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In vivo antitumor activity of by-products of *Passiflora edulis* f. *flavicarpa* Deg. Rich in medium and long chain fatty acids evaluated through oxidative stress markers, cell cycle arrest and apoptosis induction

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ABSTRACT

Antiinflammatory and antitumor activity has been reported in *Passiflora edulis* (yellow passion fruit) nevertheless the intrinsic mechanisms of action are not fully elucidated. The present study aimeds to perform a comparison between the antitumor activity involving the crude extract (HCE) and the supercritical fluid extract with ethanol as co-solvent (SFEtOH) from *P. edulis* f. *flavicarpa* Deg. The *in vitro* cytotoxicity was evaluated in MCF-7 cells, while the *in vivo* antitumor activity was assessed in male Balb/c mice inoculated with Ehrlich carcinoma cells. SFEtOH exhibited higher antitumor activity compared to HCE. Wherein, SFEtOH showed an EC_{50} of 264.6 μg/mL against MCF-7 cells as well as an increased inhibition of tumor growth of 48.5% ($p < 0.001$) in male Balb/c mice, thereby promoting an increased mice lifespan to approximately 42%. Moreover, SFEtOH caused lipid ($p < 0.001$) and protein ($p < 0.001$) oxidation by increasing glutathione redox cycle activity while decreased the thioredoxin reductase activity ($p < 0.001$). SFEtOH also induced oxidative DNA damage in Ehrlich ascites carcinoma (EAC) cells leading to G2/M cycle arrest and has increased apoptotic cells up to 48.2%. These data suggest that the probable mechanisms of antitumor effect are associated to the lipid, protein and DNA damage, leading to cell cycle arrest and triggering apoptosis via mitochondrial pathway, should be probable due to the presence of medium and long chain fatty acids such as lauric acid.

1. Introduction

The engineering development and manufacture industry are resulting in increasing environmental threats. This includes the food industry, which generates a wide variety of by-products and residues during food processing that usually have no commercial value and may cause pollution (Tilman and Clark, 2014). These by-products usually are disposed by dumping, burning or land filling, and thus is a not sustainable behavior (Gupta et al., 2015). The number of studies reported in the literature using industrial residues, as sources for additional downstream processes, enlarged considerably in the last years. Among the different by-products, the use of fruit or plant residues has economic benefits and provides positive impact on the environment (Gupta et al., 2015).

Supercritical fluid extraction (SFE) is an alternative method for extraction and fractionation of natural raw materials, performed with pressurized gases such as carbon dioxide (CO_2) as solvents. This technology is considered a green process because it provides solvent-free extracts with low thermal degradation. SFE can also be performed with the use of a co-solvent, at low concentrations, to adjust the process selectivity (Michielin et al., 2009).

The high pressure method of SFE has been widely used for recycling by-products, enabling the extraction of interesting compounds that otherwise would not be recovered from raw materials (Herrero et al.,

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2010). Also, SFE has been used to obtain potentially active molecules from plants. This approach has yielded some promising results that include the acquisition of compounds with antitumor activity (*e.g.* Kviecinski et al., 2011, Grinevicius et al., 2017).

One example of residues generated by the food industry is passion fruit seeds. They are normally discarded during the manufacturing process for the production of the fruit juice. Supercritical extraction with CO_2 on seed cakes of passion fruit yields a liquid oil extract rich in fatty acids (Shucheng et al., 2008; Oliveira et al., 2016). Barrales et al. (2015) reported the presence of linoleic, oleic, palmitic and stearic acids in the passion fruit seed oil. Kadochi et al. (2017) suggested that ketone body and medium-chain fatty acid suppressed the proliferation of colon cancer cells by remodeling of energy metabolism. Previous studies have demonstrated antitumor effects of palmitic acid in a range of colorectal cancer cell lines such as Caco-2, HTC-116 and COLO 205 (Engelbrecht et al., 2008). Lauric acid is also a fatty acid that may cause DNA fragmentation, cell cycle arrest and increases Reactive Oxygen Species (ROS) generation triggering apoptosis in several tumor lines (Takayama et al., 2009; Fauser et al., 2013). Additionally, lauric acid may cause downregulation of PINK1, which possibly causes retardation of the mitochondrial turnover, promoting imbalance in the electron transport chain complexes and resulting in ROS production, mitochondria damage, ultimately resulting in apoptosis (Kadochi et al., 2017).

The hypothesis of the current study was that SFE from passion fruit seed cakes could eventually result in a compound endowed with antitumor activity. Therefore, the aim of this work was to perform a phytochemical and biological screening for antitumor activity comparing some extracts obtained by SFE and conventional maceration.

2. Material and methods

2.1. Extraction procedures

Seed cakes of *Passiflora edulis* sp were provided by Extrair Óleos Naturais Co, Rio de Janeiro, Brazil. They were subjected to maceration at room temperature in ethanol/water (1:1 v/v) for 7 days. The vegetal material/solvent ratio was 1:5 (w/v). The solvent was eliminated under reduced pression to obtain a dried hydrothenolic HCE. The extraction yield of maceration was about 6% considering the mass of the starting material (Oliveira et al., 2016). Supercritical fluid extractions were performed using CO_2 99.9% (White Martins, Brazil) as co-solvent and a fixed bed of particle formed by 10 g of seed cake. The extractions were conducted at 40 °C for 3 h, with flow rate of 0.5 kg of CO_2/h. Different conditions of pressure were evaluated, 250 and 300 bar, performed with or without 5% ethanol as co-solvent, providing four different supercritical extracts. The SFE yield was always close to 5%. The procedure was until recovering enough extracts for the assays of biological activity (Michielin et al., 2011; Oliveira et al., 2016).

2.2. Study design

Initially, all extracts were subjected to cytotoxicity screening with MCF-7 tumor cell line in culture through the MTT Assay. The most cytotoxic SFE extract along with HCE were chemically characterized. Then, these extracts were assessed for the *in vivo* antitumor activity using male Balb/c mice, which contributed to the characterization of its mechanism of action.

2.3. MTT assay

The cytotoxicity of extracts was assessed against MCF-7 cells in culture through the tetrazolium salt or MTT assay (Mosmann, 1983). MCF-7 cell is a human breast adenocarcinoma cell line, which allows evaluating the cytotoxicity of compounds *in vitro*. The cells were cultured at 37 °C under 5% CO_2 atmosphere with 95% of air humidity. Dulbecco's modified Eagle medium was used supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL). These chemicals were purchased from Gibco, USA. After MCF-7 reaching 80% confluence, the medium was removed and replaced with the others containing the extracts (10–500 μg/mL) for 24 h, 48 h and 72 h. Only the vehicle was used for the controls (dimethylsulfoxide 0.1% from Sigma-Aldrich, USA). The results were presented as EC_{50} calculated using Graph Pad Prism 6 (San Diego, USA).

2.4. Gas chromatography and mass spectroscopy (GC-MS) on extracts

The phytochemical constitution of the extracts was assessed through gas chromatography using a chromatography system (model 7820A) endowed with a mass spectrophotometer (model 5975 MSD) (Agilent Technologies, USA). The separation of the compounds was done through split injection inside a capillary column HP5 MS (25 m × 250 μm × 25 μm, Agilent, USA) using the flow rate of 5:1 (5 mL/min). The carrier gas was helium used under controlled pressure (5 psi) and keeping the detector at 280 °C. The extracts sample volume was 1 μL (1 mg/mL). Temperature of oven column was programmed to increase from 50 up to 260 °C (4 °C/min). Acquisition of full spectra data was done through scanning mode considering intervals previously established (30–750 Da). Taking into consideration the retention times (RT) and comparing the mass spectra with Wiley 6.0 mass spectra library (USA) and literature (Adams, 2007), was possible to identify the compounds present in the extracts. The quantification of the constituents was carried out from the chromatogram profiles using relative peaks areas (%).

2.5. In vivo antitumor activity

The antitumor activity of extracts *in vivo* was assessed using male Balb/c mice. Mice (20 ± 2 g) were housed under controlled conditions, receiving water and food ad libitum. This research was conducted in accordance with internationally accepted principles for laboratory animal use and care (NIH publication # 85–23, revised in 1985). This experimental protocol was approved by the ethics committee of Universidade Federal de Santa Catarina, Brazil (CEUA-PP00784).

For dose determination, first a screening of doses was done using healthy mice. Doses of extracts above 200 mg/kg/day caused symptoms of toxicity, such as dullness and inactivity. Therefore, the doses 100 and 200 mg/kg/day were used in the following with another set of mice in which tumor was induced. The tumor induction day was considered day zero and on this date, all animals were weighed, and the abdominal circumference was measured. EAC cells (5×10^6 cells/200 μL) were inoculated into the abdomen of mice. Twenty-four hours later mice were divided into 6 groups (n = 18): one negative control group treated with excipient (dimethylsulfoxide 1% in saline), one positive control treated with doxorubicin (1.2 mg/kg) as previously reported by Hossain et al. (2012) and 4 test-groups, which received HCE or SFE extract at 100 and 200 mg/kg/day, respectively. The treatments were done daily through intraperitoneal injections for 9 days. Twenty-four hours after the last doses mice were weighed, and the abdominal circumferences were measured again. The inhibition of tumor growth was calculated using the formula according Felipe et al. (2014):

Inhibition of tumor growth (%) = [(variation in waist circumference of the treated group x 100)/variation in waist circumference of the control group] – 100.

Later, 6 animals of each group were euthanized. The ascitic fluid from mice was collected in graduated tubes. The remaining animals (n = 12) were assisted on a daily basis to evaluate survival through Kaplan-Meier method (Kaplan and Meier, 1958).

2.6. Biomarkers of oxidative stress in EAC cells

Lipid peroxidation was determined spectrophotometrically at