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Extraction of bioactive compounds from *Botryosphaeria dothidea* using supercritical carbon dioxide and compressed liquefied petroleum gas

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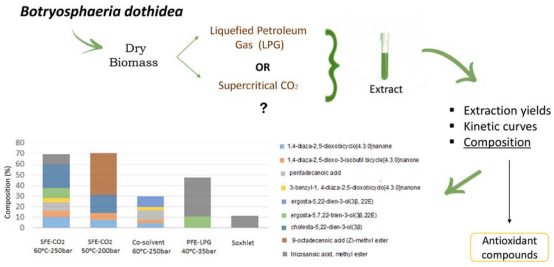
GRAPHICAL ABSTRACT



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ABSTRACT

This study presents extraction yields, kinetic curves, kinetic parameters, antioxidant capacity and composition of extract obtained from dry biomass of the fungus *Botryosphaeria dothidea*. The metabolites were produced by submerged fermentation using ultrasound as pretreatment. Thereafter, levels of pressure and temperature were evaluated for recovering the metabolites by supercritical CO_2 (150, 200 and 250 bar; 40, 50 and 60 °C) and compressed liquefied petroleum gas (LPG) (15, 25 and 30 bar; 20, 30 and 40 °C). Ethanol was also used as cosolvent, which the mixture CO_2 + 10 wt.% ethanol provided an extraction yield of 1.14 wt.%. When pure CO_2 or LPG was used, the global yields did not exceed 0.86 wt.% and 0.94 wt.%, respectively. Fatty acids, esters, sterols and fatty acid methyl esters were some types of compounds recovered. Furthermore, the antioxidant capacity was measured, with the most promising result as 38.4 ± 3.1 μmol TEAC/μg extract.

1. Introduction

Endophytic microorganisms are hosts, parasites or pathogens that colonize healthy plants, sometimes without apparent symptoms [1]. Species such as *Botryosphaeriaceae* can cause cancer in trees and fruit rot [2]. In addition to attacking plant tissues, these microorganisms can still produce secondary metabolites that stimulate growth and/or provide defense. In this way, they are considered as potential producers of bioactive compounds for several industrial fields [3]. When using colonies of the endophytic fungus *Botryosphaeria dothidea* as a potential immunosuppressive agent, for instance, the activities were satisfactory [4]. However, biological activities of extracts (metabolites concentrated) from this fungus are scarce in the scientific literature.

Among the compounds of industrial interest, antioxidants have a great highlight because they are responsible for protection against free radicals and reactive oxygen species [5] that can cause various diseases

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in humans, such as skin damage, liver damage, inflammation, among others. The use of synthetic antioxidant compounds is one of the methods used to prevent these diseases, but reports show the presence of toxic substances in this formulation. In this way, demand for natural products with the same purpose has increased.

The extraction of bioactive compounds from microorganisms, as *Botryosphaeria dothidea*, is a very interesting area, which deserves attention. The supercritical fluid extraction (SFE) and pressurized fluid extraction (PFE) are favorable technologies used to obtain bioactive compounds from natural sources. These techniques have advantages over conventional methods such as low energy consumption, extraction of thermolabile compounds, easy fluid disposal or reuse (when the solvents are in the gas phase in room pressure), excellent recovery and quality of the material to be extracted [6,7].

Carbon dioxide (CO_2) is the most used solvent for the supercritical fluid extraction of bioactive compounds from natural matrices. It is recognized as safe, non-toxic, non-flammable and allows a reduction in the use of organic solvents. LPG is a colorless liquid, which allows its handling at low temperatures and pressures, besides being a cheaper material and vastly accessible in nature. However, it is highly flammable, toxic and requires more care like attention and control, even though it can be separated from the extracts by a simple decompression [8].

Based on this context, the objective of this work was to evaluate the extraction of metabolites with antioxidant capacity from the fungus *Botryosphaeria dothidea* using supercritical fluid extraction with CO_2 (SFE-CO_2) and pressurized fluid extraction with LPG (PFE-LPG). We consider this is one of the first reports of recovering bioactive compounds from this potential fungus by promising technologies, especially related to yields, chemical composition and antioxidant capacity of extracts.

2. Material and methods

2.1. Microorganism

The microorganism *Botryosphaeria dothidea* was isolated from *Solanum americanum* plant, collected from Santiago city (Rio Grande do Sul, Brazil), and identified by Laboratory of Biochemical Phytopathology of Biological Institute (São Paulo, Brazil).

2.2. Solvents and reagents

CO_2 (purity > 99.5%) was purchased from White Martins S.A. (Santa Maria, Brazil). LPG was purchased from Liquigás S.A. (Santa Maria, Brazil) and consisted of a mixture of propane (50.3 wt.%), *n*-butane (28.4 wt.%), isobutane (13.7 wt.%), ethane (4.8 wt.%), and other hydrocarbons minor constituents (2.8 wt.%). All others reagents were purchased from Sigma-Aldrich (São Paulo, Brazil).

2.3. Obtaining the biomass

The endophytic fungus was inoculated in Potato Dextrose Agar (PDA) and incubated in Erlenmeyers for 7 days at 28 °C. Afterward, the fungus was used in the fermentation with 150 mL synthetic medium (10 g/L glucose, 7.5 g/L yeast extract, 10 g/L peptone, 2 g/L ammonium sulphate, 1 g/L ferrous sulphate, 1 g/L manganese sulphate, 0.5 g/L magnesium sulphate) [9] in a shaker at 28 °C with 120 rpm rotation during 10 days.

2.3.1. Ultrasonic pretreatment

On the second day of fermentation, the cultured fungus was treated with ultrasound (Bath, Model USC-1800A, Unique, Brazil) using 40 kHz frequency and 130 W power. The sonication time was 1 min, with a rotation change at 75 rpm, for 4–5 days. After this period, the rotation was turned to 120 rpm, concluding its 10 days of fermentation. This treatment was carried out in order to increase the production of metabolites with antioxidant capacity, according to the scientific literature [10,11].

2.3.2. Preparation and characterization of raw material

After fermentation, the biomass was filtered using a vacuum pump to separate the broth and it was frozen in the freezer for 3 days and lyophilized (L101, Liotop, São Carlos, Brazil) for 24 h. The dried biomass was macerated in a melting pot with pistil for further uses in the extractions.

2.4. Soxhlet extraction

Soxhlet extraction was performed using 1 g of lyophilized and macerated mycelium and 100 mL of *n*-hexane for 120 min and approximately 60 °C in a Soxhlet apparatus (Marconi, Model MA491/6, Brazil). At the end of the experimental run, the *n*-hexane was evaporated using a rotary evaporator under vacuum and the extracted mass was quantified by the gravimetric method. The samples were collected, suspended again with ethanol and refrigerated for further chemical analysis of compounds. The assays were performed in triplicate and the yields and composition were expressed as a mean ± standard deviation. Soxhlet extraction was used as a reference for comparing the yields and compositions obtained by SFE-CO_2 and PFE-LPG.

2.5. Supercritical CO_2 and compressed LPG extractions

The experimental assays were performed in a laboratory scale equipment [12] composed mainly by (i) a 100 mL extraction vessel (stainless steel) with internal diameter of 2.5 cm and 19.5 cm of height, supporting up to 35 MPa; (ii) a syringe pump (ISCO 500 D, Lincoln, USA); (iii) an ultrathersmostatic cooling bath (Quimis, São Paulo, Brazil) for controlling the temperatures of CO_2 and LPG at the syringe pump; (iv) an ultrathersmostatic heating bath (Quimis, São Paulo, Brazil) with thermocouples; (v) a heating electric jacket to control the temperature inside the extraction vessel; (vi) blocking valves and micrometering valves (HIP 15-11AF2 316SS, Erie, USA); and (vii) 1/8 inch tubing of stainless steel (HIP, Erie, USA).

For the extraction procedures with CO_2 and LPG, 5 g of mycelium (dried and macerated) were loaded in the extraction vessel. In the sequence, the solvent (CO_2 or LPG) was pumped in the bed and the condition of pressure and temperature was established (after 20 min). Thereafter, the micrometering valve was opened at a constant CO_2 or LPG mass flow rate to collect the extract in the collection vials. The extraction time for samples was fixed (90 min for SFE-CO_2 and 20 min for PFE-LPG) after evaluating the extraction curve obtained in preliminary tests. The flow rate of LPG and CO_2 used in all assays was 4 g/min.

The influence of temperature and pressure on the extraction yields and chemical composition was evaluated. The assays were performed using a factorial experimental design with triplicate at the central point. For the experimental assays carried out with CO_2, the extractions were performed at temperatures of 40, 50, and 60 °C, and pressures of 150, 200 and 250 bar. The density of the fluid (ρ) was obtained from the Chemistry WebBook – National Institute of Standards and Technology (NIST) [13]. For the experimental assays carried out with LPG, the extractions were performed at 20, 30 and 40 °C, and at 15, 25 and 35 bar. The collected samples were stored under refrigeration for further analyses of antioxidant capacity and chemical composition.

During the kinetic extraction curves using supercritical CO_2, the extract was collected at equal intervals of 5 min until 20 min and thereafter during 10 min, while for the compressed LPG the interval was 2 min. The curves were constructed for all samples in different experimental conditions in order to determine the yield of extract as a function of time as Eq. (1).