



# 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.

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## 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. Materials and methods

### 2.1. Chemicals

Gallic acid, Folin–Ciocalteu reagent, 2,2'-azobis(2-methylpropionamide) (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 *Paezilomyces 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 ( $9 \times 10^6$  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, Batestin, 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).

at 37 °C with excitation filter 485 nm and emission filter 520 nm. The measurements were performed in triplicate. ORAC values were defined as the difference between the area under the FL decay curve of the samples and the blank (net AUC). Regression equations between net AUC and antioxidant concentration were calculated for all of the samples and Trolox® (control). ORAC-FL values were expressed as  $\mu\text{mol}$  of Trolox®/mg of extracts.

## 2.5. Cell culture assay

### 2.5.1. Cell culture

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<sub>2</sub>. All media contained 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100  $\mu\text{g}/\text{ml}$ ).

### 2.5.2. MTT Assay

The 3T3-L1 cells ( $1.0 \times 10^5$  cells/ml) were seeded in 96-well plates and incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. Then the cells were treated with the samples (0.01–1.00 mg/ml). After 24 h of incubation, all media was removed and 10  $\mu\text{L}$  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<sub>2</sub> for 4 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.

### 2.5.3. Pre-adipocytes differentiation

The 3T3-L1 cells ( $2.0 \times 10^4$  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 10  $\mu\text{g}/\text{mL}$  insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1  $\mu\text{M}$  dexamethasone (DEX) in DMEM for another 3 days. Then, the cell culture medium was replaced with maturation medium containing 10  $\mu\text{g}/\text{mL}$  insulin in DMEM. The maturation medium was changed every 2 days, until day 12, after which mature adipocytes containing lipid droplets were formed.

### 2.5.4. Oil Red O staining

The cells were submitted to two different treatments. First, cells were exposed to the extracts sample in the differentiation medium followed by maturation medium without the samples. Using this treatment it is possible to see if the extracts could impair the pre-adipocytes differentiation.

The second treatment consisted in the addition of the extracts only in the maturation medium. This procedure intended to verify if the extracts could reduce triglyceride accumulation in mature adipocytes.

In both cases, on day 12, the 3T3-L1 mature adipocytes plated onto 24-well plates were washed once with formaldehyde 10% in PBS, and fixed with formaldehyde 10% in PBS for 60 min. After replacement of formaldehyde 10% in PBS with 60% isopropanol, the cells were stained for 30 min in freshly diluted Oil red O (Sigma) solution (2.1 mg/ml) with 60% isopropanol. Thereafter, the cells were washed four times with water and the wells were dried in room temperature. Subsequently, the Oil Red O in the stained cells was eluted with 100% isopropanol. The absorbance was measured with a microplate reader at 492 nm. Each treatment was performed in triplicate. The results are expressed as a percentage of control cells, that are fully differentiated cells without any sample treatment, according to the equation below:

$$\% \text{ of Oil Red O staining} = (\text{Abs Sample} / \text{Abs Control}) \times 100$$

### 2.5.5. Glycerol assay

On day 12 of the maturation sequence, cells were treated with the samples for 18 h, and the supernatant was collected. The amount of glycerol in the medium was determined using a Glycerol Assay Kit (Cayman, CO, U.S.A.) in accordance with the manufacturer's instructions.

## 2.6. Statistical analysis

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

## 3. Results

### 3.1. Extraction solution selection

The total phenolic content of the extracts obtained by using different extraction solvents varied from  $72.29 \pm 4.83$  to  $90.45 \pm 5.44$  mg of Gallic Acid Equivalent/ml of extract for water and ethanol 70% HCl 1%, respectively. There was no statistical difference between the samples, not being possible to use this parameter to determine the best extraction solution (data not shown).

The HPLC analysis showed that the extraction with 70% methanol, 70% ethanol acidified with 1% HCl and 50% ethanol resulted in higher content of the quantified flavanones (Fig. 1). Due to the lack of difference in methanol or ethanol as extraction solvent, it is justified the use of solutions with ethanol, since it is a food grade solvent. Still, aiming the lower solvent use, and considering the statistical similarity between the results, we selected the solution of 50% ethanol for extraction of flavanones from the "Biotransformed" residue.

Despite the widespread use of Folin Ciocalteu assay, this was not a good method for screening the best extracting solution. There was no significant difference between the samples according to this analysis, even though the HPLC results clearly indicating smaller potential of water as an extracting solvent for this system.

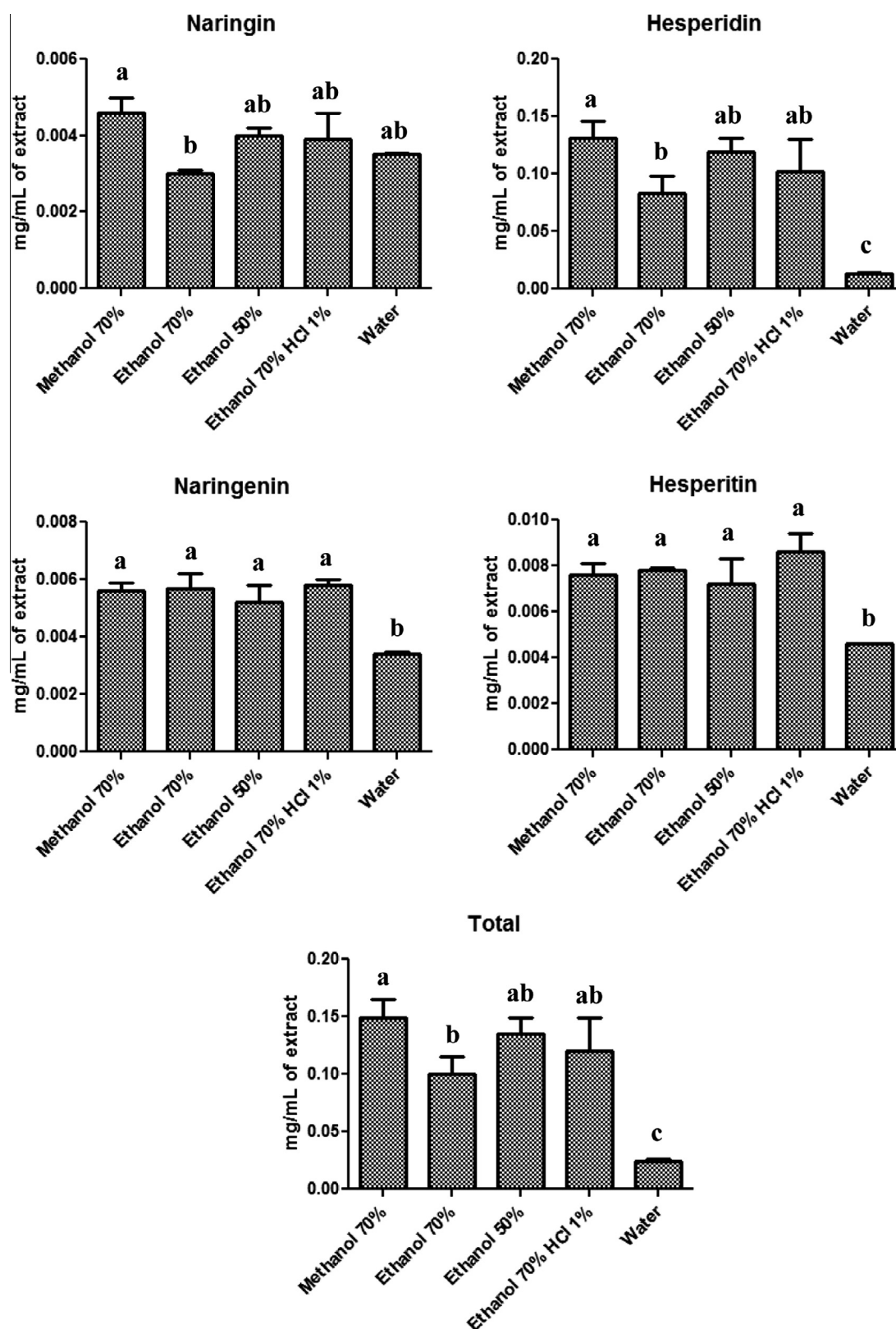
### 3.2. Characterization of "Biotransformed" residue extract obtained from the 50% ethanol solution

There was no difference in the content of total polyphenols by Folin Ciocalteu assay between the "Biotransformed" residue and the controls (Table 1).

However, once again the HPLC analysis showed difference between the samples. The "In Natura" residue had higher content of glycosides flavanones, naringin and hesperidin, while the "Biotransformed" residue had higher level of the aglycone flavanones, naringenin and hesperitin (Fig. 2). These results demonstrate that the fermentation process caused the biotransformation of the flavanones, increasing the amount of flavonoids free form.

The results in Fig. 2 indicate that the sterilization process by autoclaving degraded a certain amount of flavanones. However, this is a necessary step in the fermentation process to eliminate any microbial contamination present in the residue and allow only the reaction by the inoculated one.

Despite the fact that the "Biotransformed" residue extract presented a much smaller amount of total flavanones than the controls (Fig. 2), the antioxidant capacity of the extracts was similar according to DPPH and ORAC assays (Table 1), indicating that the flavanones presented in the "Biotransformed" residue had higher antioxidant potential.



**Fig. 1.** Flavonoids of interest quantified by High Performance Liquid Chromatography (mg/mL of extract). Different letters indicate significant differences by Tukey's test ( $p \leq 0.05$ ).

### 3.3. Cell assays

According to MTT assay, none of the extracts were toxic to 3T3-L1 cell line in the concentrations tested (Fig. 3). Since no loss of cell viability was observed in the concentration range that the

cells were exposed, it is considered safe to continue with the following cellular assays within the concentrations tested.

The analysis Oil Red O staining showed that when the samples were added in the differentiation medium, there was little effect in the total lipid accumulation (Table 2). The best result was observed



**Table 1**  
Total polyphenols, ORAC and DPPH radical-scavenging activity of the extracts.

	Total polyphenol (mg gallic acid equivalent/mg of lyophilized extract)	DPPH (μmol equivalent Trolox/mg of lyophilized extract)	ORAC (μmol equivalent Trolox/mg of lyophilized extract)
Biotransformed	35.04 ± 2.36 <sup>a</sup>	136.77 ± 5.41 <sup>a</sup>	542.93 ± 78.04 <sup>a</sup>
In Natura	36.23 ± 3.01 <sup>a</sup>	130.80 ± 11.17 <sup>a</sup>	666.99 ± 110.54 <sup>a</sup>
Autoclaved	33.31 ± 1.03 <sup>a</sup>	129.17 ± 6.71 <sup>a</sup>	658.38 ± 70.87 <sup>a</sup>

Different letters in the column indicate significant differences by Tukey's test ( $p \leq 0.05$ ).

with the addition of “In Natura” extract in the concentration of 0.5 mg/mL, presenting a lipid accumulation reduction of about 19% in relation to control cells with no treatment.

On the other hand, the addition of the extracts in the maturation medium caused a decrease in the lipid accumulation (Table 3). This reduction was dose-dependent for all the samples, reaching a reduction of 22%, 38% and 48% for “Biotransformed”, “Autoclaved” and “In Natura” extracts, respectively.

The total glycerol concentration was below the limit of detection for the cells treated with “In Natura” and “Autoclaved” extracts. Thus, the “Biotransformed” residue extract was the only that presented some effect on the amount of glycerol released, and the values observed presented dose dependent behavior ( $2.39 \pm 0.17$ – $5.24 \pm 0.29$  mg/mL of glycerol).

#### 4. Discussion

The Folin Ciocalteu assay was not useful to distinguish the samples. It is noteworthy that the color reaction the Folin Ciocalteu assay is based on not only occurs due to the presence of polyphenols, but can also be caused by other compounds with reducing power (Huang, Ou, & Prior, 2005).

The total amount of flavanones was statistically similar when using methanol or ethanol as extraction solvents. Solutions containing methanol are the most used in the extraction of polyphenols of solid materials (Hayat et al., 2010; Ramful et al., 2010; Singh et al., 2011). However, due to its toxicity (Liesivuori & Savolainen, 1991; Tephly, 1991), it is interesting the development of a process using more friendly solvents. The results presented indicate the potential of 50% ethanol solution in extraction of the flavanones naringin, hesperidin, naringenin and hesperitin from fermented citrus residue. Thereby, this process used a solvent less harmful to health, increasing the feasibility of the extract in studies with cell culture, animal models and humans. Still, using ethanol instead of methanol takes into account economic considerations imposed by the industrial context.

The HPLC analysis clearly showed the change in flavanones profile in “Biotransformed” extract. According to Madeira et al. (2014) the microorganism employed in this process probably uses naringin and hesperidin as source of carbon and energy during fermentation. In previous study of our group, it was observed that the tannase produced by *P. variotii* strain during fermentation has the ability to catalyze the flavanones deglycosylation, such as hesperidin (Ferreira et al., 2013). Also, in microbial fermentation the compounds like hesperitin and naringenin are transformed in other chemical particles, with lower molecular weight, that are used in microorganism metabolism (Justesen, Arrigoni, Larsen, & Amado, 2000). Aguilar, Aguilera-carbo, and Robledo (2008) observed that the solid-state fermentation of creosote bush leaves and pomegranate peels transformed the tannins present in the substrate into lower molecular weight phenolics, confirming the fact that the fermentation process is able to change the phenolic profile of a product.

Even using a citrus residue from pectin industry waste, the quantity of flavanones extracted was comparable to other studies. Yu et al. (2014) and Ho, Su, and Lin (2013) performed the extraction of flavonoids from fruits acquired for the research, taking care

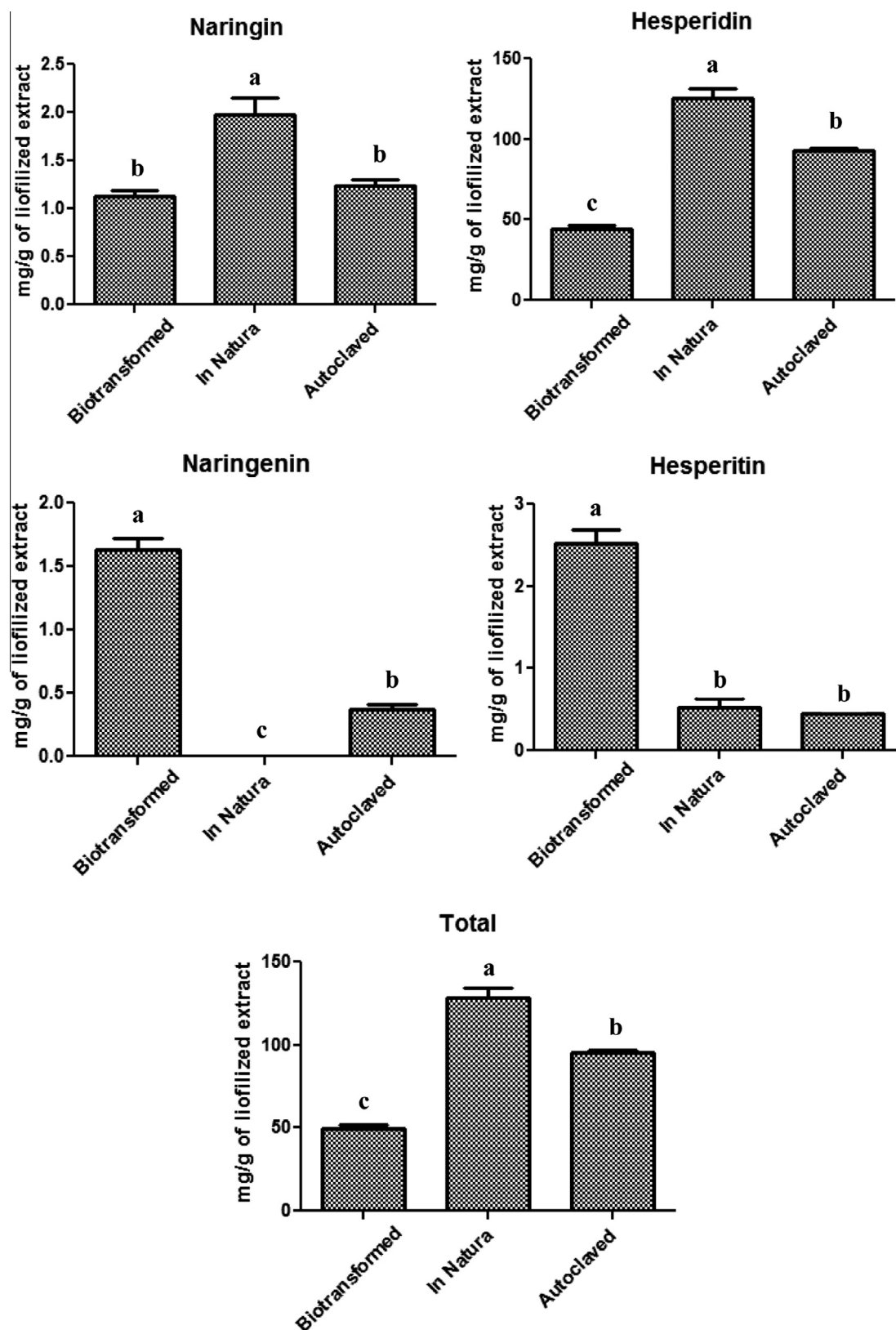
in the acquisition, transport and storage of raw materials. The first authors were able to extract 1.278 mg/g FW of naringin and 1.480 mg/g FW of hesperidin, indicating values comparable to the present work for hesperidin. In the second study, the extraction was performed in nine different citrus fruits, and for five of them the amount of hesperidin and naringin was lower than that obtained in the present study. This data reinforces the advantage of the produced extract, once it was obtained from low cost industrial waste residue, commonly used for animal feed, presenting a possibility to increase the commercial value of this product.

Despite the lower content of total flavanones in “Biotransformed” extract, antioxidant activity by DPPH and ORAC was similar between the samples. The literature indicates that in some cases hesperitin and naringenin have higher biological potential than hesperidin and naringin. Londoño-Londoño et al. (2010) demonstrated that hesperitin was more active than hesperidin in reducing lipid peroxidation in hepatic microsomes, with lower amount of TBARS. Moreover, these authors observed that at low concentrations (10 mg/mL) hesperitin was more effective in reducing oxidized LDL by peroxynitrite-oxidized LDL model. Silva et al. (2013) biotransformed orange and lime juices by enzymatic deglycosylation and observed higher antioxidant activity by DPPH method and FRAP assay after the biotransformation, indicating the higher antioxidant activity of the aglycones obtained.

According to the results, all extracts were able to reduce lipogenesis *in vitro*, however, the data found seem to indicate that each extract have a different mechanism of action. Only the “In Natura” extract showed some effect on the inhibition of pre-adipocytes differentiation (Fig. 3), and may have a promising application in preventing the formation of new mature adipocytes. Kim et al. (2012) also observed that the addition of Citrus aurantium Flavonoids extract inhibited 3T3-L1 differentiation with a reduction in the amount of lipid droplets, confirming this positive effect of flavonoids from citrus.

When the extracts were added to the maturation medium, all samples were able to reduce lipid accumulation, with a greater effect of “In Natura” extract. However, only “Biotransformed” extract, with higher content of hesperitin and naringenin, caused induction of lipolysis, observed by higher amount of free glycerol on the supernatant of the culture. It is noteworthy that “Biotransformed” extract had lower amount of flavanones per gram of lyophilized extract in comparison to “In Natura” extract (Fig. 2), and despite this great difference, the “Biotransformed” extract was able to reduce lipids content in the cells as “In Natura” extracts, and it was the only one able to cause lipolysis.

Subash-Babu and Alshatwi (2014) studied the effects of 20 μM hesperitin analytical standard in immortalized human bone marrow mesenchymal stem-cell (TERT20) differentiated with dexamethasone, IBMX, indomethacin and insulin. Hesperitin was added in two different situations: in group 1 the flavanone was administered in the differentiation medium; in group 2 the compound was added after the differentiation in the maintenance medium. In both cases, there was a reduction on lipid accumulation according to staining with Oil Red O, even though the effect was more pronounced in group 2, similar to our results. They also observed a slight stimulation of lipolysis, confirming the lipolytic activity of hesperitin.



**Fig. 2.** Flavonoids of interest quantified by High Performance Liquid Chromatography (mg/g of lyophilized extract). Different letters indicate significant differences by Tukey's test ( $p < 0.05$ ).

Some studies have been shown that hesperitin (Gamo, Shiraki, Matsuura, & Miyachi, 2014) and naringenin (Yoshida et al., 2013) seems to act as PPAR  $\gamma$  agonist. PPAR  $\gamma$  is a nuclear transcription factor that induce adipocyte differentiation (Kubota et al., 1999),

causing greater differentiation of preadipocytes to mature adipocytes when it is activated. This could explain why the "Biotransformed" extract were not able to reduce the differentiation process. Some researchers have found that when these flavonoids

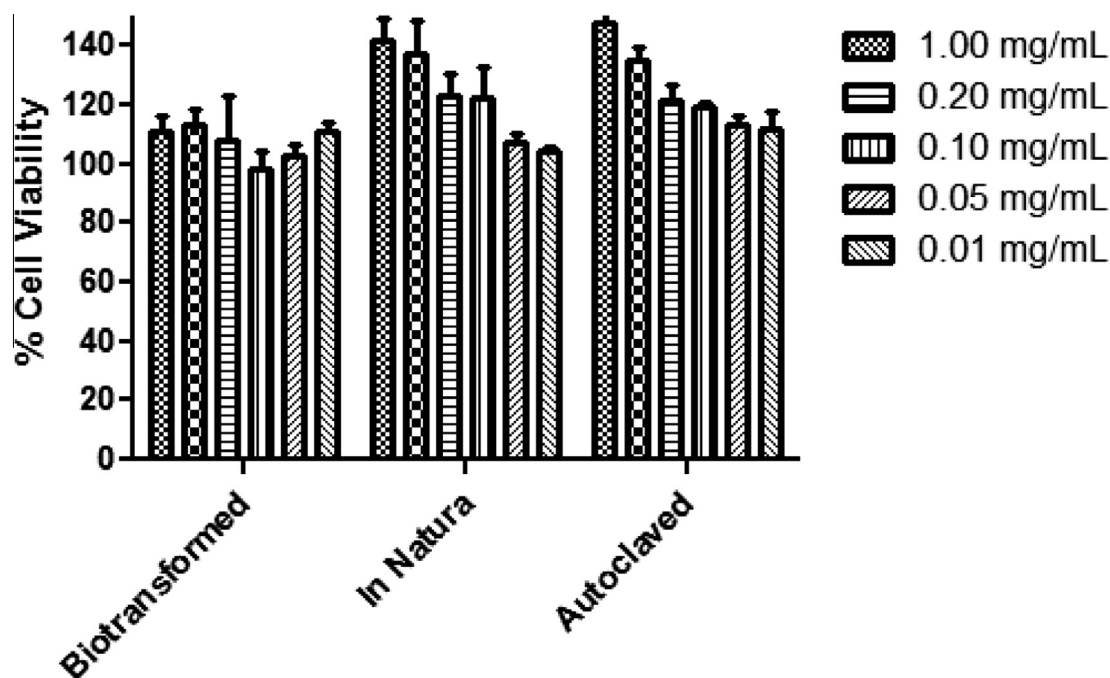


Fig. 3. Cell viability of 3T3-L1 cell line according to MTT assay.

Table 2

Oil Red O staining with samples added in the differentiation medium, % in relation to control.

Samples	Concentration			
	0.05 mg/mL	0.20 mg/mL	0.50 mg/mL	1.00 mg/mL
Biotransformed	101.52 ± 10.00 <sup>a</sup>	100.00 ± 4.50 <sup>a</sup>	103.21 ± 4.55 <sup>b</sup>	107.22 ± 4.03 <sup>b</sup>
In Natura	95.59 ± 1.52 <sup>a</sup>	93.98 ± 3.94 <sup>a</sup>	80.96 ± 4.19 <sup>a</sup>	90.46 ± 4.61 <sup>a</sup>
Autoclaved	103.45 ± 3.71 <sup>a</sup>	99.17 ± 3.26 <sup>a</sup>	96.05 ± 2.79 <sup>b</sup>	98.39 ± 2.22 <sup>a</sup>

Different letters in the column indicate significant difference between the samples in the same concentration by Tukey's test ( $p \leq 0.05$ ).

Table 3

Oil Red O staining with samples added in the maintenance medium, % in relation to control.

Samples	Concentration			
	0.05 mg/mL	0.20 mg/mL	0.50 mg/mL	1.00 mg/mL
Biotransformed	97.56 ± 1.15 <sup>b</sup>	84.67 ± 5.37 <sup>b</sup>	89.45 ± 7.70 <sup>c</sup>	78.18 ± 6.50 <sup>b</sup>
In Natura	86.91 ± 3.55 <sup>a</sup>	65.67 ± 4.30 <sup>a</sup>	55.96 ± 3.50 <sup>a</sup>	52.89 ± 2.04 <sup>a</sup>
Autoclaved	103.59 ± 1.11 <sup>c</sup>	88.86 ± 2.14 <sup>b</sup>	75.65 ± 4.12 <sup>b</sup>	62.66 ± 2.93 <sup>a</sup>

Different letters in the column indicate significant difference between the samples in the same concentration by Tukey's test ( $p \leq 0.05$ ).

were added to 3T3-L1 cell culture, it was observed a greater accumulation of lipid droplets possibly due to its agonist role (Gamo et al., 2014; Morikawa et al., 2008; Yoshida et al., 2013). However, in our study, despite the possible action of hesperitin and naringenin as PPAR  $\gamma$  agonist, there was an expressive reduction in lipid accumulation, which can be explained by the lipolytic role played by these aglycones. Still, we must consider that the afore mentioned studies were done with analytical standards, evaluating each compound alone. In the present work, we used a crude extract, and differences in response can occur due to the synergistic effect that compounds together may cause.

There are many studies indicating the potential of citrus extract in obesity treatment (Kang et al., 2012; Kim et al., 2012), however for the first time it is documented promising results with an extract rich in aglycones. The studies using aglycones usually test high cost analytical standards isolated, missing the synergistic effects of the natural extracts we propose.

Still, some authors indicate other advantages of aglycone forms in obesity treatment. Kim, Park, Kim, Lee, and Rhyu (2013) found

that hesperitin caused higher secretion of cholecystokinin (CCK) in STC-1 cells in comparison to hesperidin, indicating a possible role of this aglycone flavanone in food intake control since CCK is an anorexigen hormone (Raybould, 2009). Thus, these other information suggests the potential of the extract produced by biotransformation to other biological activities, being suitable for further studies.

## 5. Conclusions

Ethanol can replace methanol as extraction solvent of flavanones from biotransformed citrus residue. The biotransformation was able to modify the flavanones profile of the citrus residue extract, increasing the content of hesperitin and naringenin that naturally occur in low quantities in citrus fruits. Still, all extracts could be used in obesity treatment, however aiming different targets. The "In Natura" extract would be useful to reduce new adipocytes synthesis and lipid accumulation, and "Biotransformed" extract could be used to induce lipolysis on fat tissue.

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