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Improving the chemopreventive potential of orange juice by enzymatic biotransformation☆

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ABSTRACT

Among the fruit-based beverages, orange juice is the most widely consumed. The Brazilian share of orange juice in the world market is around 53%. Consumers are increasingly demanding natural ingredients expected to be safe and health-promoting. The tannase enzyme, obtained from *Paecilomyces variotti*, was applied in this study with the purpose of biotransforming the orange juice polyphenols and, in this way, modifying their biological activity. It was demonstrated that the tannase extract was able to modify the polyphenolic composition of the orange juice and act in naringin and hesperidin in the removal of glycosides. The results of the enzymatic reactions of the hesperidin, naringin and orange juice samples were products with heightened functional activity in relation to the original samples, as demonstrated with in vitro tests of antioxidant activity (ORAC and DPPH) and an antiproliferation assay of human cells. The results demonstrate the benefits of the biotechnological modification of natural food molecules, improving the nutraceutical potential of a popular beverage.

Orange juice consumption has become a worldwide dietary habit and, as a result, the consumption of frozen, concentrated juice has increased steadily over the years. The Brazilian share of the world market of concentrated orange juice is around 53%, and 98% of this production is designated to exportation to European countries. Among the fruit-based beverages, orange juice is the most widely consumed, with a participation of 35% of the fruit beverages market (Neves, 2010). Consumers are increasingly aware of diet-related health problems and therefore are demanding natural ingredients, which are expected to be safe and health-promoting. Studies demonstrated the high antioxidant capacity of orange juice, especially due to the expressive presence of vitamin C and phenolic compounds (Lee & Coates, 1999; Jayaprakasha & Patil, 2007). Concentrated orange juice has a greater flavonoid content, including polymethoxylated fresh juice (Cesar,

Polyphenols are receiving increasing interest from consumers and food manufacturers for several reasons. Epidemiological studies have suggested associations between the consumption of polyphenol-rich foods or beverages and a decreased risk of cardiovascular diseases and certain forms of cancer. In addition, these compounds have applications in food stabilization due to their ability to protect against peroxidation of oxygen-sensitive foods (Cesar et al., 2010; Benavente-Garcia, Castillo, Marin, Ortuño, & Del Río, 1997).

The polyphenols are the most regular and abundant reducing agents of our diet. Nevertheless, the molecular structure of these compounds affects their biological properties, such as bioavailability, antioxidant capacity and specific interactions with cell receptors and enzymes (Scalbert & Williamson, 2000). Most phenolics that are found in plants, however, are conjugated to sugars as glycosides. This conjugation reduces their ability to function as good antioxidants, since the availability of free hydroxyl groups on the phenolic rings is potentially important for resonance stabilization of free radicals. Lowered antioxidant capacity has direct implications on decreasing health functionality when these phenolics are ingested as food or as nutraceuticals and depend on the microbiotic enzymatic activity. Therefore, if free phenolics are released from their glycosides, the antioxidant capacity and, thus, health functionality of these phytochemicals could be improved (Vattem & Shetty, 2003).

Tannin acyl hydrolases, commonly referred to as tannases (E.C. 3.1.1.20), are inducible enzymes produced by fungi, yeast and bacteria.

Abbreviations: AAPH, 2,2′-azobis(2-methylpropionamidine); DPPH, 2,2-diphenyl-1-picrylhydrazyl; SRB, sulforhodamine B sodium salt; FL, fluorescein; AUC, area under the curve; PBS, phosphate buffered solution; EDTA, ethylenediaminetetra acetic acid.

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Tannases have mostly been characterized by their activity on complex polyphenolics and are able to hydrolyze the ester bond (galloyl ester of an alcohol moiety) and the depside bond (galloyl ester of gallic acid) of substrates such as tannic acid, epicatechin gallate, epigallocatechin gallate, and chlorogenic acid (Garcia-Conesa, 2001). Battestin, Macedo, & De Freitas, 2008, studied the tannase from *Paecilomyces variotti*, which mediated the hydrolysis of the green tea polyphenol, epigalocatechin gallate, and generated epicatechin and gallic acid. This resulted in an increased antioxidant activity of the tea extract after the enzymatic reaction.

Based on this information, the tannase obtained from *P. variotti* was applied in this study with the purpose of biotransforming the orange juice polyphenols and, in this way, modifying their biological activity.

2. Materials and methods

2.1. Materials

Naringinase, hesperidin, naringin, tanic acid, gallic acid 2,2′-azobis(2-methylpropionamidine) (97%) (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sulforhodamine B sodium salt (SRB), trichloroacetic acid and T1503 Trizma® base were purchased from Sigma-Aldrich. Fluorescein was purchased from ECIBRA, and Trolox® (97%) was purchased from ACROS Organics. Cell culture reagents were purchased from Invitrogen®. All other chemicals were purchased in the grade commercially available. The concentrated orange juice was kindly provided by Coca-Cola do Brasil®.

2.2. Enzyme

Tannase was isolated from *P. variotii* using a previously published procedure (Battestin & Macedo, 2007a). A 250-mL conical flask containing 5 g of wheat bran, 5 g of coffee husk, 10 mL of distilled water and 10% tannic acid (w/w) (Ajinomoto OmniChem Division, Wetteren, Belgium) was used for the fermentation process. The culture medium (pH 5.7) was sterilized at 120 °C for 20 min. After sterilization, the flasks were inoculated with 2.5 mL (5.0×10^7 spores/mL) of the pre-inoculum suspension and incubated at 30 °C for 120 h. After fermentation, 80 mL of 20 mM acetate buffer, pH 5.0, was added, and samples were shaken at 200 rpm for 1 h. The solution was filtered and centrifuged at 9650 *g* for 30 min at 4 °C (Beckman J2-21 centrifuge, Beckman-Coulter, Inc. Fullerton, CA, USA). The supernatant was then treated with solid ammonium sulfate (80% saturation) and incubated overnight at 4 °C. The precipitate was collected by centrifugation (9650 *g* for 30 min), resuspended in distilled water and dialyzed for 48 h against distilled water. The dialyzed preparation was freeze-dried and used as a semi-purified tannase extract.

The tannase activity was assayed according to Battestin and Macedo (2007). The substrate was a 0.7% (w/v) solution of tannic acid in 0.2 M acetate buffer at pH 5.5. The reaction was conducted by adding 0.3 mL of substrate solution to 0.5 mL of enzyme extract and incubating at 60 °C for 10 min. After incubation, the reaction was stopped by the addition of 3 mL of a 1.0 mg/mL bovine serum albumin solution (BSA) prepared in a 0.17 M sodium chloride solution in 0.2 M acetate buffer, pH 5.0. The solution was then filtered and centrifuged at 10,070 *g* for 15 min at 4 °C. The precipitate was dissolved in 3 mL of SDS-triethanolamine and 1 mL of $FeCl_3$ reagent added and held for 15 min for stabilization of the color. The absorbance was measured at 530 nm (Mondal et al., 2001) and the enzyme activity calculated from the change in absorbance at 530 nm. One unit of tannase activity was defined as the amount of tannic acid hydrolyzed by 1 mL of enzyme per minute of reaction: $Abs_{530} = Abs_{control} - Abs_{test}$.

The commercial naringinase activity was assayed according to Davis (1947). The reactional medium consisted in 0.9 mL of naringin solution 0.08% (m/v) in acetate buffer (100 mmol·L^{-1}, pH 4.0) and 0.1 mL of commercial naringenase solution (1 mg/mL) on the same buffer. The reaction occurred at 40 °C and 30 min. After that, 0.1 mL aliquot was added to 5 mL of diethylene glycol 90% (v/v). 0.1 mL of sodium hydroxe solution (4.0 mol·L^{-1}) was added at the mixture and the absorbance (420 nm) of the solutions were measured after 20 min at room temperature. One unit of naringenase activity was defined as the amount of enzyme necessary to hydrolyze by 1 mL of naringenin per minute of reaction.

2.3. Enzymatic biotransformation

The concentrated orange juice and the commercial control samples (hesperidin and naringin) were used as substrates for enzymatic hydrolysis by tannase isolated from *P. variotii* (Battestin & Macedo, 2007a). The concentrated orange juice was dissolved in a buffer with a proportion of 1 g for 5 mL of: phosphate buffer (pH 7.4, 75 mM) for the tannase reaction, and acetate buffer (pH 4.0, 100 mM) for the naringinase reaction. After that, 1 mL of the appropriated juice solution was incubated with 5.0 U of tannase and 0.85 U of naringinase at 40 °C for 60 min. The hydrolysis process was stopped by placing the reaction in an ice bath for 15 min. The hesperidin and naringin samples (5 mg) were dissolved in 1 mL of phosphate buffer (pH 7.4, 75 mM) and incubated with 5 mg of tannase at 40 °C for 60 min. The hydrolysis process was stopped by placing the reaction in an ice bath for 15 min. The biotransformed polyphenols were used for the antioxidant assay after suitable dilution with the same phosphate buffer (pH 7.4, 75 mM) for ORAC and with a 70% methanol solution for DPPH. For the cellular assays, the samples were diluted with DMEM.

2.4. Orange juice polyphenol analyses

The biotransformation products of the purified commercial polyphenols, hesperidin and naringin, were characterized by HPLC (CLAE-DAD) and mass spectrometer. The biotransformed orange juice was evaluated by HPLC (CLAE-DAD). The naringin and hesperidin biotransformed samples (50 μL) were dissolved in 600 μL of methanol and 350 μL of Milli-Q water, and then the samples were filtrated on 0.45 μm membranes. The biotransformed orange juice was centrifuged for 10 min at 6000 rpm (Daigger® model SD) and 400 μL of the supernatant was dissolved with 600 μL of Milli-Q water and filtrated on 0.45 μm membranes.

2.4.1. HPLC (CLAE-DAD)

A Dionex UltiMate 3000 (Germany) liquid chromatography, equipped with a C-18 Atlantis® (Waters, 5 μm, 4.6×150 mm) column 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 Mejía, Songa, Hecka, and Ramírez-Maresb (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 of 0.6 mL/min. The spectra were obtained at 190 and 480 nm and the chromatograms processed at 280 nm.

2.4.2. Mass spectrometer

A Synapt Q-TOF mass spectrometer (Waters, USA) was used for fingerprinting the ESI-MS analysis. The general conditions were: source temperature of 100 °C, capillary voltage of 3 kV and cone voltage of 30 V. For measurements in the negative ion mode, ESI(−)-MS, 10.0 μL of ammonium hydroxide aqueous solution was added to the sample mixture for a total volume of 1000 μL, yielding 0.1% as the final concentration. ESI-MS was performed by direct infusion with a flow rate of 10 μL/min^{-1} using a syringe pump (Harvard Apparatus). Mass spectra were acquired and accumulated over 60 s, and spectra were scanned in the m/z range between 50 and 1000.

2.5. Bioactivity potential of the biotransformed orange juice polyphenols

2.5.1. DPPH anti-oxidant assay

The potential antioxidant activity of the orange juice was assessed based on the scavenging activity of the stable 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical, as described by Macedo, Battestin, Ribeiro, and Macedo (2011). The measurements were performed in triplicate, and anti-radical activity was calculated using the linear regression equation determined by plotting the anti-radical activity of Trolox solutions of known concentrations. Antiradical activity was expressed as μmol of Trolox·L^{-1} equivalent to the orange juice solution, or μmol of Trolox·g^{-1} of the commercial naringin and hesperidin.

2.5.2. ORAC antioxidant assay

ORAC assays were performed using fluorescein (FL) as the fluorescent probe, as described by Macedo et al. (2011). The automated ORAC assay was carried out on a NovoStar Microplate reader (BMG LABTECH, Germany) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The measurements were made in a COSTAR 96 plate. The reaction was performed at 37 °C, being that it was started by thermal decomposition of AAPH in a 75 mM phosphate buffer (pH 7.4) due to the sensitivity of FL to pH. The measurements were performed in triplicate. ORAC values were defined as the difference between the area under the FL decay curve and the blank (net AUC). Regression equations between net AUC and antioxidant concentration were calculated for all of the samples. A tannase control was performed, and the ORAC value obtained was subtracted from the samples treated with the enzyme. ORAC-FL values were expressed as μmol of Trolox·L^{-1} equivalent to the orange juice solution, or μmol of Trolox·g^{-1} of the commercial naringin and hesperidin.

2.5.3. Cell culture and maintenance

The human colon adenocarcinoma grade II cell line, HT29, and the human liver hepatocellular carcinoma cell line, HepG2, were purchased from the Rio de Janeiro Cell Bank, RJ, Brazil. The other human tumoral cell lines of the antiproliferative panel (U251 (glioma, SNC); MCF-7 (breast); NCI-ADR/RES (ovary, phenotype of multidrug resistance); 786-0 (Kidney); NCI-H460 (lung, non-small cells); PC-3 (prostate); OVCAR-3 (ovary); HT29 (colon); HaCat (keratinocyte)) are from the collection of the Pharmacology and Toxicology division from CPQBA-UNICAMP. The cells were cultured in DMEM supplemented with 5% fetal bovine serum (Invitrogen©). The cells were maintained at 37 °C and 5% CO_2–95% humidified air.

2.5.3.1. Cell proliferation assay. Ninety-six-well culture dishes were inoculated with two human cell lines, HepG2 and HT29, at a density of 5×10^3 cells per well. The same methodology was applied to the nine cell lines from the antiproliferative panel. Following incubation for 24 h, the adherent cells were washed once with a PBS (phosphate-buffered solution). Cells were then incubated in DMEM containing 50–250 μg/mL of unmodified or biotransformed orange juice solution

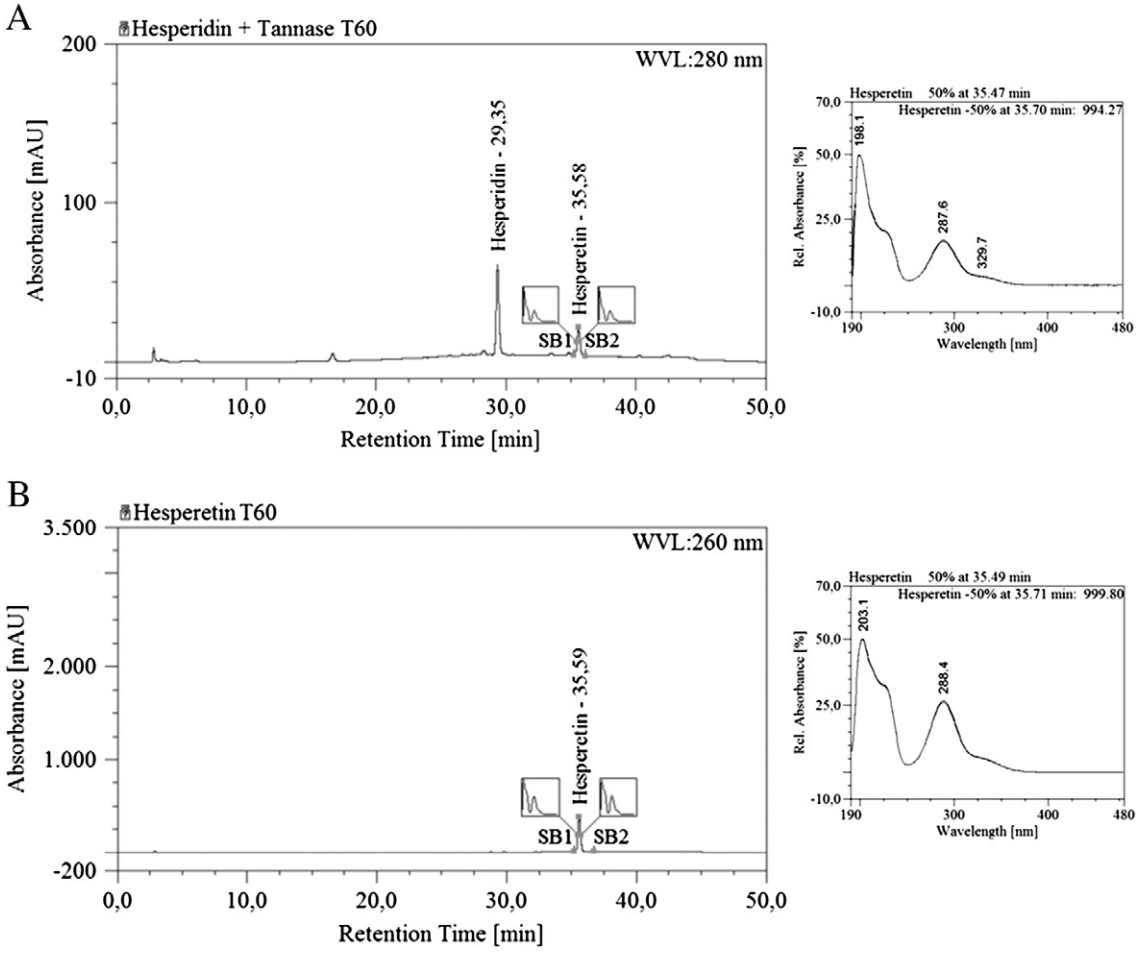

Fig. 1. CLAE chromatograms of the tannase (pH 7.4) reaction at 60 min at 40 °C of: (A) Hesperidin sample (pH 7.4) with tannase extract and (highlighted portion) absorbance spectrum of the reaction product; (B) hesperitin control.

or hesperidin and naringin. Positive and negative controls were also performed. After incubation at 37 °C in an atmosphere of 5% CO_2 and 100% relative humidity for 48 h, the cultures were assayed to detect the effects of the tested compounds on cellular proliferation.

Cellular proliferation was measured using the sulforhodamine B (SRB) assay, which has been described in detail by Monks et al. (1991). Briefly, adherent cell cultures were fixed in situ by adding 50 μL of cold 50% (w/vol) trichloroacetic acid (TCA) (final concentration, 10% TCA) and incubating the samples for 60 min at 4 °C. The supernatant was then discarded, and the plates were washed five times with deionized water and dried. One hundred microliters of SRB solution (0.4% w/vol in 1% acetic acid) was added to each microliter well, and the cultures were incubated for 10 min at room temperature. Unbound SRB was removed by washing the samples five times with 1% acetic acid. The plates were then air-dried. Bound stain was solubilized with a Tris buffer, and the optical densities were read at a single wavelength of 515 nm using an automated spectrophotometric plate reader. The results are expressed as percentages of the cell growth of the positive control (a cell line cultivated under the same conditions, without contact with the tested sample) (Macedo et al., 2012).

2.6. Calculations and statistics

Values are expressed as arithmetic means. The statistical significance of the differences between the groups was analyzed using the Tukey test. Differences were considered significant when $p<0.05$.

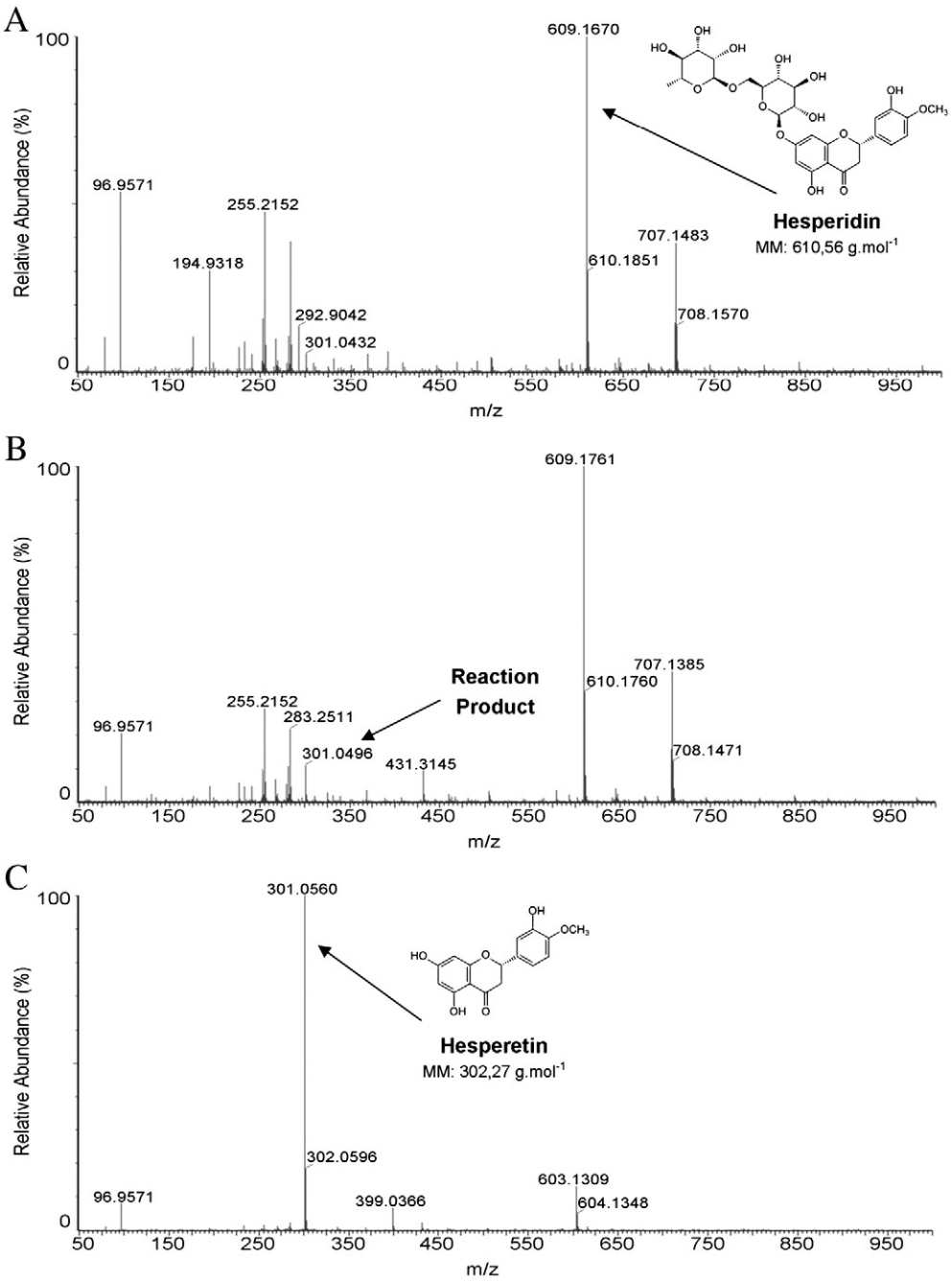

Fig. 2. Mass spectrum of the reaction at 40 °C and pH 7.4 after 60 min of: (A) Hesperidin control and (B) hesperidin with tannase extract, demonstrating the formation of hesperitin; (C) hesperitin control.

3. Results and discussion

3.1. Enzymatic Biotransformation

The capacity of the tannase to catalyze the biotransformation of commercial standards of typical citric polyphenols, hesperidin and naringin, was proven in the tests that were carried out. Fig. 1 presents the chromatogram obtained after 60 min for the hesperidin control (A) and for the semi-purified tannase extract reaction with hesperidin (hesperitin-7-O-rutinosídio) (B), both under the same conditions.

Observing Fig. 1, it is possible to see that a second compound emerges after 60 min of reaction between the tannase and the hesperidin substrate. Hesperidin is a molecule of hesperitin with a disaccharide bonded at the C7 position (rutinose, 6-O-α-L-ramnosil-D-glucose). A potential hydrolysis of this compound would lead to the formation of the aglycone molecule, hesperitin. The time of retention and the absorption spectrum of this second compound matched with these parameters for the hesperitin control tested under the same conditions (Fig. 1). For the final identity confirmation, the reaction products were analyzed with a mass spectrometer (ESI-MS). The spectra obtained for the controls of hesperidin and hesperitin, and for the compounds generated by the biotransformation reaction, are presented in Fig. 2.

By comparing the ratio of mass/charge (m/z) of the product formed with the m/z of the hesperitin control under the same conditions, it can be confirmed that hesperitin is the product generated by the reaction. The spectrum of masses obtained for the hesperidin, in reaction to the semi-purified tannase extract (Fig. 2B), presents the appearance of the compound with an m/z of 301.04, which coincides with the m/z of hesperitin under the same conditions (Fig. 2C).

Naringin (naringenin-7-O-neohesperidoside), as well as hesperidin, has a disaccharide bonded to the aglycone by way of a glycosidic bond on carbon C7; however, the disaccharide of the naringin is neohesperidoside (2-O-α-L-rhamnosyl-D-glucose). Fig. 3 presents the chromatograms obtained at 60 min for standard naringin (A) and for the reaction of naringin with the semi-purified tannase extract of *P. variotti* (B), treated under the same conditions.

As observed in Fig. 3B, a compound emerges, generated by the reaction of the naringin with the semi-purified tannase extract. Based on the similarity between the time of retention and the spectrum of absorption of the product of the reaction with the standard naringenin, there is a strong indication that the product generated is naringenin. To confirm the identity of the product obtained in the reaction, the samples were evaluated in a mass spectrophotometer (ESI-MS). The mass spectra obtained for the naringin, and for the naringin that reacted with the raw tannase extract *P. variotti*, are represented in Fig. 4, respectively, represented by designations A and B.

The analysis of the products of the reaction of the semi-purified tannase extract with naringin at 60 min (Fig. 4B) presents the formation of a product with an m/z of 271.04, which increases the indication of formation of naringenin as the product of the reaction, since the molecular weight of this aglycone is $\pm 272\ g \cdot mol^{-1}$.

Given the confirmation of the results obtained, which show the ability of the tannase to act on phenolic compounds representative of

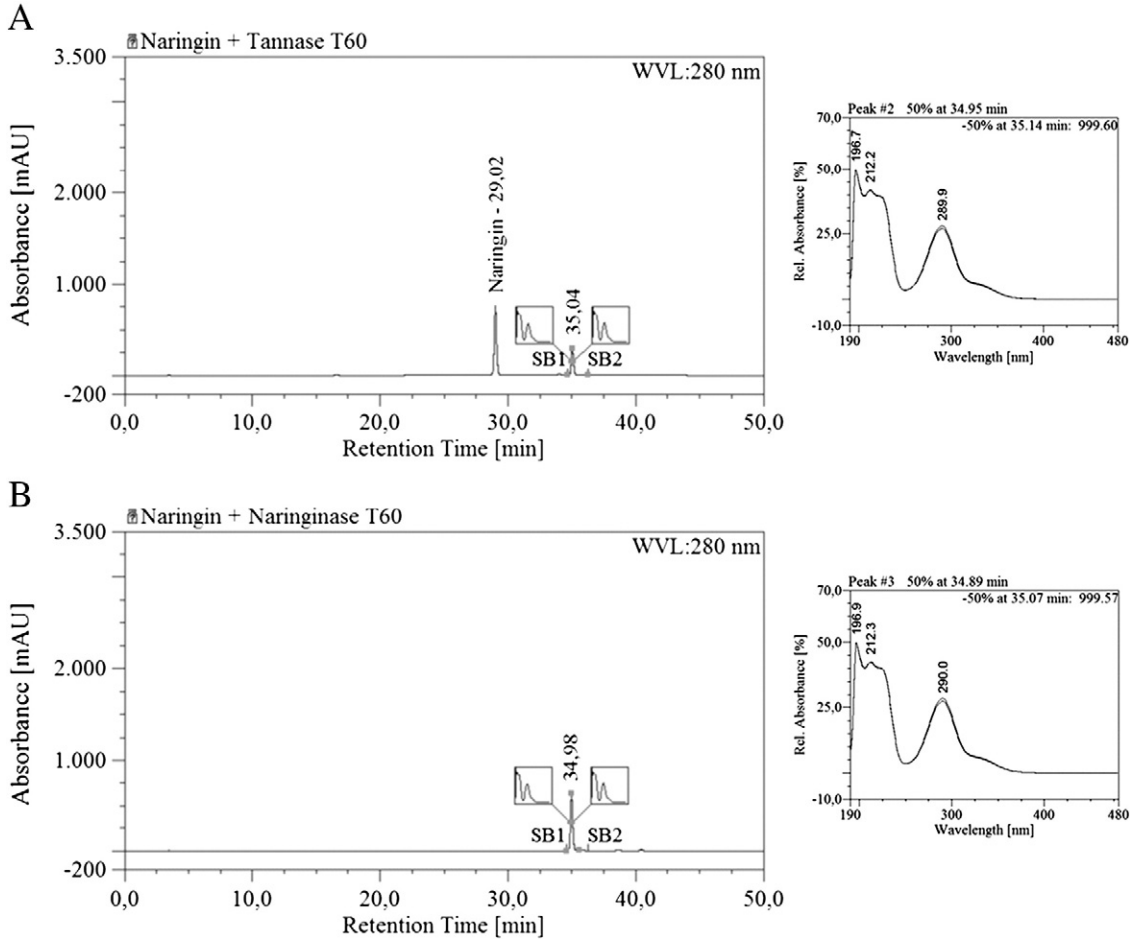

Fig. 3. CLAE chromatograms of the tannase (pH 7.4) reaction at 60 min at 40 °C of: (A) Naringin sample (pH 7.4) with tannase extract and (highlighted portion) absorbance spectrum of the reaction product; (B) Naringenin control.

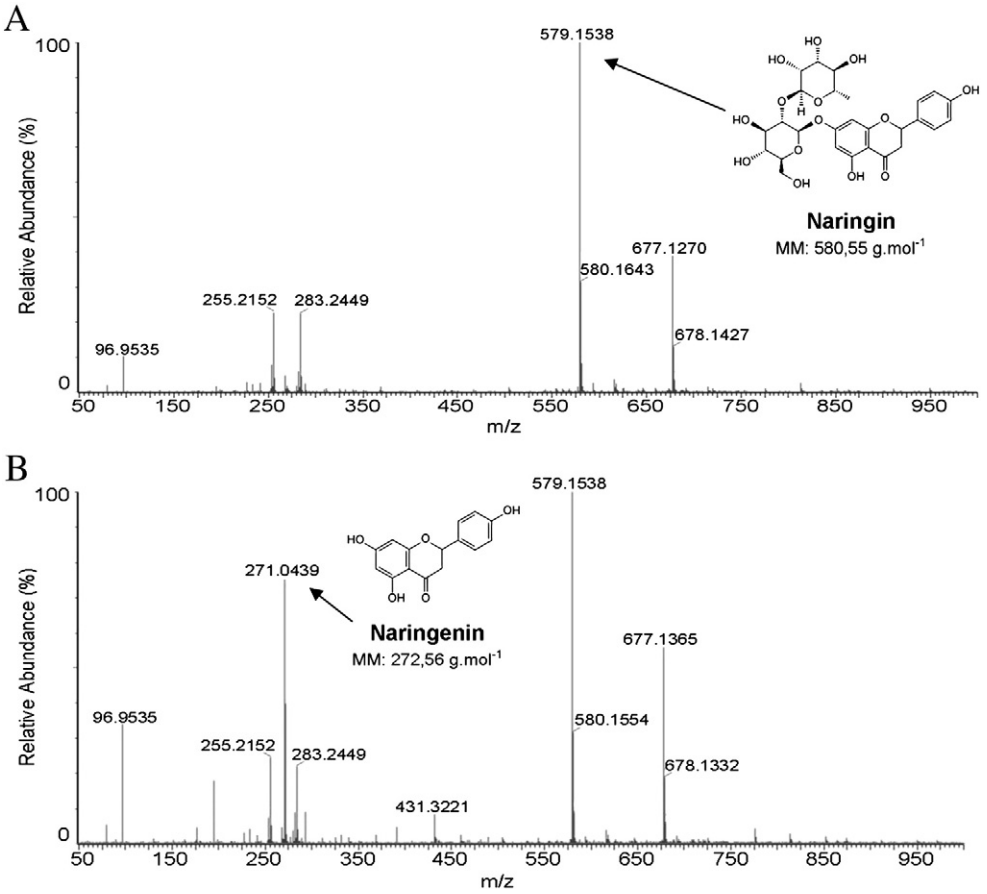

Fig. 4. Mass spectrum of the reaction at 40 °C and pH 7.4 after 60 min of: (A) Naringin control and (B) naringin with tannase extract, demonstrating the formation of naringenin; (C) naringenin control.

orange juice, the next step was to apply the enzyme in the biotransformation of the complex food matrix, that is, directly to the concentrated orange juice.

For purposes of comparison, Fig. 5 shows the chromatographic profile of the general composition in polyphenols for the orange juice sample without biotransformation (A) and for the samples treated with the semi-purified tannase extract (B), as well as for the sample treated with commercial naringinase (C).

Despite the need for more in-depth analytic studies for a detailed characterization of the changes observed in the phenolic profile of the orange juice after the activity of the semi-purified tannase extract, it can be observed that the enzyme removed peak 13 from the chromatogram (Fig. 5), which was identified as hesperidin. The compounds referenced as peaks 8 and 12, were not identified, but were greatly reduced in concentration by the activity of the tannase. Still analyzing Fig. 5C, compounds can be observed between peaks 11 and 13 on the chromatogram, unlike the results with the semi-purified tannase in Fig. 5B, in which these peaks were removed. Many times, the naringinase enzyme acted on the same compounds as the tannase, however, not to the same extent as hydrolysis.

These results make tannase an interesting option for application in complex food matrices, with a rich composition of polyphenols in which the enzyme can act in different ways in the biotransformation. In addition to the deglycosylation activity demonstrated, tannase is already known for its ability to hydrolyze substrates with the following conditions: a) those that contain at least two phenolic OH groups in the acid component; b) those in which the esterified COOH group is present in the oxidized aromatic ring; and c) those in which one of the phenolic OH groups is in a position other than the ortho position (Lekha & Lonsane, 1997). In this way, the tannase catalyzes the hydrolysis of hydrolysable tannins such as tannic acid, methyl gallate, ethyl gallate and n-propyl gallate (Aguilar et al., 2007).

3.5. Bioactivity of the samples biotransformed by tannase

The products of the reactions of standard hesperidin and naringin with tannase were assayed using two methods with different antioxidant capacities, DPPH and ORAC. The results described in Table 1 are expressed as Trolox® concentration equivalents.

The enzymatic biotransformation of naringin to naringenin resulted in an increase of 25% in the antioxidant capacity of the molecule by the ORAC method and an increase of approximately 500% by the DPPH method. In the case of hesperidin biotransformed into hesperitin, the increase was even more dramatic, reaching about 180% by ORAC and 1400% by DPPH. The reaction of the removal of esterified sugar molecules from the phenolic bodies proved to be very effective in increasing the antioxidant capacity of these compounds by the tested methods.

Although impressive, the results obtained for the antioxidant capacity of standard isolated phenolics could be reached with other commercial enzymes available on the market, with the action of specific hydrolases, like naringinase, for example. The great advantage of this study is in the application of tannase in the primary food matrix, that is, the orange juice. As was observed in the chromatographic profiles of the phenols treated with tannase and naringinase, the action of the tannase was more extensive, was not limited to the deglycosylation of flavones, and could imply more significant changes in the antioxidant capacity of these samples. The results of the tests of antioxidant capacity carried out on the naringinase, the orange juice

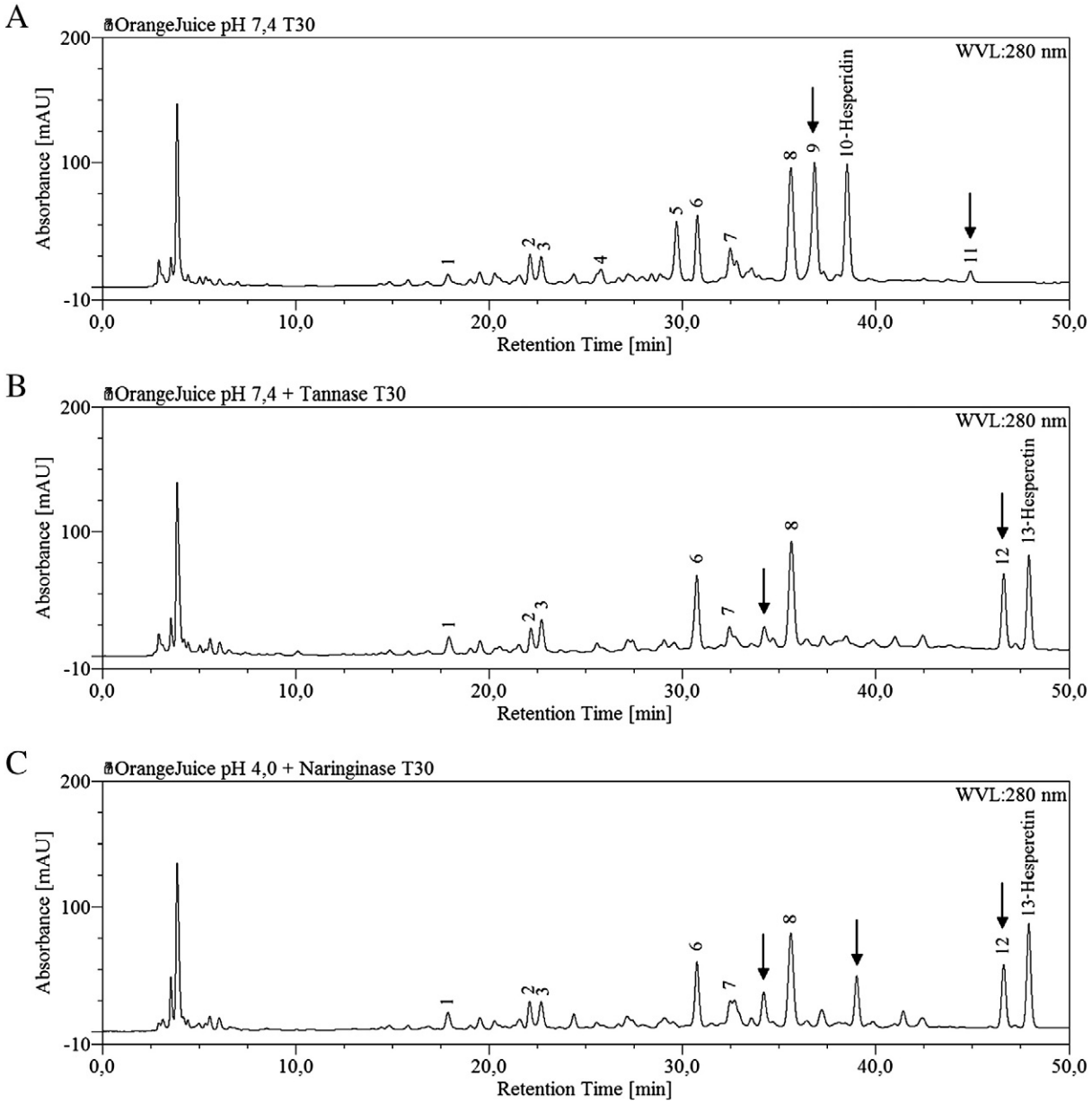

Fig. 5. CLAE chromatograms of the tannase reaction at 60 min at 40 °C of: (A) Orange juice sample (pH 7.4), (B) orange juice sample (pH 7.4) with tannase extract and (C) orange juice sample (pH 4.0) with naringenase.

samples treated with tannase and the control with no enzymatic treatment are in Table 2.

The concentrated orange juice (66°Brix) consumption data, published by Citrus Br Association (www.citrusbr.com.br), for the year of 2010, North America (EUA and Canada) had a 2.7 Kg per capita approximate consumption of 100% concentrated orange juice (carbonate drinks application were not considerate). Latin America (Brazil, Colombia, Argentina, Mexico and Chile) had a per capita consumption of nearly, 0.19 Kg. Those values represent a mean daily consumption raging between 7.4 g per person to 0.52 g per person (from North America to Latin America). Based on this information, the orange juice tested solution was prepared as 1 g of the concentrated orange juice in 5 mL of buffer. This solution was diluted at every specific assay reaching the final concentration of 50–250 μg/mL.

The orange juice treated with commercial naringinase at optimal reaction conditions of pH and reaction temperature in both the ORAC test and the DPPH test didn't show any statistically significant differences in the antioxidant capacity of the samples. However, in the biotransformation catalyzed by tannase, there was a significant increase in the antioxidant capacity of the biotransformed sample, both in the ORAC method (50% increase), as well as the DPPH method (70% increase). These results support the idea that tannase acts in a broader and more significant way than naringinase in the biotransformation of the polyphenols of the orange juice.

In addition to the deglycosylation activity of low specificity (or broad spectrum) observed in the results presented, previous research has shown tannase catalyzing the decomposition of hydrolysable tannins such as tannic acid, methyl gallate, ethyl gallate and n-propyl gallate (Lekha & Lonsane, 1997), as well as demonstrating activity in the hydrolysis of (−)-epicatechin gallate and (−)-epigallocatechin gallate (Battestin & Macedo, 2007b). Literature has also shown the activity of this enzyme in the hydrolysis of chlorogenic acid with the formation of caffeic acid and quinic acid (Aguilar et al., 2007; Yao, Fan, Lu, & Liu, 2011).

Among the many possible forms of action of natural compounds with significant antioxidant activity in the process of chemoprevention,

Table 1
Trolox equivalents and range of linearity for the results of the ORAC method and Trolox equivalents for the DPPH method, carried out at phenolic standards before and after biotransformation.

Samples	ORAC					DPPH
	Trolox equivalents (μmol/g sample)	Sample concentration range (mg/mL)	Slope	Intercept	r^2	Trolox equivalents (μmol/g sample)
Naringin	7958 ± 610[a]	0.04–0.08	247.5	3.6	0.95	67 ± 2[a]
Naringin with tannase	9955 ± 887[b]	0.04–0.08	293.4	5.4	0.99	428 ± 10[b]
Hesperidin	2333 ± 202[a]	0.04–0.08	68.5	1.3	0.99	55 ± 3[a]
Hesperidin with tannase	6552 ± 1,250[b]	0.04–0.08	110.9	8.1	0.96	865 ± 91[b]

Results are presented as the mean (n = 3) ± SD, and those with different superscripted letters are significantly different, with $p<0.05$.

Table 2
Trolox equivalents and range of linearity for the results of the ORAC method and Trolox equivalents for the DPPH method, carried out on the orange juice samples before and after the biotransformation with tannase and naringinase.

Samples	ORAC					DPPH
	Trolox equivalents (μmol/L solution)	Sample concentration range (% v/v)	Slope	Intercept	r^2	Trolox equivalents (μmol/L solution)
Orange Juice (pH 7.4)	21,279 ± 1969[a]	1.6–2.4	409.4	6.8	0.99	6100 ± 472[a]
Orange Juice with tannase (pH 7.4)	33,148 ± 2323[b]	1.6–2.4	782.8	7.7	0.99	10,349 ± 320[b]
Orange Juice (pH 4.0)	18,750 ± 1294[a]	1.6–2.4	445.0	4.3	0.99	5319 ± 185[a]
Orange Juice with naringinase (pH 4.0)	20,941 ± 1563[a]	1.6–2.4	469.0	5.4	0.99	4221 ± 731[a]

Results are presented as the mean (n = 3) ± SD, and those with different superscripted letters are significantly different, with $p<0.05$.

the antiproliferative activity is an important method of control over the development of a tumor. Initially, when the study is focused at potential functional compound in food, the tissues primarily affected by it are the epithelial tissues from the digestion system. This strategy is being reported in several recent works on the functional food field (Fang, Sha, Deng, Ling, & Xu, 2012; Kountouri et al., 2013). A promising compound should be tested on as many human tumoral cells as possible, considering that the same compound does not affect at the same manner different tissues. For this reason, the human colon adenocarcinoma cells (HT29) and the hepatocellular carcinoma (HepG2) were chosen to initial antiproliferative modulation evaluation. The results from the modulation test of the proliferation of human tumor cells (HepG2 and HT29) from the samples of naringin and orange juice, both before and after the biotransformation by tannase, are shown in Table 3. The results are expressed in relation to the growth of the same cell line, under the same conditions, in the untreated control with the tested samples (100%).

With regard to the naringin sample in the hepatic line (HepG2), the sample presented moderate pro-proliferative activity. Moreover, the sample of biotransformed naringin demonstrated significant antiproliferative activity for all concentrations tested in both tumor lines. Although the effect of naringin on the tumor line HT29 was antiproliferative and dependent on the dose, with a growth reduction of close to 45% in the greatest concentration of the sample, the antiproliferative effect of the biotransformed naringin was even greater, reaching levels of reduction of the population of cells classified as cytocidal in the greatest concentration of tested samples. In all other concentrations, the antiproliferative effects of the biotransformed naringin were more significant than the sample with no enzymatic reaction.

The sample of orange juice, however, with/without enzymatic treatment, did not present significant variations in cell growth in the hepatic line (HepG2). However, in the HT29 line, the orange juice sample presented moderate pro-proliferative activity, while the samples of juice biotransformed with both tannase and naringinase showed results of antiproliferative activity. In the samples treated with tannase, in general, the antiproliferative activity was more pronounced.

The potential for antiproliferative activity of hesperidin, before and after biotransformation with tannase, was assayed separately in a panel of nine different human tumor lines. These tumoral cell panels are an important tool to verify the antiproliferative modulation capacity of natural compounds. The high concentration of hesperidin in the orange juice and the significant rise in antioxidant activity of the compound obtained by biotransformation justified this choice. The results are presented in Fig. 6.

As seen in the graphs in Fig. 6, the antiproliferative activity of hesperidin after the biotransformation with tannase increased significantly. In comparison with the hesperidin not treated with the enzyme, the antiproliferative effects of the biotransformed compound were observed in concentrations much lower than that of the original compound, and with very significant inhibition of growth of the tumor cells. In the highest concentrations tested, the biotransformed

Table 3
Test of the proliferative activity in the samples of naringin, hesperidin and orange juice, before and after biotransformation with tannase and naringinase, in cell lines HepG2 and HT29.

Samples	HepG2					HT29				
	Samples concentration (μg/mL)									
	10	50	80	100	150	10	50	80	100	150
Naringin (pH 7.4)	101[a]	119	112	115	117	99	73	70	52	57
Naringin with tannase (pH 7.4)	37	37	35	30	25	8	44	61	37	5
Orange juice (pH 7.4)	107	106	110	110	113	72	164	158	117	152
Orange juice with tannase (pH 7.4)	114	105	107	107	99	111	103	80	97	63
Orange juice with naringinase (pH 4.0)	106	117	98	109	104	73	77	96	100	102

[a] The results are expressed as (%).

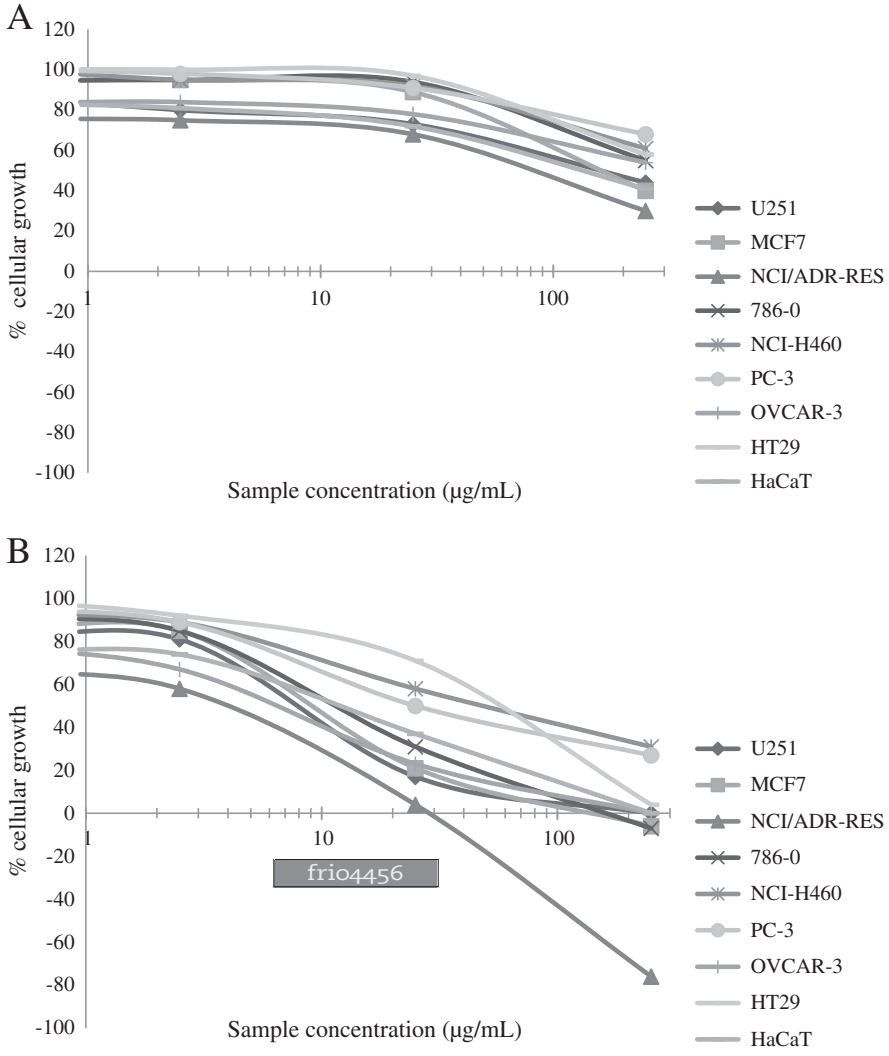

Fig. 6. Cell proliferation modulation: (A) by the hesperitin, (B) by the biotransformed hesperitin (hesperitin + tannase); results in (%) of the cell positive control growth for each cell line. Human tumoral cell lines: U251 (glioma, SNC); MCF-7 (breast); NCI-ADR/RES (ovary, phenotype of multidrug resistance); 786-0 (Kidney); NCI-H460 (lung, non-small cells); PC-3 (prostate); OVCAR-3 (ovary); HT29 (colon); HaCat (keratinocyte).

compound completely inhibited the proliferation of multiple lines tested (U251, MCF7, 786-0, OVCAR-3, HT29, HaCat), and in one of them (NCI-ADR/RES), the compound had a cytocidal effect, reducing the initial population of cells that were incubated for the test. These results make the hesperitin produced by biotransformation with tannase a promising compound for evaluation for its activity in chemoprevention.

4. Conclusion

It was demonstrated that the semi-purified tannase extract of *P. variotii* was able to modify the chromatographic profile of the polyphenolic composition of the orange juice and act in naringin and hesperidin in the removal of glycosides. Hesperitin was characterized as a phenolic compound, present in greatest concentration in the concentrated orange juice tested. As demonstrated, its enzymatic biotransformation represented an increase in bioactivity and potentially in the functionality of the compound in chemoprevention. The same occurred with naringin biotransformed in a lower concentration. The results of these reactions were products with an interesting potential of heightened functional activity in relation to the originals, as the in vitro tests of antioxidant activity and antiproliferation of tumor cells indicate. The results demonstrate the benefits of the biotechnological modification of natural food molecules, allowing the improvement of the nutraceutical potential of a beverage such as orange juice.

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