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Recovery of betulinic acid from plane tree (*Platanus acerifolia* L.)

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ABSTRACT

Betulinic acid (3β, hydroxy-lup-20(29)-en-28-oic acid) is a bioactive triterpenic acid which was identified in various botanical sources and in considerable amounts in the bark of plane tree (*Platanus acerifolia* L.). In this work, the recovery of betulinic acid from plane tree bark was studied using different liquid solvent based extraction methods, namely solid–liquid extraction (SLE), ultrasound assisted extraction (UAE) and pressurized liquid extraction (PLE). Furthermore, preliminary studies of the supercritical fluid extraction (SFE) of plane tree bark are also reported.

The liquid solvent based extraction techniques (SLE, UAE and PLE) were carried out using ethanol and ethyl acetate, and produced a recovery of betulinic acid in the range 10–15 mg/g of bark, with concentrations around 25–35% mass. A betulinic acid enrichment in the ethanolic extracts was possible by means of a simple precipitation step adding water. The precipitate contained 42–46% mass of betulinic acid and high recovery (>95%). Increasing the extraction temperature, by means of the PLE assays, has not resulted in an improvement of betulinic acid recovery.

The preliminary SFE assays produced lower recoveries of betulinic acid (0.5–8 mg/g) with respect to liquid extraction. The addition of ethanol as cosolvent produced a significant improvement of both betulinic acid recovery and concentration in the SFE extract.

1. Introduction

Triterpenic acids are secondary plant metabolites that are widespread in plants, mainly located in the peel, leave and stem bark [1]. They are part of the chemical family of isoprenoids, owning polycyclic structures of thirty carbon atoms, and presenting very low solubility in water and hydrophilic solvents. On the other hand, their solubility in organic solvents such us acetone or methanol has been demonstrated to be moderately high [2].

Betulinic acid (3β, hydroxy-lup-20(29)-en-28-oic acid) is a triterpenic acid which can be isolated from various botanical sources, including clove (*Syzygium aromaticum*), Lamiaceae herbs such as rosemary (*Rosmarinus officinalis*) and java tea (*Orthosiphon stamineus*), and the bark of several betula species (birch trees), eucalyptus (*Eucalyptus globulus*) and plane (*Platanus acerifolia*) trees [1,3–8].

Betulinic acid as well as its derivatives, have demonstrated a wide range of biological activities, including anti HIV-1 activity [9], anti-inflammatory activity [10], antimalarial activity [11], anticancer and apoptotic activity [12,13]. Additionally, it has been demonstrated that some changes in betulinic acid structure can lead to significant differences in its anticancer and antiproliferative activity [14,15].

The presence of betulinic acid at concentrations up to 3% (30 mg/g) in the external dried bark of plane tree (*P. acerifolia* L.) was previously reported [1,16,17]. These works focused in the extraction of betulinic acid from the bark of plane tree through conventional solid–liquid extraction with methanol, chloroform and heptane. Nevertheless, to our knowledge, the extraction of phytochemicals present in this botanical source has not been thoroughly studied yet.

Novel liquid solvent based extraction methods include the assistance of solid–liquid extraction using ultrasounds (UAE), and the use of high extraction temperatures by increasing also pressure to maintain the solvent in liquid state (PLE).

The use of UAE to recover triterpenic acids from different plant matrix has been recently studied [18–20] and has proved to present several advantages in comparison with conventional solid–liquid extraction. These advantages include reduction of the amount of solvent required, time and temperature, which represents an important factor when extracting thermolabile compounds [21]. Ultrasonic cavitation enhances mass transfer through its capability

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to facilitate hydrating and swelling of vegetal tissues as well as diffusion and osmotic processes [22].

Also PLE to recover triterpenic acids from different botanical sources has been previously reported [19,23]. PLE uses high pressures in order to remain solvents in liquid state beyond their normal boiling point. The combination of high pressures and high temperatures enhances mass transfer, thus facilitating the extraction process. It has demonstrated several advantages in comparison to traditional extraction procedures, mainly the decrease of both time and amount of solvent. However, the lack of industrial scale pressurized liquid extraction equipments, lead to a moderate application of this technique.

Supercritical fluid extraction (SFE) using carbon dioxide (CO_2) was also utilized to recover triterpenoid acids from different plant matrix, as reported by Domingues et al. [6–8], Felföldi-Gáva et al. [24], De Melo et al. [25] and Zhao [26] among others. Due to its low polarity, supercritical CO_2 has shown a moderate capacity to dissolve this type of compounds and thus, the use of ethanol as cosolvent has been employed as a suitable alternative to increase triterpenic acid recovery. An appropriate combination of pressure and ethanol as cosolvent may increase the yield of triterpenic acids profusely [7].

In this paper different advanced extraction techniques (UAE, PLE and SFE) and conventional solid–liquid extraction (SLE) are studied and compared, with the target of recovering betulinic acid from the bark of *P. acerifolia* L. Different GRAS (General recognized as safe) solvents were utilized (ethanol, ethyl acetate and $SCCO_2$) and different process conditions were investigated.

2. Material and methods

2.1. Chemicals

Ethanol absolute (99.5% purity), Ethyl acetate (99%, purity) was purchased from Panreac (Barcelona, Spain). CO_2 was used as the supercritical solvent with a purity of 99.9% produced by Carburos Metalicos, S.A. (Madrid, Spain). Betulinic Acid reference Standar was purchased from Extrashyntesse (Genay, Cedex, France).

2.2. Analysis

Quantification of betulinic acid (BA) in the extracts was performed by HPLC Agilent 1200 series from Agilent Technologies Inc. (Santa Clara, California, USA) according to a method previously described [19] with some modifications. Briefly, separation was carried out using a C-18 reverse phase column (250 × 4.6 mm, 5 μm), with a mobile phase consisting of HPLC grade acetonitrile-Milli-Q water–phosphoric acid (80:20:0.04, v/v/v). Elution was performed isocratically at a flow rate of 1 mL/min, at 25 °C, and with a total analysis time of 30 min. Injection volume was 10 μL and spectral data was recorded at 210 nm. Data analysis was performed by ChemStation version B.04.03. Samples were prepared using methanol at 0.7 mg/mL. Calibration curves of BA were constructed with reference standard.

2.3. Preparation of sample

4 kg of plane tree bark (*P. acerifolia* L.) were collected in the Campus of Universidad Autónoma de Madrid (Madrid, Spain) and were air dried at ambient temperature for 72 h. The final content of water in the dried sample was determined in an oven at 105 °C (48 h) and resulted 9.5% mass. The bark was ground in a grind Premil 250 (Lleal S.A., Barcelona, Spain) to a mean particles size of 500 μm and packed and stored at room temperature until utilization.

2.4. Extraction techniques

2.4.1. Solid–liquid extraction (SLE)

35 g of ground Platanus bark were extracted with 350 mL of solvent (ethanol or ethyl acetate) at 45 °C using a magnetic stirrer. Extraction time was 1.5 h. The infusion was filtrated in a vacuum flask with a Büchner funnel and the sifted material was washed with 50 mL of solvent. The liquid phase was concentrated at low temperature (35 °C) in a rotavapor (VWR from IKA Works GmbH & Co., Staufen, Germany).

In order to produce a triterpenic acid enrichment, 35 g of raw material were extracted with 350 mL of ethanol, as aforementioned, and the ethanol was removed in rotavapor until 1/3 of the initial volume. Then, an equal volume of deionized water was added, and the mixture was stored at room temperature for 30 min until a white precipitated was formed. The precipitated was collected by filtration and was dried in a freeze dryer from Labconco Corporation (Missouri, USA). The liquid mixture (water/ethanol) was concentrated in rotavapor and freeze-dried.

Extractions were carried out by duplicate and all samples were stored under refrigeration until they were analyzed.

2.4.2. Ultrasound assisted extraction (UAE)

35 g of ground Platanus bark with the corresponding solvent (ethanol or ethyl acetate) in a ratio 1:5 (bark:solvent) were submitted to ultrasounds for 15 min using a 1/2″ diameter disruptor horn probe at 70% amplitude (maximum power output of 400 W at 60 Hz) (Branson Digital Sonifier, Branson Ultrasonics, model 250; Danbury, USA) maintaining temperature at 45 °C. Sonication at the desired amplitude level was started once the set temperature was reached. The ultrasound probe was submerged to a depth of 25 mm in the sample. The input range of the selected variables was determined by preliminary experiments and the UAE conditions were selected on the basis of previous studies reported in the literature [27–30].

Extractions were carried out by duplicate. After sonication, the samples were filtrated and dried in rotavapor. In the case of ethanol experiments, the same procedure described in the case of SLE was applied after UAE in order to attain a triterpenic acid enrichment. All samples were stored under refrigeration until they were analyzed.

2.4.3. Supercritical fluid extraction (SFE)

Extractions were carried out using a pilot plant supercritical fluid extractor from Thar Technology (model SF2000; Pittsburgh, Pensilvania, USA) comprising a 2 L cylindrical extraction vessel (internal diameter = 0.07 m; height = 0.388 m) and two different separators, with 0.5 L capacity each one, independent temperature control (±2 K) and pressure (±0.1 MPa). The extraction device also includes a recirculation system where CO_2 is condensed and pumped up to the desired extraction pressure. A detail explanation of the experimental SFE device employed can be found elsewhere [31].

For each experiment, the extraction vessel was packed with 0.57 kg of ground plane tree bark (apparent density = 381.7 kg/m^3). The extraction conditions are given in Table 4, and were performed at 313 K, pressure range of 25–50 MPa and with an upwards CO_2 flow rate of 50 g/min. The overall extraction time was set to 4 h. Extractions 1 and 2 (see Table 4) were carried out in two different steps: the first step (1.5 h) at 25 MPa and without cosolvent and the second step (2.5 h) at 30 MPa and using, respectively, 10% and 20% of ethanol cosolvent. Extraction conditions were selected on the basis of previous studies reported in the literature [6].

2.4.4. Pressurized liquid extraction (PLE)

Extractions were carried out in an accelerated solvent extraction system ASE 350 from Dionex Corporation (Sunnyvale, CA,

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