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Analytical Methods

Tucumã fruit extracts (*Astrocaryum aculeatum* Meyer) decrease cytotoxic effects of hydrogen peroxide on human lymphocytes

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ABSTRACT

This study quantifies the bioactive molecules in and determines the *in vitro* protective effect of ethanolic extracts isolated from the peel and pulp of tucumã (*Astrocaryum aculeatum*, Mart.), an Amazonian fruit rich in carotenoids. The cytoprotective effect of tucumã was evaluated in lymphocyte cultures exposed to H_2O_2 using spectrophotometric, fluorimetric, and immunoassay assays. The results confirmed that tucumã pulp extract is rich in β-carotene and quercetin, as previously described in the literature. However, high levels of these compounds were also found in tucumã peel extract. The extracts also contained significant amounts rutin, gallic acid, caffeic acid, and chlorogenic acid. Despite quantitative differences in the concentration of these bioactive molecules, both extracts increased the viability of cells exposed to H_2O_2 in concentrations ranging from 300 to 900 μg/mL. Caspases 1, 3, and 8 decreased significantly in cells concomitantly exposed to H_2O_2 and these extracts, indicating that tucumã cryoprotection involves apoptosis modulation.

1. Introduction

Carotenoids are one of nature's major antioxidant pigments, found in several fruits. The reactive oxygen species (ROS) are highly reactive molecules and the body controls their degradation through two integrated antioxidant systems: an endogenous enzymatic system and exogenous control through the entry of non-enzymatic antioxidant molecules derived from the diet or produced by the body (Böhm, Edge, & Truscott, 2012). However, the biological properties of several fruits rich in carotenoids are not well characterised. This is the case of tucumã (*Astrocaryum aculeatum*, Mar, 1824), which belongs to the Arecaceae family and is distributed in the central region of the Amazon basin rom south to north, tucumã grows in Bolivia (Beni, Pando, Santa Cruz), Brazil (Acre, Amazonas, Pará, Rondônia, Roraima), Guyana, Suriname, Trinidad, and, Venezuela. This fruit has several popular names chonta, tucumo, and panima in Bolivia; tucumã, tucumã-arara, tucumã-piranga, tucumã-piririca, tucumã-uassu-rana, tucum-assu, tucum-bravo, tucum-da-serra, tucum-do-matto, and tucum-purupuru in Brazil; akuyuro palm, cuyuru-palm, and tucumou in Guyana; amana, toekoemau, and warau in Suriname; and cumare and yavaide in Venezuela (Kahn, 2008).

Tucumã is a type of palm. Average in height, it comes from the Amazon rainforest. The trunk is heavily spined, and even the inflorescences are covered by a thorny spathe. Centesimal composition analysis of the tucumã using methods described by the Association of Official Analytical Chemists showed that the mesocarp contains 412.73 ± 2.12 kcal, 44.9 ± 0.30 g wet content, 10.9 ± 0.1 g fibres, 3.5 ± 0.07 g proteins, 8.5 ± 0.6 g carbohydrates, and 40.5 ± 0.5 g fats per 100 g of pulp. Previous studies have shown that the oil extracted from tucumã consists of 74.4% unsaturated and 25.6% saturated fatty acids and is rich in omega-3, -6, and -9 fatty acids (Aguiar, 1996).

The orange-yellow fruit (about the size of a chicken egg) is an excellent source of carotenoids. This fruit contains 21 different

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types of carotenoids in which all-trans-β-carotene (47.36 μ/g), the precursor of vitamin A and which represents 75% of all carotenoids, is identified and quantified in this fruit (De Rosso & Mercadante, 2007). The vitamin A value found in tucumã is 850 RE/100 g, which is higher than other fruits such as papaya (19–74 RE/100 g), acerola (148–283 RE/100 g), and vegetables like carrots (308–625 RE/100 g) and broccoli (131–194 RE/100 g) (De Rosso & Mercadante, 2007). It is also an important source of vitamin B2 (riboflavin) and presents bioactive compounds like catechin and quercetin (Gonçalves, Lajolo, & Genovese, 2010). Additionally, the edible tucumã edible fruit is used for the production of biodiesel (Bora, Narain, Rocha, De Oliveira Monteiro, & De Azevedo Moreira, 2001).

Since DNA damage is generally regarded as an indicator of disease risk, we performed an *in vitro* study to investigate the potential cytoprotective effect of tucumã peel and pulp extracts in human lymphocytes exposed to hydrogen peroxide (H_2O_2), a prooxidant and genotoxic molecule.

2. Material and methods

2.1. Experimental design

To test the protective effects of *A. aculeatum* on cell viability and the protective effect on chromosome damage in human lymphocytes, we performed a protocol similar to the one described by Boligon et al. (2012) Briefly, we exposed the lymphocyte cell cultures to H_2O_2 (2×10^5 cells, 1 h, 37 °C). After one hour, the cells were treated with pulp and peel extracts from *A. aculeatum*. Six concentrations (100, 300, 600, 900, 1200, and 1500 μg/mL) of these extracts were tested. A cultured medium containing cells without treatment was used as a negative control. The study of the Amazonian fruit was registered and approved by the Board of Management of Genetic Patrimony (CGEN) number 530.

2.2. Chemical characterisation of tucumã extracts

This study of tucumã was part of a project that has a Brazilian Access and Benefit Sharing Permit and was registered and approved by the Board of Management of Genetic Patrimony (CGEN) (ABSP nº 010547/2013-4) Tucumã peel and pulp extracts were obtained from a composite sample representing a mixture of progenies from a native forest near Manaus City (Amazonas State, Brazil), located in the Amazonian region (3.08°S, 60.01°W). Therefore, we used tucumã fruit collected directly from native palm trees found in primary and secondary forests, pastures, and home gardens. Tucumã usually flowers from June to January and produces fruit from February to August. We obtained the fruits from Manaus city in February, 2010.

The pulp and peel were manually removed, producing 400 g of peel and 800 g of pulp, which was kept frozen at −18 °C until extraction procedures were performed one week later. The ethanolic tucumã extract was prepared from tucumã pulp and peel samples that were titrated and placed separately into sealed amber glass jars containing an absolute ethanol solution at a ratio of 1:5 (w/v). The extraction was performed over four days at room temperature.

The homogenate was filtered through Whatman No. 1 paper and then collected; the ethanol was removed using a rotary evaporator at reduced pressure, 25 °C at 115 rpm. Following this procedure, the pulp and peel extracts were lyophilized and stored at −20 °C until they were to be used. We obtained 3.3 g of peel and 6.0 g of dried tucumã pulp extract.

To perform the experimental assays, the extracts were first diluted in 40% ethanol in order to improve the extract's solubilisation. This solution was then diluted in distillated water to obtain <0.5% ethanol concentration in all tucumã concentrations tested. The ethanol was added in all control treatments to eliminate the potential influence of the ethanol in the results obtained.

Chemical characterisation of tucumã extracts was performed in triplicate, and all reagents were obtained from Sigma–Aldrich Co. (St Louis, USA). Total phenolic content (TPC) was determined using Folin–Ciocalteu's method (Chandra & Mejia, 2004). Briefly, 10 μg samples of lyophilized powdered tucumã peel and pulp extracts were dissolved in 1 mL deionized water. These solutions (1.0 mL) were mixed with 2 mL of 20% sodium carbonate (Na_2CO_3) in deionized water and 0.5 mL 1 N Folin–Ciocalteu's phenol reagent. After incubation at room temperature for 10 min, the absorbance of the reaction mixtures was measured at 730 nm against a deionized water blank plus <0.5% ethanol.

The total phenolic content of samples was calculated as gallic acid equivalents, using a calibration curve of gallic acid standard solutions (5, 10, 15, 20, 25, and 30 μg/mL of 0.2% aqueous gallic acid) and was analysed following the same method as the samples. The equation obtained for the gallic acid calibration curve was $y = 18.185x - 0.0266$ ($R^2 = 0.9783$). The results were expressed as mg gallic acid equivalents (mg GAE)/g dry plant extract material. The experiments were performed in triplicate.

The determination of the total tannin content was performed using the method described by Morrison, Asiedu, Stuchbury, and Powell (1995) with some modifications. Samples were prepared at a concentration of 0.25 mg/mL; 5 mL of solution A (1 g vanillin in 100 mL of methanol) and 5 mL solution B (8 mL HCl in 100 mL of methanol) were used in the experiment. The samples were read at 500 nm. The total tannin content was expressed as milligram equivalents of catechin per gram of sample. The equation obtained for the catechin calibration curve, ranging from 0.001 to 0.025 mg/mL, was $y = 0.00015x + 0.005$ ($r = 0.9989$). The experiments were performed in triplicate.

The alkaloid content was determined using the method described by Sreevidja and Mehrotra (2003), where Dragendorff's reagent (Bismuth sub-nitrate, 1.7 g; Glacial Acetic Acid, 20 mL; water, 80 mL; and 50% solution of Potassium iodide in water, 100 mL) precipitates alkaloids in plant materials. The samples were read at 435 nm in the spectrophotometer (UV-1100 Model, Pro-Analise CO, São Paulo, Brazil) The equation obtained for the calibration curve of bismuth nitrate pentahydrate solution in the range of 0.01–0.09 mg/mL was $y = 2.2783x + 0.0361$ ($r = 0.9997$).

Chromatographic analyses were carried out following the method described by Laghari et al. (2012), with slight modifications. The analyses were performed using a C18 column (4.6 mm × 250 mm) packed with 5 μm diameter particles. The mobile phases were water containing 2% acetic acid (A) and methanol (B). The gradient used started at 5% B and increased to 25%, 40%, 50%, 60%, 70%, and 100% B at 10, 20, 30, 40, 50, and 80 min, respectively. The flow rate was 0.8 mL/min, and the injection volume was 40 μL. The lyophilized extracts of tucumã peel and pulp were dissolved in ethanol at a concentration of 3 mg/mL. All samples and mobile phases were filtered through a 0.45 μm membrane filter (Millipore) and then degassed in an ultrasonic bath prior to use. Six different antioxidant compounds were investigated: gallic acid, chlorogenic acid, caffeic acid, quercetin, rutin, and β-carotene. Identification of these compounds was performed by comparing the retention time and UV absorption spectrum with those of the commercial standards. The wavelengths used were 254 nm for gallic acid, 325 nm for caffeic and chlorogenic acids, 365 nm for quercetin and rutin, and 450 nm for β-carotene. Stock solutions of reference standards were prepared in mobile phase at concentrations ranging from 0.020 to 0.200 mg/mL for β-carotene, quercetin, and rutin and 0.050 to 0.250 mg/mL for gallic, caffeic, and chlorogenic acids. Chromatographic peaks were confirmed by comparing the retention time of samples with those of the referenced

standards and by comparing the DAD spectra (200–600 nm). The following calibration curves were obtained: gallic acid, $y = 10523x + 1478.8$ ($r = 0.9999$); caffeic acid, $y = 12765x + 1381.7$ ($r = 0.9995$); rutin, $y = 12{,}691–1165.0$ ($r = 0.9998$); quercetin, $y = 13495x - 1092.6$ ($r = 0.9999$); and β-carotene, $y = 13681x - 1518.7$ ($r = 0.9999$). All chromatography operations were carried out at ambient temperature and were performed in triplicate.

2.3. Antioxidant capacity assays

The antioxidant capacity of tucumã pulp and peel extracts was evaluated by monitoring the ability of the extracts to scavenge the stable free radical DPPH according to a slightly modified method previously described by Choi et al. (2002). Spectrophotometric analysis was used to determine the inhibition concentration of tucumã extracts. DPPH scavenging ability was also calculated as IC50 (the concentration that yields 50% inhibition). Eleven different ethanol dilutions of each extract (1, 5, 10, 20, 40, 60, 80, 100, 140, 200, and 240 μg/mL) were mixed with 1.0 mL of a 0.3 mol/L^{-1} DPPH ethanol solution. The absorbance was measured at 518 nm against a blank after a 30 minute reaction at room temperature in a dark place. The DPPH solution (1.0 mL, 0.3 mol/L^{-1}) plus ethanol (2.5 mL) was used as a negative control. The natural antioxidants, rutin and ascorbic acid, were used as positive controls. The percentage inhibition of free radicals by DPPH (IP%) was calculated in the following way: IP% = 100 − [(ABSsample − ABSblank)/ABScontrol) × 100], where ABSsample is the absorbance of the test compound, ABSblank is the absorbance of the blank (containing 1.0 mL of ethanol plus 2.5 mL of the plant extract solution), and ABScontrol is the absorbance of the control reaction (containing all reagents except the test compound). IP% was plotted against the sample concentration, and a linear regression curve was established to calculate the IC50. Tests were performed in triplicate.

A second protocol to evaluate the antioxidant capacity of tucumã extracts was also performed. A total radical-trapping antioxidant parameter (TRAP) assay was adapted and used to determine the capacity of tucumã extracts to trap a flow of water-soluble peroxyl radicals produced at a constant rate through the thermal decomposition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (Silva et al., 2007). The assay was performed in a 96-well plate. The reaction mixture contained 300 μL of the free-radical source (10 mol/L^{-1} AAPH) in a 0.1 mol/L glycine buffer (pH 8.6), 10 μL of the test samples at different concentrations, and 10 μL of luminol (1 mol/L^{-1}) as the external probe to monitor radical production. The reactions were incubated at 20 °C. The chemiluminescence produced is directly proportional to radical generation and was recorded as counts per minute. The TRAP capacity of tucumã extracts was evaluated for instantaneous inhibition of chemiluminescence as area under the curve (AUC). The total antioxidant reactivity (TAR) was calculated as the ratio of light intensity in the absence of samples (I0) to the light intensity after *A. aculeatum* addition (I) and was expressed as a percentage inhibition.

2.4. Blood collection and lymphocyte culture

In this study, human blood was used to perform the *in vitro* experiments. The protocol was approved by the Federal University of Santa Maria Ethics Committee Board (23081.015838/2011-10) and donors signed a consent term. Peripheral blood samples were obtained from three apparently healthy volunteers (22–25 years of age), who did not smoke, drink alcoholic beverages more than tw times a week, or take prescription drugs. The samples were collected after 12 h of overnight fasting by venipuncture using a top Vacutainer® (BD Diagnostics, Plymouth, UK) and heparin tubes. The Histopaque-1077® (Sigma–Aldrich Co. (St Louis, USA)) density gradient was used to separate lymphocyte cells using 4 mL blood samples. After further centrifugation for 15 min at 2500*g*, the cells were transferred to culture media containing 5 mL RPMI 1640 with 10% foetal bovine serum, 1% penicillin and streptomycin, phytohemagglutinin, and tucumã extracts at different concentrations. After one hour, H_2O_2 was added in the cultures. The cells were cultured in a 96-well microplate at an initial density of 2×10^5 cells for 72 h at 37 °C in a 5% humidified CO_2 atmosphere (Laghari et al., 2012). In addition, the cells were counted, centrifuged for 10 min at 2000*g*, and transferred to a new culture media containing H_2O_2 treatments with and without tucumã extracts for one hour. Cell viability and DNA damage analysis were evaluated; a DNA fragmentation assay and a caspases activity analysis were also performed.

2.5. Cell viability

Cell viability was evaluated before and after treatment exposition using the MTT assay, a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase (Choi et al., 2002). Cell viability was expressed as a percentage of the control value. To perform the MTT assay, this reagent (Sigma–Aldrich Co. (St Louis, USA) was dissolved in a 5 mg/mL phosphate buffer (PBS, 0.01 mol/L; pH 7.4), added into a 96-well microplate containing the sample treatments, and incubated for four hours In addition, the supernatant was removed from the wells and the cells were resuspended in dimethyl sulfoxide (DMSO). The absorbance at 560 nm was read in a TP-Reader (Thermoplate, China). This assay was performed in octuplicate for each treatment.

2.6. DNA denaturation assay

The DNA denaturation assay was applied by a protocol previously described by Batel et al. (1999). The method is based on the ability of the specific fluorochrome dye (PicoGreen®) to make a very stable complex with dsDNA in alkaline conditions instead of ssDNA, proteins, SDS, and urea. This selectivity characteristic used to follow DNA denaturation with decreasing fluorimetric signal intensity proportionate to increasing ssDNA and mononucleotide content. Initially we evaluated the potential acute effect tucumã extracts on DNA denaturation.

Two tests were performed: one testing the acute effect o tucumã extracts on DNA damage and the other after 24 h of lymphocyte treatment with tucumã extracts. In the acute effect experiment, a kinetic of DNA denaturation (15, 30, and 60 min) was evaluated. The fluorescence was measured at an excitation of 485 nm, and an emission of 520 nm was recorded at room temperature (SpectraMax M2/M2e Multi-mode Plate Reader, Molecular Devices Corporation, Sunnyvale, CA, USA). Expression of results to DNA strand break calculations were made in relation to control values at the time of each denaturation after correction for blank readings. Blank values were measured in wells containing only the samples. Results were expressed as a percentage of dsDNA and were calculated for each treatment in relation to the untreated control samples.

2.7. Caspases activity assays

The lymphocyte were incubated under the conditions described above for 24 h. The analyses were performed using the Quantikine Human Caspases Immunoassays to measure Caspase-1, 3, and 8 in cell culture supernates using a microplate with 96 wells, according to the manufacturer. Briefly, all reagents and working standards were prepared, and the excess microplate strips were removed. The assay diluent RD1W was added (50 μL) to each well. Further-

more, 100 μL of the standard control for our sample was added per well. The sample was then covered with an adhesive strip and incubated for 1.5 h at room temperature. Each well was aspirated and washed twice, for a total of three washes. Caspase-1 antiserum was added to each well and covered, and the sample was given a new adhesive strip and incubated for 30 min at room temperature. The aspiration/wash step was repeated and the Caspase-1 conjugate (100 μL) was added to each well and the sample was incubated for 30 min at room temperature. The aspiration/wash step was repeated again, and 200 μL of substrate solution was added to each well, and the sample was incubated for 20 min at room temperature. Finally, the 50 μL stop solution was added to each well and the optical density was determined within 30 min using a microplate reader set to 450 nm. Experiments were performed in triplicate.

2.8. Statistical analysis

Data are presented as mean and standard deviation of the mean (±SD). Statistical differences between groups were evaluated by analysis of variance One-Way followed by the Tukey *post hoc* test. All statistical analyses were performed where all p values were two-tailed, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Chemical composition of tucumã extracts

The TPC, flavonoid, tannin, and alkaloid concentrations were determined in tucumã extracts (Table 1). The results showed a higher concentration of these bioactive compounds in the peel extracts than in the pulp extracts. Flavonoid and tannin content were also determined in tucumã extracts, as observed in Table 1, with the highest values occurring in the peel extract. HPLC fingerprinting of lyophilized tucumã extracts revealed the presence of gallic acid (tR = 17.83 min, peak 1), chlorogenic acid (tR = 28.14 min, peak 2), caffeic acid (tR = 34.09 min, peak 3), rutin (tR = 41.53 min, peak 4), and quercetin (tR = 48.28 min, peak 5). The last peak in Fig. 1 represents the β-carotene peak (tR = 57.64 min, peak 6).

Of the six bioactive compounds analysed here, four were found in tucumã extracts for the first time: rutin, gallic acid, caffeic acid, and chlorogenic acid. Other than gallic acid, which showed a higher concentration in tucumã pulp extract, the molecules were more concentrated in tucumã peel extract.

3.2. Antioxidant capacity assays

Tucumã peel and pulp extracts scavenging activity against DPPH radicals were determined at concentrations of 1, 5, 10, 20, 40, 60, 80, 100, 140, and 240 μg/mL. For a positive control, rutin and ascorbic acid were used at the same concentrations as the extracts. We observed that both extracts had a strong antioxidant capacity. At a concentration of 20 μg/mL, the tucumã extracts inhibited >80% of DPPH radicals. The 50% concentration to inhibit DPPH radicals (IC50) was also calculated. The IC50 was estimated to be 11.24 μg/mL for tucumã pulp extract, 8.98 μg/mL for tucumã peel extract, 5.92 μg/mL for rutin, and 4.89 μg/mL for ascorbic acid.

We also analysed tucumã antioxidant activity using a TRAP assay. Initially, this test was performed at five concentrations (1, 5, 10, 30, and 100 μg/mL), similar to the concentrations tested in the DPPH assay. However, we found higher antioxidant activity (>80% of inhibition) at all concentrations. For this reason, we changed the range of concentrations of the tucumã extracts to include several lower concentrations (1, 5, 10, 25, 50, 100, 500, 1000, 5000, and 10,000 ng/mL).

As observed in Fig. 2 and 25 ng/mL of peel extract and 50 ng/mL of pulp extract started to inhibit the pro-oxidant pulse generated

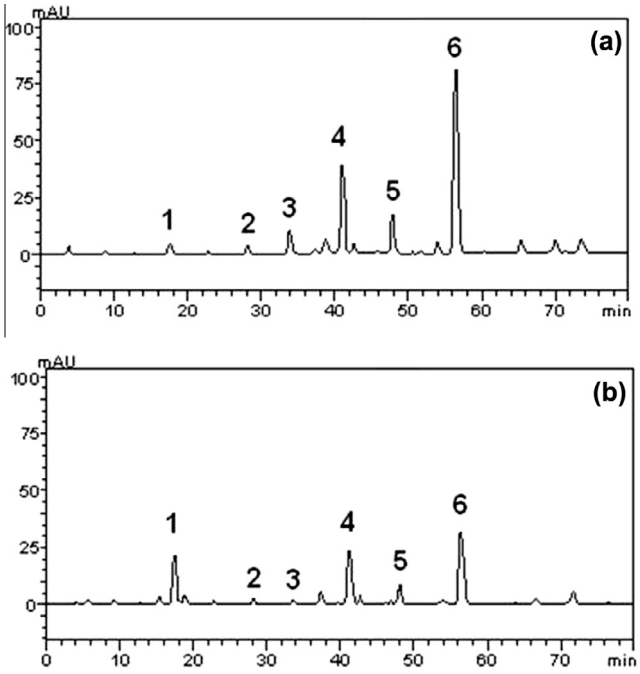

Fig. 1. Representative high performance liquid chromatography profile of *Astrocaryum aculeatum*. Lyophilized extract of bark (a) and lyophilized extract of pulp (b), detection UV was at 325 nm. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4), quercetin (peak 5) and β-carotene (peak 6). Chromatographic conditions are described in Section 2.

Table 1
Contents of total phenol contents, flavonoids tannins and alkaloids of tucumã extracts (*A. aculeatum*).

Compounds	Peel extract		Pulp extract	
	Total value	*mg/100 g	Total value	*mg/100 g
TPC (mg/GAE g)	941.8	790.95	872.1	426.35
Flavonoids (quercetin mg/g)	92.8	77.52	53.3	26.06
Tannin	31.4	26.37	8.24	4.03
Alkaloids	1.5	1.26	0.93	0.45
β-Carotene (%)	6.29	52.83	2.75	20.97
Rutin (%)	3.05	25.64	1.91	14.51
Quercetin (%)	1.27	10.68	0.65	4.97
Gallic acid (%)	0.38	3.18	1.42	10.85
Caffeic acid (%)	0.83	6.99	0.09	0.66
Chlorogenic acid (%)	0.30	2.55	0.11	0.91

GAE = gallic acid equivalent; TPC = total phenolic content expressed as extract; the total value were determined from three replicate measure performed to each compound analysis.

* mg/100 g fresh fruit.

by the free-radical-producing system of the TRAP assay. At 500 ng/mL, the inhibition levels were similar in both extracts (94.6% and 95.5%). The IC50 of the tucumã peel and pulp extracts were estimated to be 102.38 ± 4.8 μg/mL and 224.57 ± 3.9 ng/mL.

3.3. Cell viability

As expected, the H_2O_2 significantly decreased lymphocyte viability when compared to control samples and the tucumã treatments partially reverted the cytotoxicity caused by H_2O_2 in the MTT ($F = 86.727$, $p = 0.0001$) blue exclusion assay. As seen in Fig. 3, the 600 and 900 μg/mL concentrations of both extracts presented the better protective effect against H_2O_2 toxicity.

3.4. DNA denaturation assay

Quantification of plasma DNA was done using fluorescent Pico-Green dye, which is highly specific for dsDNA (Fig. 4). The H_2O_2 significantly increased the dsDNA level in supernatant medium culture when compared with an untreated control group indicating cell damage. In the presence of all concentrations of tucumã peel extract, occurred partial reversion of this damage ($F = 27.731$, $p = 0.0001$). In spite of this, the tucumã pulp extract at 100 μg/mL concentration did not revert the cell damage triggered by H_2O_2; the other high concentrations totally reverted this damage since the dsDNA values were similar to the untreated control group, and at 900 μg/mL, these values were lower than the control group ($F = 49.638$, $p = 0.0001$).

3.5. Caspases activity

The activity status of the proapoptotic proteins caspase-1, 3 and 8 was measured in lymphocytes treated with tucumã peel and pulp extracts at different concentrations exposed or not exposed to an H_2O_2 prooxidant molecule. The results are presented in Fig. 5. All caspases levels were increased in cells exposed to H_2O_2 indicating cell death induction. However, the medium supplementation with peel and pulp tucumã extracts reversed this effect. At 100 and 300 μg/mL of tucumã extracts occurred the caspase 1 levels were similar to untreated control cells. Tucumã extract also affected the caspases 3 and 8 levels of cells exposed to H_2O_2 molecule. However, this effect was partial because the caspases levels were intermediary between untreated cells and cells exposed just to H_2O_2 molecule.

4. Discussion

The present study investigated the effect of extracts of tucumã, an Amazonian fruit rich in carotenes and bioactive compounds such as quercetin, on lymphocyte cells exposed to the toxic effect

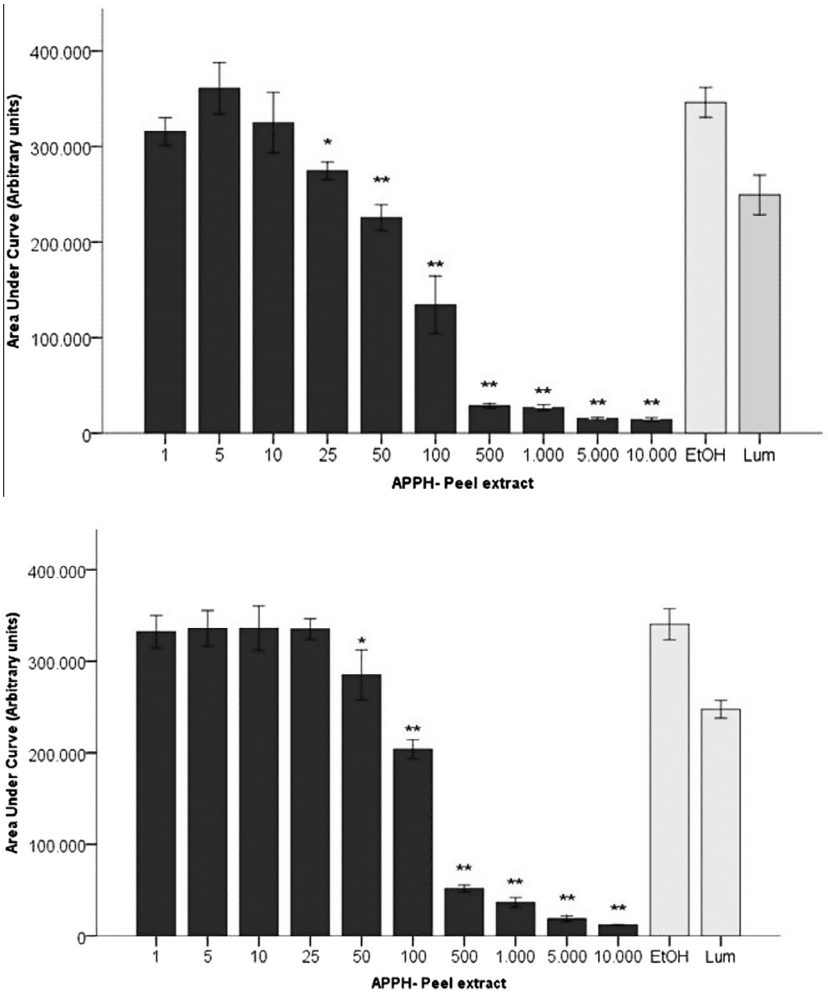

Fig. 2. Antioxidant activity determination expressed as the net area under the curve (AUC) from TRAP assay. (A) Antioxidant activity of tucumã pulp extract at different concentrations (1, 5, 10, 25, 50, 100, 500, 1000, 5000, 10,000 μg/mL). (B) Antioxidant activity of tucumã peel extract at different concentrations (1, 5, 10, 25, 50, 100, 500, 1000, 5000, 10,000 μg/mL); EtOh and Lumino control groups.

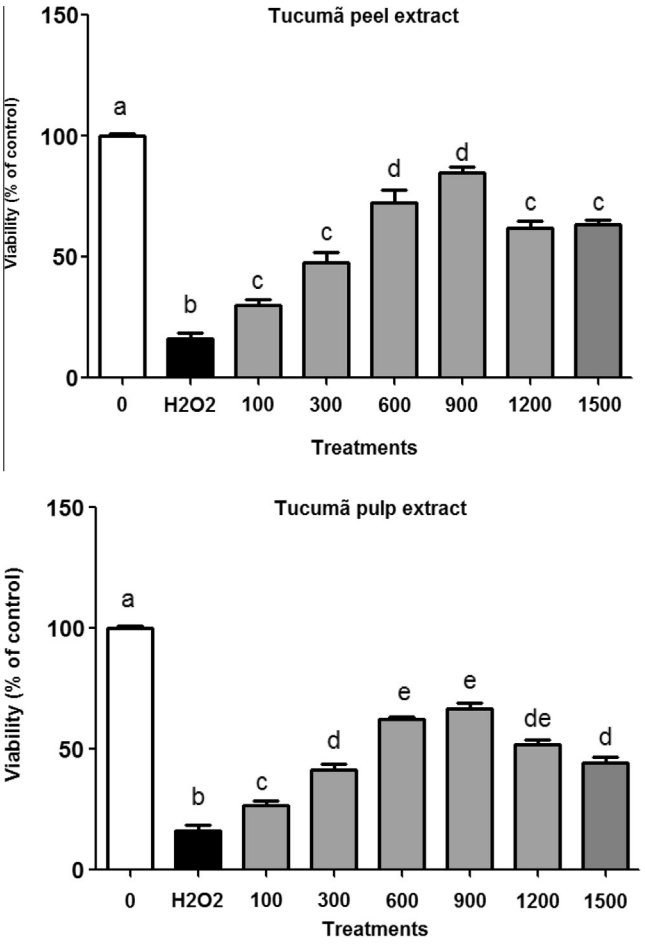

Fig. 3. Comparison of cytotoxicity. Comparison of cytotoxicity evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolic bromide (MTT) reduction among human lymphocyte cells exposed to H_2O_2 and treated to peel (A) and pulp (B) ethanolic extracts at different concentrations. Values are mean ± SE. Different letters within extract treatment indicate statistical differences compared by one-way ANOVA followed by Tukey *post hoc* tests.

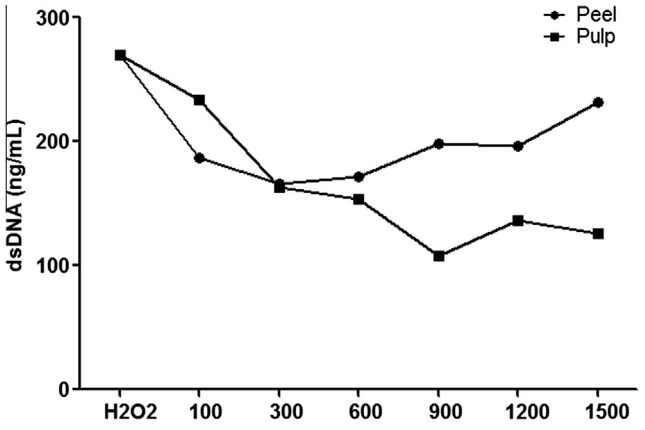

Fig. 4. DNA double-strand denaturation of human lymphocyte cells. DNA double-strand denaturation (dsDNA, ng/mL) of human lymphocyte cells after exposure to H_2O_2 and treated to tucumã peel (A) and pulp (B) ethanolic extracts at different concentrations. Values are mean ± SE. Different letters within extract treatment indicate statistical differences compared by one-way ANOVA followed by Tukey *post hoc* tests.

of H_2O_2. In general, the results showed a beneficial effect of tucumã extracts on the cyto and genotoxicity lymphocytes exposed to H_2O_2.

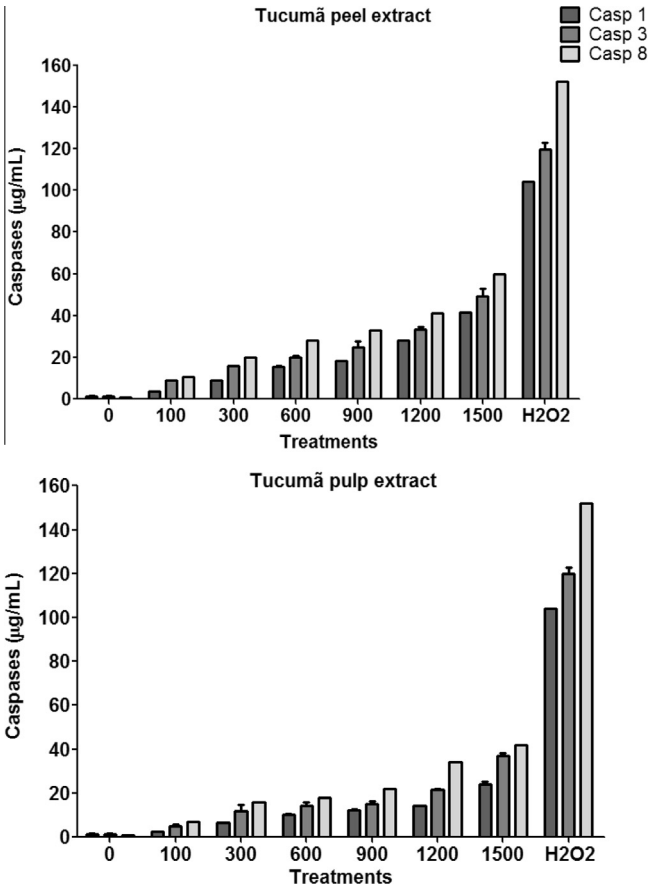

Fig. 5. Activation of caspases 1, 3, and 8. Activation of caspases 1, 3, and 8, analysed by immunoassay tests of human lymphocyte cells after exposure to H_2O_2 and treated with ethanolic tucumã extracts at different concentrations. Peel extracts (3A = caspase 1, 3B = caspase 3, 3C = caspase 8); Pulp extracts (3D = caspase 1, 3E = caspase 3, 3F = caspase 8) (Values are mean ± SE). Different letters within extract treatment indicate statistical differences compared by one-way ANOVA followed by Tukey *post hoc* tests.

The results observed here are probably related to antioxidant molecules found in the peel and pulp extracts. The tannin and alkaloid contents found in tucumã peel and pulp extracts are lower when compared to extracts of other fruits, such as red grapefruit and pomegranate. A lower tannin concentration found in the extracts analysed here confers some safety in tucumã consumption, because an excess of tannins can lower protein digestibility and cause gastric enzymatic inhibition. β-Carotene and quercetin were previously measured in tucumã fruit (De Rosso & Mercadante, 2007; Gonçalves et al., 2010). Therefore, data from these two important compounds in our extracts served to validate our protocol that described new chemical elements in the tucumã fruit. Quercetin and rutin have been reported to carry out numerous biochemical and pharmacological activities, including free-radical scavenging and ultraviolet-light protection. These molecules also have effects on immune and inflammatory cell functions (Mosmann, 1983).

Tucumã pulp extract contained a higher gallic acid concentration than the peel extract. We estimate that the ingestion of 100 g of fresh tucumã gives a similar amount of gallic acid as the ingestion of approximately 130 mL of red grape juice. This juice is considered one of the predominant sources of gallic acid in the human diet. Although caffeic and chlorogenic acids were found at low concentrations in tucumã, the levels of these compounds were higher in peel than in pulp extracts.

The bioactive molecules present in the tucumã extracts probably contributed to their antioxidant capacity, and this was confirmed by DDPH and TRAP assays. The DPPH assay of tucumã pulp extract had been previously reported by Gonçalves et al. (2010). However, to the best of our knowledge, the antioxidant effect of tucumã peel extract has not been reported before. However, the important concentration of antioxidant molecules in tucumã extracts is not a guarantee that these extracts would be able to revert oxidative stress in biological systems exposed to prooxidant molecules, such as the H_2O_2 molecule.

We chose H_2O_2 as the prooxidant molecule since, along with the superoxide radical and hydroxyl radical, H_2O_2 is one of the three major reactive oxygen species (ROS): metabolic by-products continuously generated by the mitochondria in growing cells as a consequence of aerobic metabolism (Halliwell, 1992). Nevertheless, at low levels, H_2O_2 should be considered a signalling molecule in several cell processes because at high levels, H_2O_2 causes damage to organelles, particularly the mitochondria. Potentially damaging oxidative stress can be generated by excess ROS, and dysfunction may result in energy depletion, accumulation of cytotoxic mediators, and cell death (Lima et al., 2008). This cell injury process occurs since, in a high concentration, ROSs like H_2O_2 cause damage to cellular proteins and lipids or form DNA adducts that can promote carcinogenic activity (Choquenet, Couteau, Paparis, & Coiffard, 2008) 18. In these terms, the consequence of cell oxidative stress reversion indicates the potential preventive role of the cell oxidative stress by the tucumã extracts. It is important, however, to point out that we did not find a linear response related to different tucumã extract concentrations on lymphocyte cell cyto- and geno-toxicity caused by H_2O_2. This fact needs to be discussed in greater depth.

Two potential explanations should be considered for the interpretation of these results. Firstly, in respect to an occurrence of chemical compounds in tucumã, higher concentrations can decrease the positive effect of other compounds, such as antioxidant molecules also present in the extracts. This is the case with alkaloid compounds. Some alkaloid compounds are toxic to several organisms and others are used as pharmacological drugs (Castro & Freeman, 2001). In the extracts tested here, we found a higher total alkaloid concentration in the peel when compared to tucumã pulp extracts.

Another possible explanation is related to the increase of antioxidant concentrations present in the extracts that were tested. This is the case with carotenoids that are present in high concentrations in tucumã fruit as described in previous studies and in our results (Morrison et al., 1995; Sreevidja and Mehrotra (2003); Laghari et al., 2012). De Rosso and Mercadante (2007) described 24 carotenoids of which 21 were chemically identified in tucumã fruit. The all-trans-β-carotene was found to be the principal carotenoid, representing 75% of the total carotenoid content in tucumã, followed by 13-cis-β-carotene, all-trans-α-carotene, and all-trans-β-cryptoxanthin, each representing between 2.0% and 2.8% of the total carotenoid content. The other 19 carotenoids represent 15% of the total content. The analysis showed that tucumã offers one of the highest concentrations of pro-vitamin A (β-carotene), with 52 mg/100 g pulp. This concentration is approximately eight times higher than that which is found in carrots (6.6 milligrams/100 g pulp). The mean consumption of 30 g of tucumã pulp can supply three times the daily vitamin A requirement for an adult (Morrison et al., 1995).

Carotenoids are naturally occurring organic pigments and are precursors to vitamin A, which is indispensable for cell differentiation, embryonic development, and vision, as well as having many other roles, including potential therapeutic benefits in the treatment of several morbidities due to their antioxidant properties (Lee, Giordano, & Zhang, 2012). There is little to no doubt that carotenoids and their oxidation products exhibit important bioactivities in cell lines and that the use of cell cultures to interrogate the mechanisms of action may well help in designing sensible clinical trials. However, prospective randomized trials have failed to demonstrate a consistent benefit for the carotenoid β-carotene in patients at risk for CVD. The basis for this apparent paradox is not well understood, but may be attributed to the distinct antioxidant properties of various carotenoids resulting from their structure-dependent physicochemical interactions with biologic membranes (Böhm et al., 2012). Therefore, we cannot discard the idea that high tucumã extract concentrations that present higher carotenoids levels could interrupt the cytoprotective effects observed in lower concentrations.

Similar results were observed when the potential protector effect of tucumã peel extract on DNA damage of lymphocytes exposed to H_2O_2 was analysed. The treatments with 300 and 600 μg/mL presented a better protective action than treatments with tucumã peel extracts in both lower and higher concentrations. However, all pulp extract treatment at concentrations >100 μg/mL reverted the DNA damage caused by H_2O_2 to a similar level observed in untreated control cells. These results are in concordance with some previous studies, which observed that some carotenoids, such as β-carotene, increased DNA resistance to oxidative damage at relatively low concentrations. However, this protection was rapidly lost with an increasing carotenoid concentration (Astley, Hughes, Wright, Elliott, & Southon, 2004; Lowe, Booth, Young, & Bilton, 1999).

On the other hand, compared with the peel, the pulp extract had approximately 50% lower concentration of β-carotene. Perhaps the differences in the concentration of this and the other compounds like alkaloids can explain the different results on DNA damage of lymphocytes exposed to H_2O_2 since tucumã concentration >100 μg/mL presented a genoprotective effect (Fig. 4). Independent of the causes leading to any differences between tucumã peel and pulp extracts, the results suggest that the edible pulp presents genoprotective properties.

H_2O_2 has the capacity to destroy cells, such as neurons, by inducing apoptosis (Chandra, Samali, & Orrenius, 2000). Apoptotic signalling mainly converges in the activation of intracellular caspases, also known as cysteine aspartate specific proteases, which is a family of intracellular proteins involved in the initiation and execution of apoptosis. Therefore, activation of a caspase cascade is a central effector mechanism promoting apoptosis in response to death-inducing signals from cell surface receptors or from mitochondria (Toshiyuki et al., 2000). Initiator caspases, like caspase 8, are able to activate effector caspases like caspase 1 and 3 (Budihardjo, Oliver, Lutter, Luo, & Wang, 1999). Investigations as performed by Jiang et al. show that H_2O_2 induces apoptosis, triggering a caspases pathway. The authors have shown that H_2O_2 treatment caused a time-dependent increase in caspase-1 and -3 proteolytic activities. Based on this previous evidence, we performed an analysis to determine if tucumã extracts could act on caspase pathways involved in the apoptosis process of lymphocytes caused by H_2O_2 exposition. Our results confirm the activation of caspases activity in the presence of H_2O_2 previously described, and tucumã extracts were able to totally or partially revert the caspases activation (Fig. 5).

To the best of our knowledge, this is the first study to describe the cytoprotective effects of tucumã fruit. The results obtained from tucumã peel extracts open the option of using the parts of this fruit that are less edible (for example, only riverine people eat the peel of the tucumã fruit) as a food supplement. However, *in vivo* investigations need to be performed to evaluate the tucumã extract's effects on biochemical and physiological markers related to the prevention of morbidities like cancer and cardiovascular diseases.

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