BIOTRANSFORMED CITRUS RESIDUE EXTRACTS ACT AS AN ANTI-INFLAMMATORY IN CO-CULTURE OF ADIPOCYTES AND MACROPHAGES

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RESUMO – Na obesidade, ocorre maior infiltração de macrófagos no tecido adiposo, aumentando a secreção de citocinas pró-inflamatórias, contribuindo para o desenvolvimento de doenças crônicas não-transmissíveis, sendo positivo a redução dessas citocinas. Assim, avaliou-se o efeito de extratos de resíduos de cítricos (Biotransformado, In Natura, Autoclavado) na secreção de TNF-α, IL-6 e adiponectina em co-cultura de adipocitos (3T3-L1) e macrófagos (RAW264.7). Células 3T3-L1 foram cultivadas até diferenciação. Posteriormente, um transwell foi colocado em cada poço e células RAW264.7 foram inoculadas na camada superior. Após 24 horas, as células foram incubadas com os extratos durante 24 horas. O tratamento com 1,0mg/mL de extrato Biotransformado e In Natura reduziu a secreção de TNF-α (30,7% e 14,9%) e IL-6 (43,4% e 42,7%) comparado a control. O extrato Biotransformado nessa concentração promoveu ainda, maior aumento de adiponectina em relação ao In Natura (66,0% e 35,3%, respectivamente), permitindo concluir que o extrato Biostranformado causou um melhor perfil antiinflamatório. 150 palavras.

ABSTRACT – In obesity, more macrophage infiltrate on adipose tissue, increasing pro-inflammatory cytokines secretion, contributing to development of chronic non-communicable diseases. Thus, reduction in cytokines would be beneficial. We evaluated the effect of citrus residue extracts (Biotransformed, In Natura, Autoclaved) on TNF-α, IL-6 and adiponectin secretion in 3T3-L1 and RAW264.7 co-culture. 3T3-L1 was cultivated for 12 days for differentiation, after that a transwell was added in the well and RAW264.7 was grown in upper layer. After 24 hours, cells were incubated with citrus residue extracts for 24 hours. Treatment with 1.0mg/mL of Biotransformed and In Natura extracts reduced secretion of TNF-α (30.7% and 14.9%) and IL-6 (43.4% and 42.7%) compared to control. Still, Biotransformed extract at a concentration of 1.0mg/ml promoted greater increase in adiponectin in relation to In Natura (66.0% and 35.3% respectively), so we conclude that Biostranformed extract caused a better anti-inflammatory profile. 150 palavras

PALAVRAS-CHAVE: extrato de resíduo de cítrico, biotransformação, obesidade, inflamação, cultura celular.

KEYWORDS: citrus residue extract, biotransformation, obesity, inflammation, cell culture.
1. INTRODUCTION

Obesity is a disease characterized by excess body fat, associated with a chronic subclinical inflammatory condition caused by increased secretion of pro-inflammatory adipokines (Balistreri et al., 2010). This increase in adipokines circulation also appears to be responsible for the development of chronic non-communicable diseases associated with obesity, causing insulin resistance, blood pressure increase, change in serum lipids, increased inflammatory response and thrombus formation (Grundy et al., 2004).

The phenolic compounds are a class of substances that has been investigated for use in prevention and treatment of these diseases. An interesting source of phenolic compounds is citrus fruits. Among the most commercially important citrus, there is orange, with Brazil being the largest producer in the world, reaching a production of 18,012,560 megatons in 2012, according to estimates of the Food and Agriculture Organization (FAO). However, it is important to observe that most of oranges are destined to juice production, and about 50% of the waste generated is composed of peel and pulp, commonly used as animal feed component. However, it is known that citrus peel has a high content of polyphenols, and several studies have shown the positive effects of peel extracts in the treatment of chronic non-communicable diseases (Ding et al., 2012; Kim et al., 2012; Kang et al., 2012; Raasmaja et al., 2013).

In this sense, this work aims to assess the biological potential of an extract rich in phenolic compounds, obtained by biotechnological processes, from citrus residue from the pectin extraction industry. It is noteworthy that the value of this waste is very low, being a by-product of two subsequent industrial processes: first, the orange juice extraction; and second the pectin extraction.

In addition to the use of industrial residue with low value as a source of phenolic compounds, an important innovation proposed by the work is the biotransformation of polyphenols from citrus residue, by solid state fermentation with the microorganism Paecilomyces variotii, generating a product with different polyphenols profile composition from those obtained by simple extraction. This Biotransformed extract of citrus phenolics can be a viable source of bioactive phenolic compounds, which to date has no source for commercial extraction, since it is present naturally in very low concentrations in plant sources. Furthermore, it is expected to find a synergistic effect of the various polyphenols present in the extract, showing a higher biological effect than an isolated polyphenol analytical standard.

Considering these, the study aimed to test citrus residue extracts for its anti-inflammatory on adipose tissue activity in vitro, evaluating adiponectin, IL-6 and TNF-α concentration in 3T3-L1 adipocyte and RAW 264.7 macrophage co-culture.

2. MATERIAL E METHODS

2.1 Citrus Residue Extracts

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 50% ethanol extracts were prepared from the “Biotransformed” residue, after fermentation process, and two control residues. The first control was the unfermented residue consisting of the extract from 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. 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).

### 2.2 Cell Culture Assays

3T3-L1 murine pre-adipocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM) at 37°C in a humidified atmosphere with 5% CO₂. All media contained 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 µg/ml).

The 3T3-L1 cells (2.0 x 10⁴ cells/mL) were seeded in 24-well plates and grown until confluence. Two days after confluence, designated as day 0, the cells were switched to differentiation medium containing 10µg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1µM dexamethasone (DEX) in DMEM for another 3 days. Then, the cell culture medium was replaced with maturation medium containing 10µg/mL insulin in DMEM. The maturation medium was changed every 2 days, until day 12, after which mature adipocytes containing lipid droplets were formed. On day 12 of the maturation sequence, transwell inserts (0.45 µm - Milipore, Ireland) were placed in each well and RAW 264.7 were inoculated in the upper layer. After 24 hours, cells were treated with the extracts for 24 hours, and the supernatant was collected. The amount of adiponectin, IL-6 and TNF-α in the medium was determined using a Milliplex® MAP Mouse Adipocyte Luminex assay in accordance with the manufacturer’s instructions.

### 2.3 Statistical Analysis

Results were expressed as means ± standard deviation (SD). The statistical difference between the groups was analyzed using analysis of variance (ANOVA). Post hoc comparison was performed by Dunnet’s and Tukey’s test. Differences were considered significant when p ≤ 0.05. All analyses were performed using the software GraphPad Prism 5 for Windows version 5.00 (GraphPad Software Inc.).

### 3. RESULTS E DISCUSSION

The cell treatment with all extracts increased the amount of adiponectin in comparison to control (non-treatment cells). Autoclaved extract caused greater increase in adiponectin concentration, however it had no effect on IL-6 in the two concentrations tested, not showing favorable improvement in the inflammatory state (Table 1).

On the other side, the addition of 1.0 mg/mL of Biotransformed and In Natura extract reduced the concentration of IL-6, in comparison to control, by 43.4% and 42.7%, respectively. And assessing TNF-alpha we can observe a reduction of 30.7% with the addition of Biotransformed extract and 14.9% with In Natura extract (Table 1). Still, at this concentration Biotransformed extract caused
greater secretion of adiponectin in relation to In Natura. Thus, the Biotransformed extract showed better anti-inflammatory activity in adipose tissue stimulated by the presence of macrophages.

Table 1 – Cytokines and NO concentration in 3T3-L1 and RAW 264.7 co-culture

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control Conc mg/mL</th>
<th>Biotransformed</th>
<th>In Natura</th>
<th>Autoclaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiponectin (pg/mL)</td>
<td>1391.95±434.80 0.2</td>
<td>1937.69±312.13ab</td>
<td>2246.59±318.04ab</td>
<td>2926.31±421.84ab</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2311.23±554.79a</td>
<td>1883.43±139.61a</td>
<td>2691.94±606.68a</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>2190.54±1061.05 0.2</td>
<td>2174.09±721.92a</td>
<td>2172.84±180.38a</td>
<td>2237.48±284.59a</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1239.58±728.43b</td>
<td>1255.74±189.67b</td>
<td>2187.16±807.58a</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>529.29±56.48 0.2</td>
<td>488.57±49.60a</td>
<td>767.87±136.78b</td>
<td>680.64±113.93ab</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>366.73±44.77a</td>
<td>450.65±95.61a</td>
<td>452.60±118.43a</td>
</tr>
</tbody>
</table>

Asterisk indicates the statistical significant difference compared to control by Dunnett’s test (p ≤ 0.05). Different small letters indicate significant difference between the samples in the same concentration by Tukey’s test (p ≤ 0.05).

In view of the flavonoids profile, In Natura extract contains mainly hesperidin and naringin, and because of the biotranformation process, the Biotransformed extract contains hesperitin and naringenin (Nakajima et al., 2016). Some studies show that analytical standards of these flavanones have anti-inflammatory activity. Yoshida et al. (2010) found that in 3T3-L1 adipocytes cell culture, hesperetin and naringenin analytical standards showed anti-inflammatory effect by inhibiting the activation of NFkB through TNF-α, with a consequent reduction in the secretion of IL-6. Pinho-Ribeiro et al. (2016) studied RAW 264.7 macrophages stimulated with LPS, and verified that naringenin treatment reduced NFkB activation, and thereafter reduced TNF-α and IL-6 secretion. In another study, naringenin analytical standard (1%) was supplemented in mice fed a high fat diet, and reduced expression of TNF-α, MCP-1 (monocyte chemotactic protein-1), and TLR 2 in adipose tissue (Yoshida et al., 2013), promoting protection against chronic non-communicable diseases. In the present study, the extracts also caused reduction in inflammatory cytokines in vitro, and considering the aforementioned researches they probably acted in NFkB pathway.

If these results are also observed in vivo, this could be an alternative way for producing a nutraceutical with anti-inflammatory activity at lower cost than an analytical standard.

4. CONCLUSIONS

The different flavanone profile of the Biotransformed extract caused a better anti-inflammatory activity in vitro, being a product with promising results to combat inflammation associated with obesity.
5. REFERENCES


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