Food Research International 97 (2017) 37–44

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Biotransformed citrus extract as a source of anti-inflammatory polyphenols: Effects in macrophages and adipocytes

Vânia Mayumi Nakajima [a,*], Tais Moala [a], Cintia Rabelo e Paiva Caria [b], Carolina Soares Moura [a], Jaime Amaya-Farfan [a], Alessandra Gambero [b], Gabriela Alves Macedo [a], Juliana Alves Macedo [a]

[a] Department of Food and Nutrition, Faculty of Food Engineering, State University of Campinas – UNICAMP, Rua Monteiro Lobato, 80, Cidade Universitária Zeferino Vaz, CP 6121, CEP 13083-862 Campinas, SP, Brazil

[b] Clinical Pharmacology and Gastroenterology Unit, São Francisco University Medical School, Bragança Paulista, SP, Brazil

ARTICLE INFO

Article history:
Received 12 January 2017
Received in revised form 14 March 2017
Accepted 19 March 2017
Available online 22 March 2017

Keywords:
Citrus residue extract
Polyphenols
Obesity
Anti-inflammatory activity
Cell culture

ABSTRACT

Chronic non-communicable diseases such as obesity are preceded by increased macrophage infiltration in adipose tissue and greater secretion of pro-inflammatory cytokines. We evaluated the anti-inflammatory potential of Biotransformed extract, and two control extracts: In Natura and Autoclaved. The assays were performed using a cellular model with RAW264.7, 3T3-L1 cells, and RAW264.7 and 3T3-L1 co-culture. The innovation of the study was the use of Biotransformed extract, a unique phenolic extract of a bioprocessed citrus residue. LPS stimulated RAW264.7 cells treated with the Biotransformed extract exhibited lower secretion of TNF-α and NO and lower protein expression of NFκB. In RAW264.7 and 3T3-L1 co-culture, treatment with 1.0 mg/mL of the Biotransformed extract reduced secretion of TNF-α (30.7%) and IL-6 (43.4%). Still, the Biotransformed extract caused higher increase in adiponectin in relation to control extracts. When the co-culture received a LPS stimulus, the Autoclaved extract at 1.0 mg/mL reduced IL-6 and TNF-α concentrations, and raised adiponectin. However, it was noteworthy that the Biotransformed extract was also able to significantly reduce IL-6 concentration while the Natural extract was not. The Biotransformed citrus extract evaluated in this study showed anti-inflammatory activity in macrophages and in co-culture, indicating that bioprocess of citrus residue can contribute to new product development with anti-inflammatory potential.

1. Introduction

Obesity is a disease characterized by excess body fat, associated with a chronic subclinical inflammatory condition caused by an increase in the secretion of pro-inflammatory adipokines (Balistreri, Caruso, & Candore, 2010). This increase in circulating adipokines also appears to be responsible for the development of chronic non-communicable diseases associated with obesity, which may involve insulin resistance, blood pressure increase, changes in serum lipids, higher inflammatory response and thrombus formation (Grundy, Brewer, Cleeman, Smith, & Lenfant, 2004).

Phenolic compounds are a class of substances of vegetable origin that has been investigated for a variety of properties ranging from protecting the plant against natural predators to the prevention and treatment of chronic diseases in humans. An interesting source of phenolic compounds because of its wide distribution throughout the world is citrus fruits.

Among the commercially most important citrus, oranges stand out in Brazil. This country is the largest world producer, according to estimates of the Food and Agriculture Organization (FAO). However, it is important to observe that most of this total is pressed for juice making, and that about 50% of the waste generated is composed of peel and pulp, commonly channeled to the animal feed industry. Moreover, it is known that citrus peels have high contents of polyphenols, and that several studies have shown the positive effects of peel extracts in the treatment of chronic non-communicable diseases (Ding et al., 2012; Kang et al., 2012; Kim et al., 2012; Raasmaja et al., 2013). Still, some studies have demonstrated the anti-inflammatory role of citrus flavonoids, mainly hesperetin (Giménez-Bastida, González-Sarrías, Vallejo, Espín, & Tomás-Barberán, 2016; Ma, Feng, & Ding, 2015; Ren et al., 2016) and naringenin (Karuppagounder et al., 2016; Yu, Ma, Yue, Yao, & Mao, 2014). However, these studies often test analytical standards of high cost.

Researches with citrus extracts have commonly used sources rich in hesperidin and naringin, with low amounts of aglycones. However evidence shows that the aglycone forms have higher antioxidant capacity (Hirata, Murakami, Shoji, Kadoma, & Fujisawa, 2005; Silva et al., 2013), higher bioavailability (Li et al., 2008; Miyake et al., 2006), and

* Corresponding author.
E-mail address: vania_nakajima@yahoo.com.br (V.M. Nakajima).

http://dx.doi.org/10.1016/j.foodres.2017.03.034
0963-9969/

better influence in hunger and satiety control (Kim, Park, Kim, Lee, & Rhyu, 2013) than the glycosides. Besides, some studies with hesperitin showed positive effects in hypertension (Takumi et al., 2012; Yamamoto et al., 2013) and diabetes treatment (Kumar et al., 2012, 2013; Urios et al., 2014). Thus, a residue extract containing both glycosidic and aglyconic polyphenols in a more adequate composition for higher biological potential could be an innovation with commercial interest.

Biotransformation by fermentation is one way to produce extracts with increased aglycone contents. Our research group has been studying biotransformation processes to increase the production of more bioactive polyphenols from industrial orange residues (Ferreira, Macedo, Ribeiro, & Macedo, 2013; Madeira, Nakajima, Macedo, & Macedo, 2014; Madeira, Speranza, & Macedo, 2012; Nakajima, Madeira, Macedo, & Macedo, 2016). Among the extracts already developed, we have reported that biotransformation can produce extracts with in-vitro lipolytic activity, contrasting with unprocessed controls that show no activity (Nakajima et al., 2016).

In the present work, we intended to determine whether the fermentative biotransformation of this residue could result in an extract with an improved biological profile. Thus, this study aimed to evaluate the anti-inflammatory potential on obesity scenarios of different citrus residue extracts in RAW 264.7 macrophages and in co-culture of RAW 264.7 macrophages and 3T3-L1 adipocytes.

2. Materials and methods

2.1. Chemicals

Insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), lipopolysaccharide (LPS) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). All the other chemicals used were of analytical grade.

2.2. Biotransformed citrus residue

The citrus residue was supplied by CP Kelco Industry Headquarters, Limeira - SP - Brazil, a company 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 aspore suspension of the microorganism (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 polyphenol extracts from citrus the 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 of 50% ethanol, placed in ultrasonic bath at 30 °C for 15 min, and after in a shaker at 200 rpm. Lastly, the product was filtered through No. 1 Whatman paper.

Three extracts were prepared: one from the Biotransformed residue and two other for control from the intact residue. 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 filtrate was then concentrated on a rotary evaporator at 40 °C to remove the organic solvent. The aqueous solution was finally frozen and freeze-dried.

These extracts have been previously analyzed and the polyphenol profiles published (Nakajima et al., 2016). In summary, the Biotransformed extract had higher content of the aglycones hesperetin (2.5 ± 0.2 mg/g of lyophilized extract) and naringenin (1.6 ± 0.1 mg/g of lyophilized extract), whereas the In Natura showed higher amounts of the glycosides hesperidin (125.7 ± 5.7 mg/g of lyophilized extract) and naringin (2.0 ± 0.2 mg/g of lyophilized extract), and the Autoclaved consisted of a mixture of the four flavonoids, yet with lower concentrations of all.

2.4. Cell culture assay

2.4.1. Cell culture

RAW 264.7 murine macrophages and 3T3-L1 murine pre-adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in a humidified atmosphere with 5% CO_2. All media contained 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL).

2.4.2. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay

RAW 264.7 cells (1.0×10^5 cells/mL) were seeded in 96-well plates and incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. Then the cells were treated with the samples (0.01 mg/mL to 1.00 mg/mL). After 24 h of incubation, all media was removed and 10 μL of MTT solution (5 mg/mL) was added to the cell culture. The cells were further incubated at 37 °C in a humidified atmosphere with 5% CO^2 for 3 h. The MTT formazan crystals were dissolved in SDS 10% in HCl 0.01 M for 18 h. The optical density of formazan solution was measured with a microplate reader at 540 nm. The results are expressed as a % of control cells that are cells without any sample treatment.

The same protocol was used for 3T3-L1 cells. However, after addition of MTT solution, the cells were further incubated for 4 h.

2.4.3. RAW 264.7 inflammatory assay

RAW 264.7 macrophages were grown in 24-well plates (1×10^5 cells/well) for 24 h. Extracts (0.2 mg/mL and 1.0 mg/mL) plus LPS (100 ng/mL) was added to the treatment group of plates, while medium or LPS alone was added to the control group of plates. After a 24-h LPS-stimulation, the cell-free supernatants were collected and assayed for TNF-α levels using the enzyme-linked immunosorbent assay (ELISA) kit Mouse TNF (Mono/Mono) ELISA Set (BD OptEIA™), in accordance with the manufacturer's instructions. The optical density of each well was read at 450 nm.

Nitric oxide (NO) concentration was determined in the cell-free supernatant as an indicator of NO production (Green et al., 1982). Nitrite concentration, in turn, was determined using Griess's reagent (1% sulfanilamide, 5%phosphoric acid, 0.1%N - (1-naphthyl) ethylenediamine). The absorbance was measured at 540 nm, and the nitrite concentration in the samples was determined by comparison with a standard curve of sodium nitrite (5–320 mM).

For western blotting, RAW 264.7 macrophages were grown in 6-well plates (1×10^5 cells/well) for 24 h. Extracts (0.2 mg/mL and 1.0 mg/mL) plus LPS (100 ng/mL) were added to the treatment group of plates, while medium or LPS alone was added to the control group of plates. After LPS-stimulation for 18 h, the cells were washed with ice-cold PBS and then lysed with 150 μL cell lysis buffer [100 mM Tris–HCl, 10 mM EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluorete, 10 mM sodium orthovanadate, 2 mM PMSF, 0.1 mg/mL aprotinin] per well of a six-well plate for 40 min. The lysate was centrifuged at 12,000 rpm for 15 min at 4 °C, and the protein quality of each sample was determined using the Lowry protein assay. Samples of 20 μg protein were electrophoresed by SDS–PAGE and transferred to nitrocellulose membrane. After blocking the non-specific site with blocking solution (5% BSA for HSP70 and 5% non-fat milk for NFκB) for 2 h, the membrane was incubated with antibodies (1:1000) against