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The protective effects of guaraná extract (*Paullinia cupana*) on fibroblast NIH-3T3 cells exposed to sodium nitroprusside

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ABSTRACT

The antioxidant effects of the hydro-alcoholic guaraná extract (*Paullinia cupana* var. *sorbilis* Mart.) on nitric oxide (NO) and other compounds generated from the degradation of sodium nitroprusside (SNP) in an embryonic fibroblast culture (NIH-3T3 cells) were evaluated. The guaraná bioactive compounds were initially determined by high-performance liquid chromatography: caffeine = 12.240 mg/g, theobromine = 6.733 mg/g and total catechins = 4.336 mg/g. Cells were exposed to 10 μM SNP during a 6 h period because the cells exhibited >90% mortality at this concentration. Guaraná was added to the cultures in five concentrations (0.5, 1, 5, 10 and 20 mg/mL). The guaraná antioxidant effect was evaluated by viability assays, biochemical oxidation [lipid peroxidation, catalase and superoxide dismutase (SOD) activity] and genotoxicity (DNA Comet assay) analysis. Additionally, oxidative stress was evaluated by a 2,7-dihydrodichlorofluorescein diacetate fluorescence assay. Guaraná reverted the SNP toxicity mainly at lower concentrations (<5 mg), which decreased cell mortality, lipid peroxidation, DNA damage and cell oxidative stress as well as increased the SOD levels. These results demonstrate that guaraná has an antioxidant effect on NO metabolism in situations with higher cellular NO levels.

1. Introduction

Guaraná, an Amazonian Brazilian fruit, used to prepare an energetic beverage recognised as a safe food by the Food Drug Administration (FDA) (Ducan et al., 2010) and by the Brazilian agency that regulates and registers medicines and foods (ANVISA). Unfortunately, when compared to other food stimulants such as coffee and ginseng, the number of scientific studies of guaraná properties published in the literature is quite small, suggesting the need for additional work.

Previous investigations described several biological proprieties of guaraná, including antioxidant (Mattei et al., 1998; Basile et al., 2005), antimicrobial (Basile et al., 2005), antiplatelet aggregation (Ravi Subbiah and Yunker, 2008), anti-obesogenic (Opala et al., 2006), antimutagenic and anticarcinogenic (Fukumasu et al., 2011) effects.

Studies have also shown that guaraná protects against testicular damage caused by cadmium exposition (Leite et al., 2010), improves memory (Kennedy et al., 2004), and displays antidepressive (Campos et al., 2005), anti-fatiguing and energetic properties (Haskell et al., 2007). On the other hand, safe studies have indicated low toxicity with guaraná ingestion (Santa Maria et al., 1998; Mattei et al., 1998).

Recently, our research team described for the first time a case-control study related to habitual ingestion of guaraná (*Paullinia cupana*, Mart. Var. sorbilis) by an elderly population sample ($n = 637$) residing in the Amazon Riverside region of the Maués municipality (Brazil). The study evaluated the effect of habitual guaraná ingestion on variables related to cardiometabolic functions such as anthropometry and biochemical biomarkers of lipid, glycemic and oxidative metabolism. The subjects were classified as either those who habitually drank guaraná (GI) or those who never drank guaraná (NG) based on their self-reported intake of guaraná. We observed that the prevalence of hypertension,

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obesity and metabolic syndrome in the GI group was lower than the prevalence found in the NG group. Additionally, a significant association between lower levels of advanced oxidative protein product (AOPP) and habitual guaraná consumption were observed (Costa Krewer et al., 2011).

According to traditional understanding, guaraná also exhibits aphrodisiacal properties, and a study performed by Antunes et al. (2001) described the physiological effects of guaraná on rabbit cavernosal tissue that suggested a possible vascular relaxing effect (Antunes et al.,2001).

Because oxide nitric (NO) is a molecule, which is virtually produced by all cells and is implicated in many physiological processes such as neurotransmission, smooth muscle relaxation, immune regulation and the host defense against microbes (Thomas et al., 2008), we tested here the guaraná potential protective effect on cytotoxicity caused by sodium nitroprusside (SNP), which releases cyanide and/or nitric oxide using the embryonic fibroblast culture (NIH-3T3 cells) as an experimental *in vitro* model.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were purchased from Gibco® Life Technologies Inc., Grand Island, NY, USA and Sigma® St. Louis, MO, USA.

2.2. Plant material

P. cupana powder that is produced and supplied by EMBRAPA Oriental (Agropecuary Research Brazilian Enterprise) located in the Western Amazon in Maués, Amazonas-Brazil was used in all experiments. The guaraná powder produced by EMBRAPA present high quality and integrity has already been used in previous studies such as Angelo et al. (2008). The guaraná powder used in this study was produced from seeds obtained and processed in 2012 and the cultivation and processing is briefly described: Guaraná is harvested by hand in the dry season. If the entire fruit bunch is ripe, it is either snipped off with scissors or small pruning shears, or broken off manually. If only a few berries are ripe, they are picked individually. Before roasting the seeds, the red skin must be removed. The fruits are skinned by hand, left to soak in water, or simply stored for several days until the skin softens. After this process guaraná seeds are roasted in an open-sided hut or in machinery similar to that used for processing coffee and then the toasted seed are pounded. Guaraná powder ground particles ranging in size from 0.630 to 1 mm, 0.315 to 0.630 mm, and 0.315 to 2 mm. The guaraná powder was stored in dry conditions at ±4 °C and was protected from light until the extract preparations.

2.3. Hydro-alcoholic guaraná extraction and bioactive compound determination

The hydro-alcoholic extract of *P. cupana* was produced using 70:30 alcohol and water to 100 mL of extraction fluid prepared at a concentration of 300 mg/mL.

After 24 h of extraction, the preparation was centrifuged at 3000 rpm for 10 min, and the supernatant was isolated. The resulting solution was filtered through Whatman No. 1 paper, and the ethanol was removed using a rotary evaporator at reduced pressure, 25° at 115 rpm, and further was lyophilized to determine xanthine and catechin compositions as well as to perform the experimental procedures.

Chromatographic analysis was performed with detection by UV absorbance at 272 nm on an HPLC system consisting of a Shimadzu Prominence LC-20A, an LC-20AT quaternary pump, a SIL 20 auto sampler A, a DGU-20A5 on-line degasser, a CBM-20A integrator and a SPD-20AV DAD detector according to Andrews et al. (2007). A 150 × 4.6 mm i.d. ODS-3 column (Phenomenex Prodigy ODS-3 100A, 5-μm particle size; Torrance, CA, USA) was used for the separation.

The Bempong and Houghton (1992) guaraná bioactive compound analysis was used as a reference. A stock solution of caffeine (250 μg/mL) was prepared and stored at 5 °C. Working level standards were prepared by diluting the stock solution in mobile phase at the following ratios: 200 μL to 100 mL, 400 μL to 100 mL, 2 mL to 100 mL, 4 mL to 100 mL and 8 mL to 100 mL. The least concentrated standard was designed to achieve a limit of detection of 0.005% based on a 1 g sample diluted to 100 mL (LOD = 0.05 mg/g). The guaraná sample extract was filtered through a 0.45-μm filter into an autosampler vial for analysis. The HPLC conditions were: flow rate, 1 mL/minute; mobile phase A, 0.1% H_3PO_4 in water; mobile phase B and, 100% ACN. The chromatographic system was calibrated with at least a five-point standard curve for each set of samples analysed. Standards were run after every fourth sample. Excellent reproducibility was seen in the standards; typically the *R* value for the calibration curve was 0.9999 or better. From these results, we prepared the guaraná compound to add to the culture medium according to the Santa Maria et al. (1998) protocol. The lyophilised extract was diluted in distilled water and prepared at a concentration of 200 mg/mL. The mixture was infused for 7 min by boiling, centrifuged (1500 rpm, 15 min) and filtered. The solution was sterilised by filtration (0.20 μM) before adding to the culture medium.

The estimation of condensed tannins in the guaraná extract was measured spectrophotometrically (Morrison et al., 1995). The contents were expressed as milligram equivalents of gallic acid/mL of grape juice. The equation obtained for the standard curve of gallic acid in the range of 2.5–20 mg/mL was $y = 0.0434x + 0.1359$ ($R^2 = 0.9819$).

2.4. Cell line and culture conditions

The embryonic fibroblast NIH-3T3 cell line was obtained from ATCC (ATCC® Number: CRL-1658™) and maintained in DMEM supplemented with 10% FBS, 0.1% gentamicin and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

2.5. Experimental protocol

The cells were distributed in 25-cm^2 culture flasks at initial densities of 2×10^5 cells and maintained in the same incubation conditions until 90% confluent. The flasks were identified as follows: (C) sample cell culture without SNP and guaraná used as a negative control, (SNP) sample with 10 μM SNP as a toxicity positive control. The groups supplemented with guaraná plus SNP were identified by concentrations of the guaraná added to the culture medium. Five guaraná extract concentrations (0.5, 1, 5, 10 and 20 mg/mL) of aqueous solution plus 10 μM SNP were added to the culture medium to test the protective effect. After a 6-h incubation time, cell viability was analysed. Cell aliquots were harvested, counted and frozen for biochemical analysis. Additionally, cell aliquots of sample treated with different guaraná powder extracts concentrations, control group and treated with SNP were separated and used for a genotoxicity assay. The replicated tests ranged from three to eight according to the protocol performed.

2.6. Cytotoxicity assays

The effects of guaraná with SNP exposition on the viability of NIH-3T3 embryonic fibroblasts were analysed by MTT 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide and trypan blue assays. Briefly, the cells were harvested and seeded in octuplicate at a density of 2×10^4 cells/well in DMEM medium and added to each well of a 96-well tissue culture microplate. Plates were incubated for 24 h at conditions described previously for cellular adherence. Then, the medium was replaced by 200 μL aliquots of the extract solutions plus SNP as follows: control, 10 μM SNP, 0. 5 mg/mL (*P. cupana* extract) plus 10 μM SNP, 1 mg/mL plus 10 μM SNP, 5 mg/mL plus 10 μM SNP, 10 mg/mL plus 10 μM SNP and 20 mg/mL plus 10 μM SNP followed by incubation for 6 h.

After the exposition period, cells were stained for 4 h at 37 °C with 10 μL/well MTT reagent (10% concentration) and 5 mg/mL in DPBS. Then, 100 μL of DMSO was added per well to solubilise the purple formazan crystals produced. The absorbance of each well was measured at 570 nm and 630 nm with a Microplate Reader Benchmark Bio-Rad®. The results were expressed as the average percentage of concentration compared to the control.

A trypan blue assay was performed to confirm a possible alteration in cell populations. The cells (2.5×10^5/well) were grown for 4 days in a six-well culture microplate. After this period, the cultures were treated with the same procedure as described by the MTT protocol, trypsinised and counted in a hemocytometer.

2.7. Oxidative and antioxidant biomarkers analysis

We analysed four oxidative metabolism biomarkers in the cell culture exposed to guaraná at different concentrations: lipoperoxidation (TBARS), DNA damage (genotoxicity), superoxide dismutase (SOD) and catalase (CAT) activity, and thiol group levels. All tests were standardised as described in a previous study performed by our research team (Montagner et al., 2010). To perform each assay, we used a cell density of 3×10^6 cells. TBARS was quantified by measuring its formation (Ohkawa et al., 1979). Total superoxide dismutase (SOD, E.C.1.15.1.1) activity was measured spectrophotometrically according to the method described by Boveris and Cadenas (1997). Catalase activity (EC 1.11.1.6.) was determined according to Aebi. Thiol groups were determined as described by Ellman (1959).

A single cell gel electrophoresis (Comet assay) assay was used to test the protection of guaraná on NIH 3T3 cells after NP exposure. The assay was performed as described by Singh et al. (1995) in accordance with general guidelines. Overall, 100 cells (50 cells from each of the three replicate slides) were selected and analysed. Cells were visually scored according to tail length and received scores from 0 (no migration) to 4 (maximal migration). Therefore, the damage index for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). The slides were analysed under blind conditions by at least two different researchers.

To determine potential antioxidant effects of guaraná on SNP exposition we performed an additional analysis using the dichlorofluorescein acetate assay (DCF-DA) (Halliwell and Whitemann, 2004). We used a cell density of 2.5×10^5