J. of Supercritical Fluids 100 (2015) 86–96

Contents lists available at ScienceDirect

The Journal of Supercritical Fluids

journal homepage: www.elsevier.com/locate/supflu

Copaifera langsdorffii supercritical fluid extraction: Chemical and functional characterization by LC/MS and in vitro assays

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ARTICLE INFO

Article history:
Received 21 January 2015
Received in revised form 21 February 2015
Accepted 23 February 2015
Available online 1 March 2015

Keywords:
Copaifera langsdorffii
Supercritical fluid extraction
Response surface methodology
Flavonoids
Kaurenoic acid
Quercitrin
Antioxidant activity

ABSTRACT

Copaifera langsdorffii is a native Brazilian plant containing important amounts of polyphenols and diterpenes with biological activity. To obtain extracts enriched in these two types of compounds, supercritical fluid extraction (SFE) conditions have been optimized by using a response surface methodology (RSM) and considering as factors: extraction pressure and temperature and percentage of co-solvent (ethanol). The response variables selected were the concentration of the two most representative compounds in *C. langsdorffii*, kaurenoic acid (diterpenic acid) and quercitrin (glycosylated flavonol), total flavonoids content, and antioxidant activity (measured by DPPH and TEAC assays). According to the statistical analysis of the experimental design, extraction temperature and ethanol percentage were the main factors influencing the selectivity towards the extraction of the target compounds. Two optimized extracts were obtained: (1) containing high amount of total flavonoids, high concentration of quercitrin and low EC_{50} for the DPPH assay, named HQE—High Quercitrin Extract (100 bar, 70 °C and 50% of ethanol, in the region of gas-expanded liquids, GXLs) and (2) containing high value of kaurenoic acid extract, named HKAE-High Kaurenoic Acid Extract, (390 bar, 70 °C and pure CO_2). Chemical characterization by LC/MS of HQE showed the presence of galloylquinic acids and flavonoids while, on the other hand, HKAE presented only nonpolar compounds such as kaurenoic acid. Moreover, a solid–liquid extraction (SLE) with methanol was carried out as a benchmark protocol for comparative purposes. SFE presented approximately 10-fold higher content of total flavonoids than the extracts obtained by SLE.

1. Introduction

The *Copaifera* sp. genus belongs to the botanical family of *Leguminaseae Juss.*, sub-family *Caesalpinioideae Kunth.*, but it can be also found in the literature by its previous classification: Fabaceae Lindley. *Copaifera* is a native Brazilian plant known popularly as *C. langsdorffii*; only in Brazil more than 20 species from the *Copaifera* sp. genus have been described, all of them with similar therapeutic claims [1,2]. Several scientific studies on *Copaifera* sp. reported important pharmacological activities, among them: neuroprotective, anti-inflammatory, analgesic, antimicrobial, antitumor, antioxidant, wound healing, gastro protective, anti-helminthic, leishmanicide, trypanomicide and muscle relaxant [1–7].

Among the different species of *Copaifera*, *C. langsdorffii* has been suggested as a promising natural source of products for the pharmaceutical market. The oral use of *C. langsdorffii* leaves was reported to be effective in relieving pain and eliminating renal calculi in nephrolitiasic patients [8]. The *in vitro* disruption of oxalate crystals and the *in vivo* assay using ethylene glycol-induced nephrolithiasis in rats was reported by Oliveira and co-workers employing *C. langsdorffii* leaves' extracts [9]. An *in vivo* study reported a decrease of the number and weight in calcium oxalate calculi previously introduced in the bladder of rats treated with hydroethanolic extract of *C. langsdorffii* leaves [10].

Phytochemical investigations on *C. langsdorffii* leaves reported 6 to 10% of phenolic compounds, 3% of the amino acid *N*-methyl-trans-4-hydroxy-L-proline and sesquiterpenes as α-cubebene, α-copaene, ciperene, β-copaene, caryophyllene, β-humulene, muurolene, β-selinene, δ-cadinene and γ-cadinene [11–13]. Furthermore, recent works reported the isolation and identification of kaurenoic acid, caryophyllene oxide, kaurenol, hydroxy-labdaenoic

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http://dx.doi.org/10.1016/j.supflu.2015.02.028
0896-8446/

acid, quercitrin and afzelin [14]. Quercitrin (quercetin-3-*O*-rhaminoside) is a glycosylated flavonol with a molecular weight of 448.38 g/mol, practically insoluble in cold water, moderately soluble in hot water and freely soluble in ethanol [15]; several activities have been described for quercitrin, such as antioxidant [16,17], anti-inflammatory and anti-complement system [18,19], enzymatic inhibition of HMG-CoA reductase and angiotensine-I converser [20], tyrosinase [21], cyclooxygenase-2 [16], myeloperoxidase [22] and inhibition of substances such as nitric oxide [16]. Moreover, in a recent study [23] galloylquinic acids have been identified as compounds present in *C. langsdorffii* leaves. It is well know that galloylquinic acids are very good antioxidant compounds since they can present from one to four molecules of gallic acid linked to a molecule of quinic acid.

On the other hand, diterpene acids, such as kaurenoic acid (also known as ent-kaur-16-en-18-oic acid), are also an important part of *C. langsdorffii* chemical compounds. Kaurenoic acid (Fig. 1) presents a molecular weight of 302.45 g/mol and a p*K*a value of 4.70 ± 0.40, being soluble in hexane and ethyl acetate [24]; diterpenes from kaurane series present many biological effects such as antimicrobial, antifungal [25], anti-inflammatory [26], vasodilatory [27], wound healing [28] and anti-psoriasis [29]. Besides, some previous studies on the oil, extracts, fractions and isolated substances, such as kaurenoic acid, from the *Copaifera* sp. genus reported antitumor activity [30–36].

Extraction protocols for plant antioxidant phenolic compounds are very diverse due to the variation and complexity of phenolic substances and to the variety of vegetable matrices. The extraction yield, phenolic content and antioxidant activity of the extracts are strongly dependent not only on the specific plant material and its bioactive components, but also on the solvent and extraction method employed [37]. There are several extraction processes described in the literature, ranging from more traditional such as maceration, percolation and Soxhlet extraction [38] to modern extraction methods such as those based on the use of compressed fluids (including supercritical fluids (SCF), gas-expanded liquids (GXLs), pressurized liquids and subcritical water extraction) [39]. Supercritical fluid extraction (SFE) offers considerable advantages compared to traditional extraction processes because it provides higher selectivity, shorter extraction times, higher efficiency and do not use toxic organic solvents avoiding the environment and sample contamination [40,41]. SFE has been largely applied to extract different chemical constituents from plants including low [42] and high to medium polarity compounds (such as phenolics) [40,41,43]. Furthermore, to our knowledge this extraction process has never been used to evaluate the compounds' extraction profile from *Copaifera* sp. leaves. Moreover, since the *Copaifera* genus presents polyphenols and diterpene compounds, both with important biological activities, the goal of the present work has been the study and optimization, *via* experimental design, of the supercritical fluid extraction of two representative compounds of these groups: kaurenoic acid and quercitrin, from the *C. langsdorffii* leaves.

2. Materials and methods

2.1. Materials

The leaves of the *Copaifera langsdorffii* Desf., Fabaceae, tree were collected at the Ribeirão Preto *Campus* of the University of São Paulo, SP, Brazil in July, 2012 and a voucher specimen (SPFR 10120) was deposited in the herbarium of the Faculdade de Filosofia, Ciência e Letras de Ribeirão Preto, University of São Paulo. The herbal material was cleaned and dried in ventilated atmosphere of 30 ± 5 °C temperature, milled in a cutting mill to obtain particles between 0.5 and 1.0 mm and stored in a closed plastic bag in fresh and dried environment at 25 °C until its use.

The kaurenoic acid standard was kindly given by the Laboratório de Farmacognosia from Faculdade de Ciências Farmacêuticas de Ribeirão Preto and the quercitrin standard was acquired from Sigma Aldrich (Madrid, Spain).

Acetonitrile (LC–MS quality, LabScan, Gliwice, Poland), water (purified using a Milli-Q system, Millipore Corporation, Billerica, USA) and acidified water with phosphoric acid (Sigma-Aldrich, Madrid, Spain) were used as mobile phases for HPLC analysis.

The solvents used in the supercritical extraction process were CO_2 premier quality provided by Carburos Metálicos (Air Products Group, Madrid, Spain) and ethanol absolute 99% provided by Panreac Quimica S.A. (Barcelona, Spain). Methanol was purchased from Sigma-Aldrich (Madrid, Spain).

2,2-Diphenil-1-pycril hydrazyl hydrate (DPPH, 99%), gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, ≥97%), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS, ≥99%) and $AlCl_3$ anhydrous were purchased from Sigma-Aldrich (Madrid, Spain).

2.2. Solid–liquid extraction

A solid–liquid extraction (SLE) was performed as a reference extraction in order to compare the results of yield, total flavonoids, kaurenoic acid and quercitrin concentration and antioxidant activities with the extracts obtained by SFE. For that, 1 g of the dried milled leaves of *C. langsdorffii* was weighed in a flask and added of 50 mL of methanol. The extraction was kept for 24 h at room temperature under orbital shaking of 150 rpm. After this time, the extracts were filtered and dried under vacuum.

2.3. Supercritical fluid extraction

Supercritical fluid extractions were performed in a homemade supercritical fluid extractor using pumps for CO_2 and ethanol PU2080 from Jasco (Tokyo, Japan) and a manual back pressure regulator LF-540 from Pressure Tech (Hadfield, United Kingdom). Three factors were considered for the optimization of the SFE process: pressure (100–400 bar), temperature (40–70 °C) and the amount of ethanol (0–50%) that was used as co-solvent. Optimization was performed using a Box–Behnken experimental design. Experiments were carried out in randomized order. A kinetic study of the extraction process at the central conditions of the experimental design was carried out to select the extraction time that was set at 120 min. The other factors were maintained constant: carbon dioxide flow rate, 1 mL/min (measured at 50 bar) and mass of plant, 1 g.

Once obtained the optimized conditions according to the results of the Box–Behnken design and the statistical analysis, the scale up of the extraction conditions to obtain the extract enriched in kaurenoic acid was performed in a Helix Spe-ed SFE equipment (Applied Separations, Inc, Allentown, PA, USA) which was loaded with 50 g of *C. langsdorffii* dried and milled leaves. The other conditions as pressure, temperature and co-solvent were selected as provided by the experimental design.

2.4. Physico-chemical extract characterization

2.4.1. Yield (θ)

To obtain the soluble solids (SS) extracted in the experiments, a thermogravimetric method was used. For that, the liquid extracts were dried in an evaporator under vacuum and, after full dryness,