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Copaíba (*Copaifera* sp.) leaf extracts obtained by CO_2 supercritical fluid extraction: Isotherms of global yield, kinetics data, antioxidant activity and neuroprotective effects

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ABSTRACT

Copaíba (*Copaifera* sp.) is a tree with a wide application in Brazilian folk medicine. The copaíba leaf extract obtained using supercritical CO_2 might be applied to afford neuroprotection following experimental stroke. This study aimed to obtain leaf extracts of *Copaifera* sp. via supercritical fluid extraction, assessing the global yield isotherms, process variables through the kinetic parameters of extraction, as well as antioxidant activity and neuroprotective effects. Three isotherms (40 °C, 50 °C and 60 °C) were studied in the pressure range of 10–30 MPa. The overall higher mass on dry basis yield (~3.8%) and antioxidant activity (~64%) were observed at 20 MPa and 60 °C. For the kinetic study of extraction, the extracts obtained with higher and lower antioxidant activity were chosen, and the models of Martinez et al., Tan and Liou and Sovová showed good and similar adjustments, as presented values of residual sum of squares in the order 10^{-2}. The results of histological and immunohistochemical analyzes suggest that the extract obtained at 60 °C and 20 MPa has anti-inflammatory and neuroprotective effects on experimental stroke induced by microinjections of endothelin-1 (ET-1) into the rat brain.

1. Introduction

Stroke is a devastating neural disorders and a main cause of neurological disability. Thrombolytic recombinant tissue plasminogen activator (tPA) is available as a treatment, but only a few patients have a good response to tPA, which can be used about 3 h after symptom onset [1]. Stroke is caused by rupture or obstruction (ischemia) of blood vessels followed by a number of symptoms including numbness or weakness in the face, arm, or leg, especially on one side, confusion or trouble understanding other people, trouble speaking, vision alterations and global mortality rate about 40% [2].

Copaíba is a native tree from Latin America and West Africa [3] and its most famous product is the oil resin taken directly from the stem. In Brazilian folk medicine, this oil has curative potential for many diseases.

It has been shown that the oil extracted from the trunk of certain varieties of copaíba has anticancer activities in the case of pulmonary metastasis of melanoma [4], mammary carcinoma [5,6], antiparasitic activity against Chagas disease [7], anti-inflammatory and neuroprotective after acute injury in the rat central nervous system [8] and copaíba leaf extracts has protective effects against colon carcinogenesis [9]. Diterpenes isolated from the oleoresin of copaíba display significant antimicrobial activity [10] and psoriasis patients showed improvements after topical and oral administration of oil resin [11].

In this work, extracts of *Copaifera* sp. leaves obtained by CO_2 supercritical extraction were studied in terms of global yield isotherms and kinetic parameters, as well as the antioxidant

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Nomenclature

k_d	desorption coefficient (s^{-1}) from Tan and Liou model [16]
b_i and t_{mi}	temporal dimensions parameters (s) from Martínez et al. [17]
K_m	the mass-related partition coefficient, (kg plant) (kg solvent)$^{-1}$ from Sovová model published in 2012 [19]
$t_{comb,f}$	combined characteristic time of mass transfer from Sovová model published in 2012 [19]
t_r	the residence time from Sovová model published in 2012 [19]

activity and neuroprotective effects after experimental stroke induced by microinjections of endothelin-1 (ET-1) into the rat brain. Hydrodistillation and Soxhlet extraction with ethanol were also observed for comparison purposes.

2. Materials and methods

2.1. Copaíba leaf samples

The copaíba leaves were purchased from the Ver-O-Peso market (Belém, Pará, Brazil). The humidity of the sample was determined by the method of distillation with xylol (Ecibra, PA—ACS, São Paulo, Brazil) [12]. The leaves were comminuted in a knives mill (Tecnal, model TE-631/3, Brazil) at 2251.5 rad/s for 10 s. The particle size analysis was performed using Tyler sieves (WS Tyler, USA) −18 + 60 mesh. The samples used in the experiments were selected in the range −24 + 48 mesh.

2.2. Extraction procedures: Supercritical fluid extraction (SFE), hydrodistillation and ethanol extraction

The experiments were performed in LASEFI (UNICAMP, Campinas, Brazil) using a SFE unit built at the University of Hamburg–Harburg (TUHH, Hamburg, Germany) which is operated according to the methodology described by Zetzl et al. [13]. The extraction column of this equipment has a capacity of 10^{-4} m^3. 99.8% Purity CO_2 (White Martins Gases Industrial LTDA, Campinas, Brazil) was used as solvent.

The global yield isotherms were determined using 0.019 kg of dried and ground copaíba leaves, using a factorial design combining the temperatures of 40, 50 and 60 °C with pressures of 10, 15, 20, 25, 30 MPa. The CO_2 flow rate was maintained at 8.33×10^{-5} kg/s. The extraction was performed in two stages: static period (supercritical CO_2 and copaiba leaves were in operation conditions closed in extraction vessel) of 1800 s and a dynamic period (system was opened and just CO_2 and extract were exhaust continuously from vessel to collector flask) of 7200 s. The experiments were performed in duplicate. The global yield were calculated by ratio of extract mass and copaiba leaves mass on dry basis.

Overall extraction curves (OECs) were obtained at 60 °C/20 MPa and 60 °C/30 MPa using 0.09329 kg of sample in each experiment. The densities of the supercritical CO_2 were calculated using TermoDi software, developed by LASEFI (UNICAMP, Campinas, Brazil).

The hydrodistillation was performed in accordance with the AOAC 962.17 [14] with extraction time of 7200 s, ratio of boiling water and a sample of 10:1 (v/w).

In the Soxhlet extraction, ethanol (99%, Nuclear, São Paulo, Brazil) was used as solvent in a ratio of 1:10 (copaíba leaves:ethanol) and reflux for 7200 s. Then, ethanol was evaporated from extract in a rotary evaporator (Heidolph, model Laborota 4001 Schwabach, Germany) under the control of vacuum (Heidolph, model Rotavac/Rotavac control, Schwabach, Germany). This extract was diluted with ethyl acetate and analyzed by thin layer chromatography on a silica gel plate (Merck KGaA, Darmstadt, Germany) as stationary phase and hexane (96%, Merck KGaA, Darmstadt, Germany) with ethyl acetate (99.5%, Merck KGaA, Darmstadt, Germany) as mobile phase at a ratio of 7:3 (hexane: ethyl acetate). For resolution of substances after elution, the plate was sprayed with a solution of anisaldehyde (100 ml glacial acetic acid, 2 ml of sulfuric acid and 1 ml of anisaldehyde) and heated at 100 °C.

2.3. Antioxidant activity

The samples obtained in global yield isotherms were subjected to determination of antioxidant activity according to the method used by Leal et al. [15], in which the method of beta-carotene and linolenic acid is modified to the characteristics of SFE extracts obtained.

2.4. Modeling of the OECs

The OECs 60 °C/20 MPa and 60 °C/30 MPa were fitted to models of Tan and Liou [16], Martínez et al. [17] and the model Papamichail et al. [18] modified by Sovová in 2012 [19]. The adjusted parameters were: the coefficient of desorption (k_d) of Tan and Liou model [16], b_i and t_{mi} of Martínez et al. model [17], the mass-related partition coefficient (K_m), the combined characteristic time of mass transfer ($t_{comb,f}$) and is the residence time (t_r) in Sovová model published in 2012 [19]. Residual sum of squares and standard error were used to verify the qualities of mathematical fitting and parameters values.

2.5. Experimental model of focal ischemia

2.5.1. Animals and surgical procedures

Six male Wistar rats (Biotério Central, UFPA, Brazil) weighing approximately 0.250 kg at time of surgery were used in this study. The animals were socially housed in standard cage with food and water provided ad libitum. All procedures were in accordance with guidelines suggested by the Ethics Committee on Animal Research of the Federal University of Pará (Brazil).

Animals were anesthetized via intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) and held on a stereotaxic frame. Stereotaxic measurements for craniotomy were relative to bregma and with depth determined from skull surface. ET-1 (E7764 Sigma-Aldrich, USA) was diluted in sterile saline and colanyl blue dye and injected into the motor cortex according to the following coordinates: mesolateral: 2.3, anteroposterior: 1.2, dorsoventral: 0.6 [8].

After surgery, animals were housed in standard cage with food and water ad libitum for 24 h and deeply anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and then submitted to intracardiac perfusion of 0.9% saline and 4% paraformaldehyde.

2.5.2. Microtomy and immunohistochemical analysis

Brains were cryoprotected in different concentrations of glycerol solution with 30% sucrose and sectioned using a cryostat (Zeiss HM505E) at 2×10^{-5} m thickness.

Inflammatory cells were immunolabed using MBS-1 antibody (1:500, CNS Inflammation Group, UK) which detects epitopes present in neutrophils [8].

2.5.3. Treatment with extract from Copaifera sp. leaves by SFE (SFE-Cl)

The copaíba leaf extract was obtained in the operating conditions of SFE at 60 °C and 20 MPa. Copaíba leaf extract (18.5 mg/ml) diluted in 5% ethanol was intraperitoneally administered in the