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Recovery of high purity plumbagin from *Drosera intermedia*

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ABSTRACT

In this study a simple process encompassing an efficient extraction and fractionation method to obtain high purity plumbagin from micropropagated *D. intermedia* plants is described. Plumbagin is a naphthoquinone derived from a diverse but restricted group of plant species that includes the *Drosera* spp. and is in demand for pharmacological research. It was brought initially to the attention of researchers due to its broad antimicrobial and antitumor properties but has continued to find application against new pharmacological targets. The procedure described in this work involved testing four extraction methods to maximize product recovery and separating the 30–50% acetonitrile in water fraction (v/v) on a SPE column followed by lyophilisation. By applying ultrasonic treatment to the plant matrix leached in *n*-hexane followed by a single step purification process, 2.74 mg of plumbagin per gram of plant material could be obtained with a recovery of 86.3% and over 99% purity.

1. Introduction

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone; Fig. 1) is the most important secondary metabolite isolated so far from carnivorous plants (Eilenberg et al., 2010). Despite the high cytotoxicity of this naphthoquinone, which has limited its medical application, it has been shown recently that at subtoxic concentrations, plumbagin is effective against several pharmacological targets and has regained the interest of researchers in the field of drug discovery (Checker et al., 2010; McKallip et al., 2010; Son et al., 2010).

Many higher plants are major sources of useful secondary metabolites that are used in pharmaceutical, agrochemical, flavour and aroma industries. Attempts to synthesise plant-derived active principles have largely been successful, but in most cases this has proved to be uneconomic in comparison to isolation from plant material (Wink et al., 2005). Isolation of high purity phytochemicals from complex plant matrixes can be a demanding task (Kale et al., 2007; Vismaya et al., 2010), therefore, the development of simple and cost effective downstream processes are imperative to achieve overall economical viability. *Drosera intermedia* Hayne is a carnivorous plant species known to produce plumbagin, which can easily be produced *in vitro* at large scale (Grevenstuk et al., 2010). There are a few reports on the extraction of plumbagin from *Drosera* plants for analytical purposes (Marczak et al., 2005; Wang and Huang, 2005). However, a downstream process for recovering plumbagin applicable to industries has not been studied yet.

In this contribution, four methodologies were compared to maximize the recovery yields of plumbagin from *Drosera* plants, including maceration, Soxhlet extraction (SE), ultrasound-assisted extraction (UAE) and supercritical fluid extraction (SFE). Furthermore, the viability of solid phase extraction (SPE) for product recovery and purification was evaluated.

2. Experimental

2.1. Plant material and extraction

All extraction procedures were performed in triplicate with 5 g of fresh micropropagated plants of *D. intermedia* produced according to Grevenstuk et al. (2010). The plants were authenticated by Dr. A.I. Correia from the Botanical Garden of the University of Lisbon where a specimen voucher has been preserved under the number LISU231581. Prior to extraction, the plant material was ground in a mortar with liquid nitrogen to reduce particle size. Solvent extractions were performed with *n*-hexane using the same volume of solvent (150 mL) to compare extraction efficiency. Supercritical CO_2 without modifiers was the solvent of choice for SFE.

Abbreviations: SE, Soxhlet extraction; UAE, ultrasound-assisted extraction; SFE, supercritical fluid extraction; SPE, solid phase extraction.

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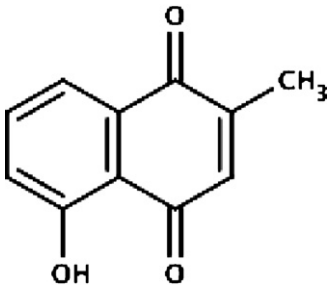

Fig. 1. Structure of plumbagin.

Plant material was extracted twice for 24 h by maceration at room temperature under mechanical stirring. Soxhlet extraction was performed until exhaustion to prevent eventual thermal degradation of plumbagin, which coincided with 1 h extractions. For UAE, the plant–solvent mixture was placed in an Erlenmeyer flask and immersed into a Bandelin Sonorex Super RK103H (Bandelin Electronic, Berlin, Germany) ultrasound bath operating at 35 kHz. A period of 1 h sonication at room temperature was chosen to guarantee maximum product recovery. The water of the bath was renewed after each extraction to prevent overheating.

SFE procedure was carried out in a semi-batch flow extraction apparatus built at IST, Technical University of Lisbon (Esquível and Bernardo-Gil, 1993). The operation parameters were based on an optimized protocol for extraction of plumbagin (Rodrigues et al., 2006). In brief, plant material was extracted at 20 MPa, 40 °C and at an approximate flow rate of 3.62 g/min during 2 h. The CO_2 was supplied by ARLIQUIDO-Portugal in bottles as a 99.5% pure fluid.

2.2. Sample treatment

All extracts, except the one obtained by SE, were filtered (Whatman no 1, Springfield Mill, England), centrifuged (Heraeus Megafuge 1.0R, Osterode, Germany) and the supernatants were evaporated under vacuum on a rotary evaporator at 40 °C. The extraction yields were registered and the dry samples were dissolved in a 50% aqueous acetonitrile solution at 2.5 mg/mL for further analysis.

2.3. SPE procedure

A SPE column (SUPELCLEAN™ C18 Packing; 60 mL; 10 g) was used to clean an aliquot of each sample. The distinct yellow coloration of plumbagin makes it possible to elute plumbagin selectively from a SPE column using an appropriate gradient. Each sample was loaded onto a pre-conditioned column and the most polar impurities were eluted with 100 mL of 30% aqueous acetonitrile solution. A volume of 100 mL of 50% aqueous acetonitrile solution was used to elute plumbagin and collected. The fraction was evaporated under vacuum to remove the organic solvent and the water removed by lyophilisation. The recovery yields were obtained by weighing the dry sample.

2.4. Plumbagin quantification

To determine the content in plumbagin, the obtained samples were analyzed using high performance liquid chromatography with diode array detection (HPLC–DAD) with a setup as described by Silva et al. (2009). In brief, the setup consisted of an Agilent 1100 series LC system (Agilent Technologies, Waldbronn, Germany), composed by the following modules: vacuum degasser (G1322A), quaternary pump (G1311A), autosampler (G1313A), thermostated column compartment (G1316A), and diode array detector (G1315B). Data acquisition and instrumental control were performed using LC3D ChemStation (Agilent Technologies) software. Analyses were performed on a Mediterranean Sea 18 column (150 × 4.0 mm, 5 μm particle size; Teknokroma, Barcelona, Spain) adapted with an Ultraguard Sea 18 precolumn (10 × 3.2 mm; Teknokroma, Barcelona, Spain) and the mobile phase consisted of acetonitrile (A) and water. The applied gradient was: 0–30 min, 10–90% A; 30–35 min, 90–100% A; 35–40 min, hold at 100% A; 40–45 min, 100–10% A; and hold at 10% A for 15 min. The flow rate was 0.5 mL/min and the injection volume 10 μL. The analyses were performed at 25 °C and the detector was set at 254 nm. The crude extracts and SPE products were injected at 1.0 mg/mL and 0.5 mg/mL, respectively. The plumbagin concentration of the samples was determined by plotting a standard curve, using calibration solutions of plumbagin (0.2, 0.4, 0.6 and 0.8 mg/mL) (Sigma, Steinheim, Germany) prepared in 50% aqueous acetonitrile solution and injected in triplicate. Plumbagin concentration was used to calculate sample purity by determining the mass fraction of plumbagin of each injected sample.

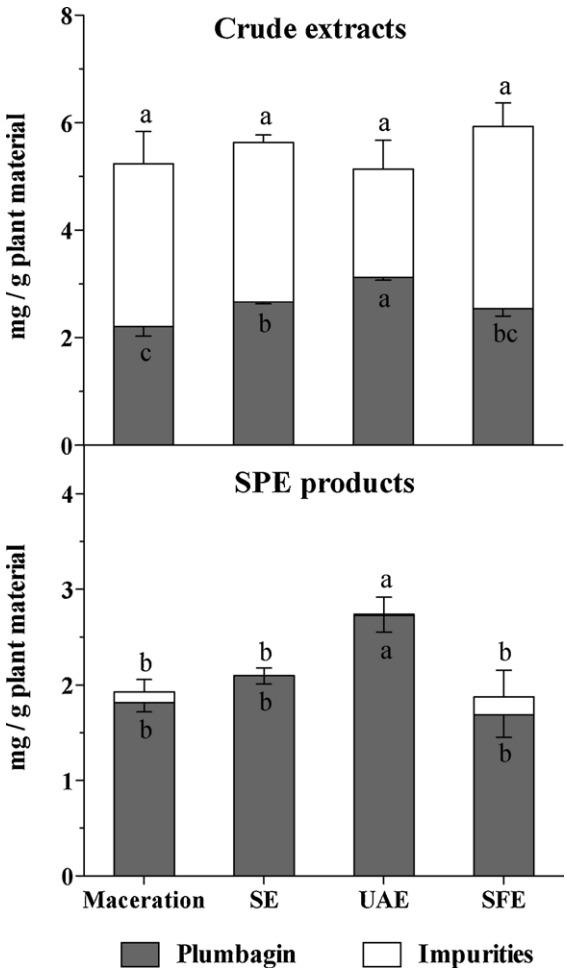

Fig. 2. Plumbagin content of crude extracts and SPE products obtained from *D. intermedia* using different extraction methods. Each value represents mean ± standard error of 3 repetitions. For each crude extract, SPE product or plumbagin content, values followed by the same letter are not significantly different according to Duncan's test ($P < 0.05$).

2.5. Statistical analysis

The data were subjected to analysis of variance (ANOVA) to assess if there were significant differences between the extraction procedures. Significant differences between means were