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Supercritical carbon dioxide extraction of compounds from *Schinus terebinthifolius* Raddi fruits: Effects of operating conditions on global yield, volatile compounds, and antiproliferative activity against human tumor cell lines

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ABSTRACT

Schinus terebinthifolius Raddi is a South American plant with medicinal properties. We report the extraction of compounds from *S. terebinthifolius* fruits using supercritical CO_2 ($scCO_2$), with emphasis on the effects of $scCO_2$ pressure (100–300 bar) and temperature (40–60 °C) on global yield, volatile compounds, and antiproliferative activity against nine human tumor cell lines. Supercritical extracts obtained at 50–60 °C, independently of pressure, showed potent activity against kidney cancer with total growth inhibition < 3.9 μg/mL. Furthermore, extracts obtained at 200 bar and 50 °C showed potent activity against multidrug-resistant ovarian, prostate, and ovarian tumor cell lines, and glioma. Gas chromatography-mass spectrometry revealed the following volatile compounds: δ-3-carene, α-phellandrene, limonene, germacrene D, and caryophyllene. The results suggest that sesquiterpenes may be the metabolites responsible for the antiproliferative activity. However, future work involving fractionation of the extracts might shed light on the chemical compounds responsible for each antiproliferative activity.

1. Introduction

Brazil has one of the greatest biodiversity worldwide, being a rich source of bioactive compounds of commercial value. Among several Brazilian native plants, *Schinus terebinthifolius* Raddi (Anacardiaceae family) stands out for presenting great potential for exploitation and commercial use. All parts of *S. terebinthifolius* are used in folk medicine for the treatment of several pathologies; in addition, the dried fruits of *S. terebinthifolius* are widely employed as a culinary spice, known as pink pepper [1–3]. Many authors have reported biological activities of compounds from *S. terebinthifolius* fruits, such as antioxidant activity of aqueous and alcoholic extracts [4]; use of essential oil (EO) in the larval control of *Stegomyia aegypti* [5]; and insecticidal activity of EO against African malaria vectors [6]. Although several studies show that *S. terebinthifolius* fruits are a promising source of bioactive compounds, few studies have investigated its antiproliferative activity. In particular, Bendaoud et al. [7] investigated the in vitro antiproliferative activity of *S. terebinthifolius* fruit EO and demonstrated a promising activity against a human breast cancer (MCF-7) cell line; these authors suggested that sesquiterpenes may be the active metabolites responsible for the antiproliferative activity.

Considering the methods already employed to extract compounds from *S. terebinthifolius* fruits, several studies have focused on conventional techniques such as hydrodistillation [5], Soxhlet extraction [8], dynamic maceration [4], and steam distillation [7]. Despite the simplicity of the conventional extraction processes, these technologies have a number of disadvantages; e.g., the use of high temperatures (hydrodistillation) that can degrade thermolabile compounds, or the use of toxic organic solvents (Soxhlet and dynamic maceration) that can contaminate the plant extract and/or the environment [9]. Thus, an

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attractive alternative extraction process is supercritical fluid extraction (SFE). The application of SFE is quite advantageous as it allows the use of less environmentally aggressive solvents such as supercritical CO_2 ($scCO_2$), which is non-flammable, inert, and with low toxicity. Another advantage is the ease of solvent separation from the final product. In addition, the selectivity of supercritical fluids can be tuned by pressure, temperature, and cosolvent content manipulation [10–13].

S. terebinthifolius fruit extracts obtained by $scCO_2$ extraction can be purchased from suppliers such as Blue Marble Biomaterials (United States of America), Flavex® Naturextrakte GmbH (Germany), and Albert Vieille SAS (France). Moreover, Smith et al. [14] mention that the award-winning perfume "Estée Lauder *Pleasures*", created by Firmenich (Switzerland), contains supercritical extract (obtained by $scCO_2$) from pink pepper. It is worthwhile to mention that few scientific studies have investigated the extraction of compounds from *S. terebinthifolius* fruits using SFE. Andrade et al. [15] investigated the encapsulation process of supercritical extract (obtained by $scCO_2$) from *S. terebinthifolius* fruits. Nonetheless, no report was found on the antiproliferative effect of *S. terebinthifolius* fruit supercritical extract, which has the advantage of being solvent-free. The objective of this study was therefore to investigate the $scCO_2$ extraction of compounds from *S. terebinthifolius* fruits with emphasis on the effects of SFE operating conditions on global yield, volatile compounds, and antiproliferative activity against nine human tumor cell lines.

2. Materials and methods

2.1. Characterization of the raw material

Ripe *S. terebinthifolius* fruits were manually collected in Aracruz, Espírito Santo, Brazil (19°55′15″ Latitude South; 40°10′11″ Longitude West; Altitude 32 m), during June 2014. The material was stored in closed plastic bags at −18 °C until the extraction procedure. A voucher specimen (number 188116) was deposited in the Herbarium of the Institute of Biology, University of Campinas, Brazil. Moisture content on dry base (U) was determined by keeping the samples in a vacuum oven (Quimis®, Q819V2) at 70 °C for 24 h [16]; these conditions led to final constant weights. The fresh fruits (U = 69.4 ± 0.6%) were subjected to drying in an oven (Quimis®) at 40 °C for three days. Sequentially, the dried fruits were ground in an industrial blender (Waring Commercial®) and classified according to size using vibrating screens (Tyler sieve series, Bertel®) with sieve sizes from 10 to 42 mesh for 20 min. The mean particle diameter (geometric mean diameter, d_{gm}) was determined by the method recommended by ASAE [17]. Real density (ρ_r) was determined by helium gas pycnometry (Ultrapyc 1200e pycnometer, Quantachrome). The bed apparent density (ρ_a) was calculated considering the extraction cell volume and the mass of feed; bed porosity was calculated using real and apparent densities.

2.2. Supercritical CO_2 extraction

A full factorial design with seven experiments (Table 1) was developed to investigate the influence of pressure (100–300 bar) and temperature (40–60 °C) on $scCO_2$ extraction. Therefore, for two factors (pressure and temperature), the experimental design consisted of factorial assays (2^2 = 4 assays at levels of −1 and +1) and 3 replicates in the central points (at level 0). The experimental design allowed the study of a wide range of $scCO_2$ densities (0.298–0.928 g/cm^3), with the fewest number of experiments, which is fundamental for extract production with different characteristics. All assays were performed in duplicate and in random order. The experimental data were analyzed by Statistica® software using a significance level of 0.05.

A schematic representation of the experimental apparatus used for supercritical extraction is shown in Fig. 1. The equipment was supplied with CO_2 (99.5%, White Martins®), which was stored in a CO_2 cylinder (1). In all the equipment, needle valves (2) (Autoclave Engineers®) were used to block the CO_2 flow. The CO_2 was cooled in a thermostatic bath (3) (Marconi®, model MA184) before entering the high-pressure pump (4) (Thar SFC, model P-50A) to avoid cavitation. The high-pressure pump controlled the desired pressure of the solvent by a pressure controller (5). Before beginning the extraction, the extraction cell (6), with 2-cm inner diameter, was packed manually forming a particles bed; 20 g of raw material were used to determine the global yield. The remaining volume of the extraction cell was filled with 5-mm diameter glass beads (bed inlet). The extraction cell was installed inside an oven with a temperature controller (7) (Quimis®).

Table 1
Operating conditions, global yield, and δ-3-carene mass content of supercritical extracts (assays 1–7), essential oil (EO), dichloromethane extract (DE), and ethanolic extract (EE) from *S. terebinthifolius* fruits.

Full factorial design				*Output variables*	
Assay	Pressure (bar)[a]	Temperature (°C)[a]	$scCO_2$ density (g/cm^3)	Global yield (%)[b]	δ-3-carene mass content (%)[c]
1	100 (−1)	40 (−1)	0.562	5.70	6.24
2	300 (+1)	40 (−1)	0.928	13.0	2.23
3	100 (−1)	60 (+1)	0.298	3.08	7.47
4	300 (+1)	60 (+1)	0.831	13.7	2.16
5	200 (0)	50 (0)	0.762	12.5	2.59
6	200 (0)	50 (0)	0.762	11.8	2.72
7	200 (0)	50 (0)	0.762	12.8	2.57
EO	–	–	–	4.20	7.29
DE	–	–	–	16.6	1.12
EE	–	–	–	42.6	0.00

[a] Coded values in parentheses.
[b] Global yield according to Eq. (1).
[c] (kg of δ-3-carene/kg of extract) × 100.

After leaving the extraction cell, the fluid phase (CO_2 and supercritical extract) passed through a micro-metering needle valve (8) (Autoclave Engineers®) that allowed adjusting the solvent flow rate and reducing the pressure. To avoid freezing and obstruction of the line, caused by the Joule-Thomson effect, the micro-metering valve had a heating system (9). This system was comprised of an aluminum structure that covered the valve and an electrical resistance that was connected to a temperature control system (10) (COEL®, model HW500). After passing through the micro-metering valve, the reduced pressure resulted in the separation between solutes and solvent. The extract was collected in a collection flask (11) (100-mL penicillin flask), which was previously weighed and partially immersed in a dry-ice bath to minimize the loss of volatile compounds. A total extraction time of 360 min was used to determine the global yield. This period resulted in a S/F (solvent mass/dry mass of raw material) of about 121. The mass flow of solvent (6 g/min) was continuously measured by a Coriolis mass flow meter (12) (Siemens, Sitrans FC Mass 6000) during each extraction. Dichloromethane (Sigma-Aldrich®) was used to recover the extract adhered in the tubing between the extraction cell and the micro-metering valve. The solvent was evaporated using a rotatory evaporator (Buchi®, model R-200) under vacuum at 40 °C. The global yield (X_o) was calculated according to Eq. (1).

$$X_o = \frac{m_E}{m_d}100 \quad (1)$$

where m_E is the mass of extract, and m_d is the mass of dry raw material fed into the extraction cell. After the extraction, the extracts were preserved in sealed dark vials at −18 °C until further analysis.

2.3. Conventional extractions

Extraction of EO from *S. terebinthifolius* fruits was accomplished by hydrodistillation in a Clevenger apparatus, according to the procedures described in official pharmacopoeias for determining EOs in aromatic