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Biotransformation effects on anti lipogenic activity of citrus extracts

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ABSTRACT

Citrus peel is a good source of flavonoids, with higher content in relation to pulp. This study proposed to investigate the anti-lipogenic potential of a newly developed citrus flavonoids extract, obtained from citrus industrial residue, bioprocessed in order to generate a commercial source of some flavonoids naturally found in low quantity. The results showed that the citrus peel extract obtained after biotransformation was a good source of hesperitin and naringenin, flavonoids that has no source for production on a large scale, as in supplements or medicines. Still, the results showed that all extracts could be used in obesity treatment. The original extract, "In Natura", would be useful to reduce new adipocytes synthesis and lipid accumulation, and the extract bioprocessed, "Biotransformed" extract could be used to induce lipolysis on fat tissue.

1. Introduction

Citrus fruits are source of many bioactive compounds, as flavonoids, coumarins, limonoids and carotenoids (Turner & Burri, 2013). The main class of citrus flavonoid are the flavanones, but there are also considerable amounts of flavones, flavonols and anthocyanins (Benavente-García, Castillo, Marin, Ortuño, & Del Río, 1997). The most frequent types of flavonoids found in citrus are hesperidin, naringin, narirutin, eriocitrin, nobiletin and tangeritin (Sun et al., 2013).

The positive effects of citrus flavonoids in obesity treatment (inducing lipolysis and reducing lipid accumulation), and its complications (causing anti-inflammatory response, reducing serum lipids, and improving blood pressure) are demonstrated in several studies in cell culture (Kang et al., 2012; Kim et al., 2012; Yoshida et al., 2010, 2013), biological assays (Alam, Kauter, & Brown, 2013; Um, Moon, Ahn, & Youl Ha, 2013) and clinical trials (Dallas, Gerbi, Tenca, Juchaux, & Bernard, 2008; Dallas et al., 2013). It is noteworthy that citrus peel has higher content of polyphenols and antioxidant activity in comparison to pulp, indicating that citrus residues are a promising source of bioactive compounds (Barros, Ferreira, & Genovese, 2012).

In most of the studies, citrus peel is obtained from the fruit acquired particularly for the research, and we aim to evaluate the potential of a citrus residue from industrial waste as a commercial source of bioactives. In this context, Brazil is the world's largest orange producer, according to estimates from the Food and Agriculture Organization (FAO). Of the total produced, it is estimated that 85% is destined for juice industry. In juice production, about 50% of the waste generated is composed of peel and pomace, indicating that there is a rich source of this raw material.

Still, citrus extracts commonly used in researches are rich in hesperidin and naringin, with low amount of aglycones. Studies developed to test the aglycones forms commonly use high cost analytical standards. Thus, a residue extract containing the

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biotransformed polyphenols on a unique composition with biological potential would be an innovation with commercial interest.

Our research group have been studying alternatives of bioprocesses to increase the production of more bioactive polyphenols from these industrial arrange residues. Madeira, Nakajima, Macedo, and Macedo (2014) observed that the fermentation process of citrus peel resulted in an extract rich in flavanones aglycones, often found in low amounts in the nature. This is an advantage because some evidence have shown that the aglycones form have higher antioxidant capacity (Hirata, Murakami, Shoji, Kadoma, & Fujisawa, 2005; Silva et al., 2013), and higher bioavailability (Li et al., 2008) in comparison to glycosides. Besides, recent evidences are highlighting the importance of synergism among bioactive compounds in complex matrix with better effect than isolated compounds.

These polyphenols from plant material are commonly extracted with methanol (Hayat et al., 2010; Ramful, Bahorun, Bourdon, Tarnus, & Aruoma, 2010; Singh, Sood, & Muthuraman, 2011). However this is a toxic solvent (Tephly, 1991), being of interest the development of a extraction procedure using a food grade solvent.

Considering these, the study aimed to test a biotransformed citrus peel extract for its antioxidant activity *in vitro*, and the ability to reduce lipogenesis and induce lipolysis in adipocyte cell culture.

2. 2-Materials and methods

2.1. Chemicals

Gallic acid, Folin–Ciocalteu reagent, 2,2′-azobis(2-methylpropionamidine) (97%) (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox®, analytical standards hesperidin, hesperitin, naringin and naringenin, insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), Oil Red O were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Fluorescein was purchased from ECIBRA. All the other chemicals used were in an analytical grade.

2.2. Biotransformed citrus residue

The citrus residue was supplied by CP Kelco Industry Headquarters, from Limeira – SP – Brazil, specialized in pectin production. The residue was dry and contained citrus peel (flavedo and albedo). The material was crushed, and passed through a 10 mesh sieve (Bertel Metallurgical Industries LT). The residue was biotransformed by solid-state fermentation using the microorganism *Paecilomyces variotii* (Brazilian Collection of Environmental and Industrial Microorganisms-CBMAI 1157) according to Madeira et al. (2014). Briefly, the fermentation medium was prepared in 250 ml Erlenmeyer flasks containing 10 g of the residue and 10 ml of water. The medium was sterilized by autoclaving for 15 min at 121 °C. After cooling, the flasks were inoculated with 1 mL of the microorganism spore suspension (9×10^6 spores/mL) and incubated at 30 °C with 90% relative humidity (Climate Camera 420 CLD – Nova Ética, SP, Brazil) for 48 h.

2.3. Preparation of polyphenols extracts from citrus residue

The extraction of phenolic compounds was carried out according to a process adapted from Hayat et al. (2010). One gram of the biotransformed material was mixed with 25 mL 70% methanol. The solution was treated in ultrasonic bath at 30 °C for 15 min, in shaker at 200 rpm for 15 min, and then filtered on Whatman paper (No. 1). Different extraction solvents were tested instead of 70% methanol, in order to reduce costs and toxicity of the final extract. The tested extraction solvents were: 70% ethanol (v/v), 70% ethanol (v/v) acidified with 1% HCl (v/v), 50% ethanol (v/v) and water.

After the definition of the extraction solution, the extracts were prepared from the "Biotransformed" residue and two control residues. The first control was the unfermented residue consisting of the product without any processing ("In Natura"), and the second control was the sterilized residue ("Autoclaved"). The sterilized residue was used as a control of process to verify the modifications that occurred in the extract after the sterilization by autoclaving.

After filtration, the product obtained was concentrated on a rotary evaporator at 40 °C to remove the organic solvent. Then the aqueous solution was frozen and freeze-dried.

2.4. Extracts characterization

2.4.1. Total phenolic content

Total phenolic contents of the extracts were measured using the Folin–Ciocalteu assay according to Singleton, Orthofer, and Lamuela-Raventós (1999). Gallic acid was used as a standard and a calibration curve was plotted in a concentration range of 25–200 μg/mL. All analyses were performed in triplicate and results were expressed as mg of gallic acid equivalents (GAE)/mL or mg of extract.

2.4.2. Determination of main flavanone compounds by High Performance Liquid Chromatography (HPLC)

A DionexUltiMate 3000 (Germany) liquid chromatography, equipped with a C-18 Acclaim® 120 column (Dionex, 3 μm, 4.6 × 150 mm) maintained at 30 °C by a thermostat, was used. The detection was carried out using a UV/VIS (DAD-3000). The method was adapted from Caridi et al. (2007), and De Mejía, Song, Heck, and Ramírez-Mares (2010). The solvents were: A (water/formic acid, 99.9:0.1 v/v) and B (methanol/formic acid, 99.9:0.1 v/v), with a flow rate of 0.6 mL/min. The spectra absorption were obtained at 190 and 480 nm, and the chromatograms were processed at 280 nm. The standard flavanones detected and quantified were naringin, naringenin, hesperidin and hesperitin.

2.4.3. DPPH radical-scavenging activity

The potential antioxidant activity of the extracts was assessed based on the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, as described by Macedo, Battestin, Ribeiro, and Macedo (2011). The reaction mixtures, consisting of 50 μl of test samples and 150 μl of 0.2 mM DPPH in methanol, were carried out on a NovoStarMicroplate reader (BMG LABTECH, Germany) with absorbance filters for a wavelength of 520 nm. The decolorizing process was recorded after 90 min of reaction. The DPPH solution and reaction medium were freshly prepared and stored in the dark. The measurement was performed in triplicate. The antioxidant activity was calculated from the equation obtained by the linear regression after plotting known concentration solutions of Trolox®. Antiradical activity was expressed as μmol of Trolox® equivalent/mg of extracts.

2.4.4. ORAC

The ORAC (Oxygen Radical Absorbance Capacity) assay was performed using fluorescein (FL) as the fluorescent probe, as described by Dávalos, Gómez-Cordovés, and Bartolomé (2004), and adapted by Ferreira, Macedo, Ribeiro, and Macedo (2013). Briefly, 20 μL aliquots of the sample, Trolox® solution or buffer (blank) were distributed in black-walled 96-well plate, followed by the addition of 120 μL fluorescein sodium salt solution 0.38 μg/mL (Ecibra, São Paulo, Brazil) diluted in sodium phosphate buffer 75 mM (pH 7.4). The reaction was initiated by addition of 60 μL AAPH solution (Sigma–Aldrich, Steinheim, Germany) at a concentration of 108 mg/mL dissolved in sodium phosphate buffer 75 mM (pH 7.4). The fluorescence was monitored every 56 s during 75 min using a Novo Star Microplate Reader (BMG LABTECH, Germany)