report of the chemical screening of the major secondary metabolites of *P. lusitanica* by HPLC–SPE–NMR without using field specimens for obtaining plant material.

2. Materials and methods

2.1. Plant material

In vitro plantlets of *P. lusitanica* were produced according to the protocol described by Gonçalves et al. (2008). The original plant material, specimen number LISU215272, was authenticated by Dr. A. I. Correia from the Botanical Garden of the University of Lisbon.

2.2. Sample preparation

In vitro cultured *P. lusitanica* plantlets (50 g) were extracted twice for 24 h with methanol (HPLC-grade, Fluka, Buchs, Switzerland). The obtained extract was filtered (Whatman no 1, Springfield Mill, England), concentrated under rotary vacuum evaporation and dissolved in water. After freeze drying, 760 mg of extract was obtained. A solid-phase extraction column (SUPELCLEAN™ LC-18 Packing; 60 mL; 10 g) was used for sample preparation to remove the most apolar and fatty constituents of the extracts and served as a sample cleanup. For analysis, 25 mg of cleaned extract were dissolved in 500 μL of 50% methanol solution in water.

2.3. HPLC–MS/HPLC–SPE–NMR

The HPLC–SPE–NMR/HPLC–MS measurements were performed with a Agilent 1200 quaternary solvent delivery pump equipped with a Spark Prospekt 2 solid-phase extraction (SPE) device, containing HySphere resin SH cartridges (10 × 2 mm, 25–35 μm), a Bruker Avance III 600 spectrometer, a Bruker Daltonics MicrOTOF ESI mass spectrometer and HyStar 3.2 software. Topspin 2.0 software was used for NMR experiments. The chromatographic separation was conducted with an Alltima HP 3 μm (150 × 4.6 mm i.d.) column using a binary eluent consisting of water containing 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B), with a 0.6 mL/min flowrate. The following linear gradient was applied: at 0 min 10% B; at 5 min 15% B; at 43 min 25% B; at 45 min 95% B; at 47 min 95% B; and at 50 min 10% B followed by a 5 min conditioning step. The injection volume was 20 μL. The HPLC eluate was monitored by DAD, and for each compound one absorption threshold at 220 (**1**), 280 (**2**–**7**), or 312 (**8**) nm was defined in order to provide start and stop signals for the SPE trappings. A total of four cumulative trappings were performed for each peak selected for analysis. The cartridges were dried with nitrogen gas and the analytes were eluted with methanol-d_4 to the NMR flow probe. ^{1}H NMR spectra were recorded at 600 MHz using standard pulse sequences delivered by Bruker. After NMR experiments, the cartridge content was flushed from the probe in to vials and analyzed by ESI–MS. Ions were detected in negative mode in the range of m/z 100–1500. The MS working conditions were as follows: the nebulizer pressure was 3.0 bar, the drying gas flow equalled 8.0 L/min, the drying gas temperature was 190 °C, and the capillary spray was 4.2 kV.

3. Results and discussion

The main compounds identified in this study in the *P. lusitanica* extract were iridoid glucosides and caffeoyl phenylethanoid glycosides. Eight peaks were separated by chromatography and selected for analysis (Fig. 1). The identified iridoid glucosides were mussaenosidic acid (**1**) (Çaliş et al., 1993), scutellarioside II (**4**) (Çaliş et al., 1993), and scutellarioside I or globularin (**8**) (Damtoft et al., 1985). Another five caffeoyl phenylethanoid glycosides, namely verbascoside or acteoside (**7**), R and S campneoside II (**5**,**6**) and R and S campneoside I (**2**,**3**) were identified by comparison with literature data (Wu et al., 2004). The chemical structures of the identified compounds are shown in Fig. 2.

Caffeoyl phenylethanoid glycosides are characteristic for most taxa in Lamiales, and the most common representative, verbascoside, has been reported in all the families of Lamiales. Only two examples are known from outside the order (Jensen, 1992; Taskova et al., 2005). Iridoid glucosides are characteristic for Lamiales, but they are more widespread than caffeoyl phenylethanoids. However, specific compounds can be systematically very useful. Aucubin and catalpol, for instance, are characteristic for most taxa within Plantaginaceae (Taskova et al., 2005).

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