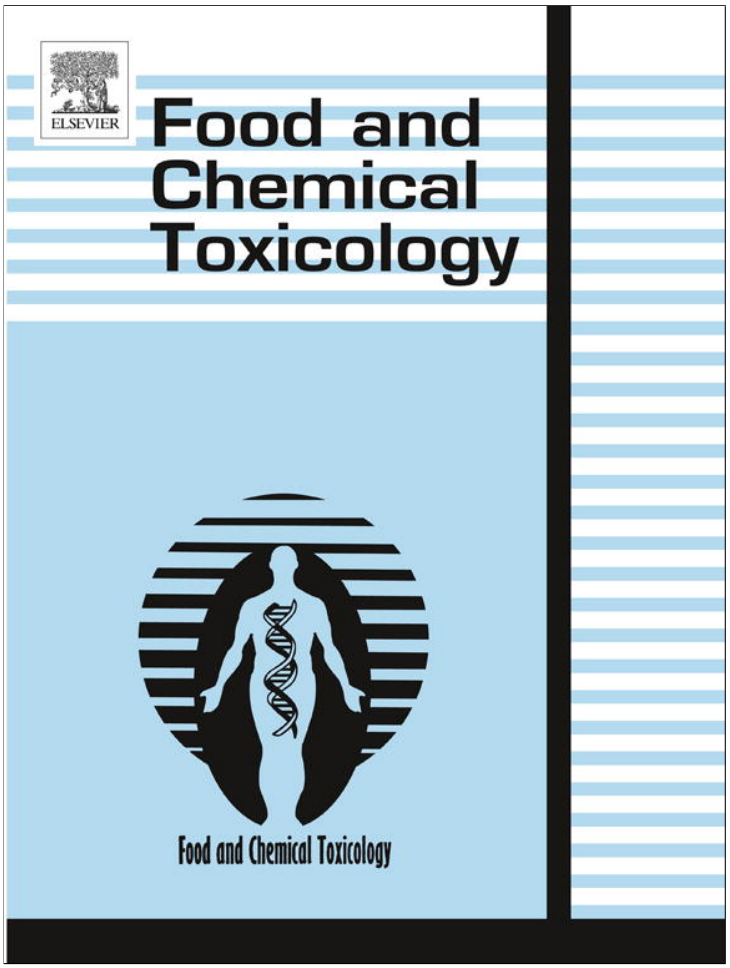

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The protective effects of guaraná extract (*Paullinia cupana*) on fibroblast NIH-3T3 cells exposed to sodium nitroprusside

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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

cells/well in a 24 well microplate. After adherence, the cells were exposed to same guaraná powder extract at concentrations previously described. After guaraná exposition, cells were washed and incubated with DCF-DA (10 μM) at 37 °C for 40 min in the dark. They were then washed twice with DPBS pH 7.4, and then the localisation of the fluorescence in living cells treated with DCF-DA was examined using a Nikon grid-confocal microscope. The images were captured at 40X magnification using the Improvision (PerkinElmer®) software. Data were normalised to values obtained from the untreated controls.

2.8. Statistical analysis

All analyses were performed using the statistical package for social studies SPSS version 18.0 (SPSS Inc., Chicago, IL). The results were presented in mean ± standard error (SE). Initially we tested if the variables presented a normal distribution using a Kolmogorov test. Because we observed data normality, the quantitative variables were compared among guaraná powder extract at different concentrations by analysis of variance followed by Tukey's *post hoc* test. The analysis of oxidative stress by DCF-DA fluorescence was performed using the Digimizer image analysis software (MedCalc Software, Mariakerke, 2005–2009 Belgium) by evaluating the average intensity of the cells (pixels units). At least 30 cells for each experimental group were analysed, and the observed fluorescence intensity was compared using a Kruskall–Wallis non-parametric variance test followed by a Mann–Whitney *post hoc* test. All p-values were two-tailed. The alpha value that was considered to be statistically significant was $p = 0.05$.

3. Results and discussion

3.1. Guaraná bioactive compounds and the effect on SNP cytotoxicity

A total of 92 mg which equals 30.6% of guaraná extract was obtained and used to perform all experiments. The bioactive compounds of guaraná were determined to be caffeine = 12.240 mg/g, theobromine = 6.733 mg/g and total catechins = 4.336 mg/g of guaraná extract (Fig. 1). The guaraná's condensed tannin concentration was 16 mg/g. These results corroborate previous studies describing the presence of these bioactive compounds in guaraná extract that present biological activity (Bellinardo et al., 1985; Yamaguti-Sasaki et al., 2007; Smith and Atroch, 2010). However, other molecules are present in guaraná as suggested by study performed by Angelo et al. (2008). These authors investigated 15,000 RNA transcripts (EST sequences) from guaraná in three different developmental stages obtained from EMBRAPA and observed that presence of transcripts related to secondary metabolism molecules as flavonoids and carotenoids. The analysis show some levels of identity among guaraná other species such as *Camelia sinensis* (green tea), *Malus domestica* (apple), *Vitis vinifera* (grapefruit), *Theobroma cacao* (cocoa) and *Coffea arabica* (coffee). Possibly, the substances evaluated here as well as others present in guaraná seeds could be at least partially responsible for the guaraná effects.

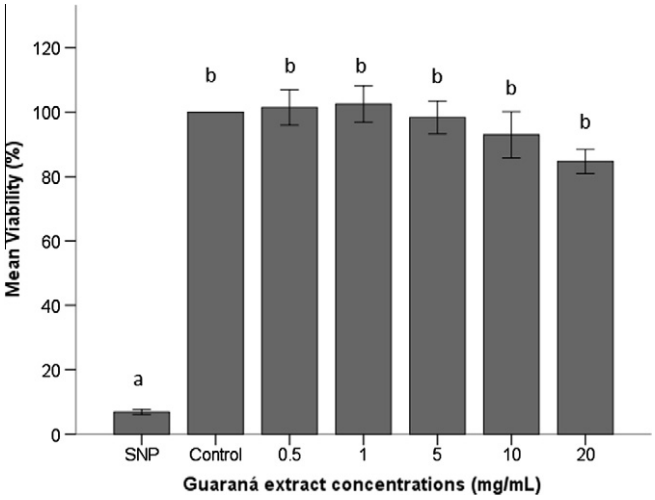

Fig. 1. The viability cell measured by the reduction of MTT in NIH 3T3 cells after 6 h of co-incubation with different concentrations of guaraná extracts and 10 μM SNP. Data are presented as mean values ± standard error (SE) and express the viability as a percentage of the control value. Different letters indicate statistical differences among guaraná powder extract at different concentrations, SNP and control groups by ANOVA one-way analyses followed by Tukey's *post hoc* test at $p < 0.05$.

The effect of guaraná exposition on cytotoxicity caused by SNP exposition was evaluated considering five concentrations. The guaraná powder extract at 0.5, 1.0, 5.0 and 10 mg/mL concentrations restored cell viability measured by MTT assay ($F = 159.36$; $p = 0.0001$; Fig. 1). However, at a guaraná concentration of 20 mg/mL an intermediary viability was observed between negative (without SNP) and positive (10 μM SNP) groups.

The trypan blue assay presented similar protective results as observed in the MTT assay; however, in this test, the frequency of cell viability in 20 mg/mL was also similar to the negative control group (SNP = 7%, 0.5 mg/mL = 101%, 1.0 mg/mL = 100%, 5.0 mg/mL = 98%, 10.0 mg/mL = 92.8% and 20.0 mg/mL = 82%; $F = 86.74$, $p = 0.0001$).

3.2. Guaraná effect on SNP lipid peroxidation and antioxidant enzymes levels

The lipid peroxidation showed a significant association with cell exposed to different guaraná power extract concentrations ($F = 209.32$; $p < 0.0001$). As seen in Fig. 2, the 10 μM SNP exposure doubled the level of lipid peroxidation when compared to the control group. The presence of guaraná powder extracts decreased the lipid peroxidation induced by SNP ($F = 35.78$; $p < 0.001$) until the SNP concentration reached 10 mg/mL. However, cells exposed with 20 mg/mL guaraná plus 10 μM SNP exhibited the lowest peroxidation levels when compared to the other guaraná concentrations.

The CAT activity observed in the control group was 1.59 ± 0.08 μmol/min/mg protein. However, in the presence of 10 μM SNP with or without guaraná supplementation, the catalase activity was completely inhibited. On the other hand, the cells that received only SNP exposition had an increase in SOD activity when compared to the negative control group (Fig. 2). The SOD activity further increased when 0.5, 1 and 5 mg/mL guaraná concentrations were added in the cells exposed to SNP, whereas the guaraná concentrations at 10 and 20 mg/mL guaraná concentrations presented SOD activity similar to cells treated only with SNP.

3.3. Guaraná effect on SNP genotoxicity

The effects of the different guaraná concentrations on genotoxicity of cells exposed to SNP are compared in Table 1. In the presence of SNP, all analysed nuclei presented some DNA damage. However, the lower guaraná concentrations (0.5 and 1.0 mg/dL) reverted the genotoxicity caused by this oxidant compound because the mean of the DNA damage index was similar to the untreated control group. In addition, guaraná concentrations ranging from 5 to 20 mg/mL presented a DNA damage index similar to SNP exposition, indicating no genoprotective effect at these concentrations.

3.4. Guaraná effect on SNP cell oxidative stress evaluated by DCF-DA fluorescence assay

We observed significant differences among the cellular oxidative states evaluated by DCF-DA fluorescence on the cells exposed at different guaraná extracts concentrations ($F = 22.41$; $p < 0.001$). As shown in Fig. 3, cells exposed to SNP exhibited higher oxidative stress when compared to the control group. Guaraná supplementation decreased this oxidative stress at the highest concentration of 5 mg/mL. Therefore, high guaraná concentrations (10 and 20 mg/mL) did not revert the oxidative stress caused by SNP.

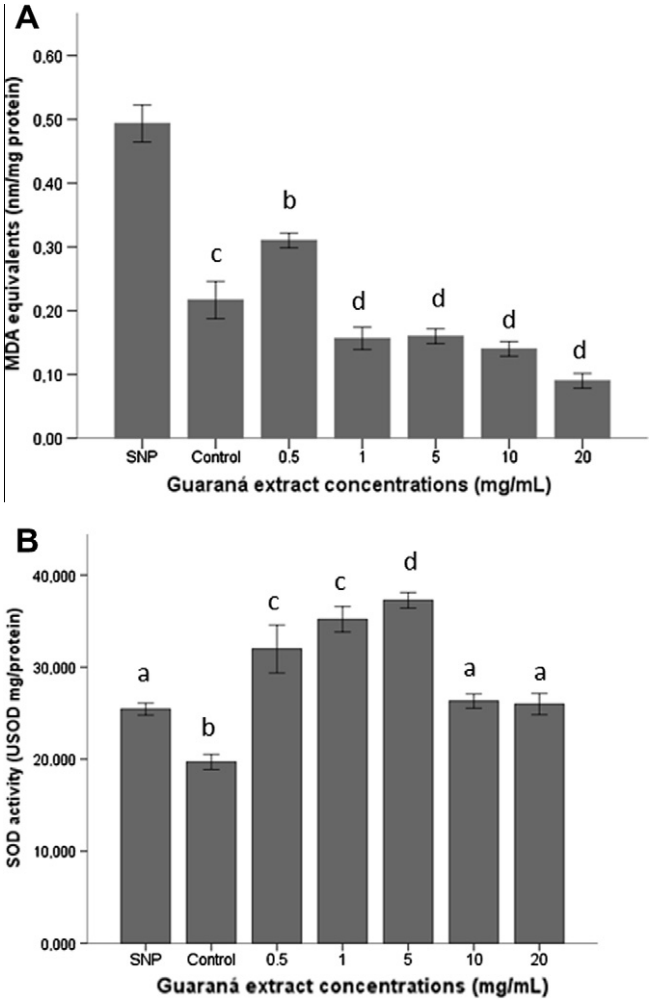

Fig. 2. Effect of different guaraná concentrations plus 10 μM SNP induced TBARS production and SOD activity in NIH 3T3 cells homogenates in comparison to control cultures and 10 μM SNP cultures. Data show means ± SE values. In A and B graphs, different letters indicate statistical differences by ANOVA one-way analyses followed by Tukey's *post hoc* test at $p < 0.05$.

In general, the guaraná exposition demonstrates *in vitro* protective effects against cytotoxicity and oxidative stress in NIH-3T3 embryonic fibroblasts cells induced by SNP exposure. However, these effects were dependent on the guaraná concentration with the most effective extract concentrations ranging from 0.5 to 5 mg/mL.

These results suggest that guaraná has an *in vitro* bioactive action on NO modulation because SNP acts as an in situ NO donor. In some pathological conditions, such as chronic inflammation, stroke, diabetes, inflammation, neurodegeneration, and cancer, peroxynitrite generation occurs primarily from an increase in NO levels (Ahmad et al., 2009). In these terms, it is important to identify foods that have an effect on NO or RNS compounds generated by an excess of NO concentration in the organism and its mechanism of action (Halliwell, 2008).

From the these results, we believe that guaraná presents an antioxidant effect on NO generated from SNP or on peroxynitrite produced by an excess of NO that reacts with the superoxide ion that is continuously generated by the mitochondrial respiratory machinery. The potential guaraná antioxidant function could explain the decrease in cell death caused by exposure to SNP in cells that were supplemented with guaraná. In both cell viability analyses (MTT and trypan blue assays), we observed that guaraná at concentrations ranging from 0.5 to 10 mg/mL after 6 h of exposure to SNP restored the viability to similar percentages observed in untreated control cells. However, the higher guaraná concentration (20 mg/mL) presented an intermediary effect on viability between the untreated control cells and the cells exposed to SNP. These results suggest that the "chemical protection provided by guaraná" is dependent on the balance between its bioactive compounds and other oxidations or biological products present in the cells. Accordingly, an increase that is higher than the required amount of antioxidant compounds can lead to an imbalance that is not beneficial to the cell.

Since we use a fairly high concentration of SNP that induces a mortality rate above 90% after 6 h of exposure, the protective effects of guaraná during this period are most likely related to necrotic cellular events. However, investigations to determine the protective nature of guaraná (in apoptotic or necrotic cellular events) need to be conducted. Moreover, this study did not address the question of which mechanism of cell death (apoptosis or necrosis) is inhibited by guaraná in low concentrations.

Additional biochemical and cytological analyses indicated that the guaraná protection is related to the differential modulation of cellular oxidative stress indicators. From 1 mg/mL concentration of guaraná in the cell medium, we observed a significant reduction on lipid peroxidation even in the presence of SNP. These results reinforce the idea that guaraná acts directly on peroxynitrite molecules because these radicals can oxidise proteins, nitrate tyrosine residues and induce cell membrane lipid peroxidation (Denicola and Radi, 2005).

We found an unexpected result when we analysed the effects of cells exposed to different guaraná power extract concentrations on SOD activity. When we compared untreated cells and cells treated only with SNP, we observed an increase in SOD levels in presence

Table 1
DNA migration in the comet assay for the genotoxicity assessment of embryonic fibroblast NIH/3T3 cell line cultures exposure to 10 μM SNP with and without guaraná extract (*Paullinia cupana*) supplementation.

Treatments	Comet class frequency (Mean ± SE/100 nucleus analysed)					Damage index
	0	1	2	3	4	
Control	52 ± 2	26 ± 1	11 ± 2	9 ± 0.6	2 ± 0.01	0.47 ± 0.02[a]
10 μM SNP	0	1 ± 0.7	7 ± 1	63 ± 2	29 ± 2	1.00 ± 0.0[c]
Guaraná (mg/dL)						
0.5	47 ± 4	12 ± 2	8 ± 3	13 ± 3	19 ± 2	0.53 ± 0.14[a]
1.0	60 ± 3	13 ± 0.6	7 ± 2	10 ± 2	9 ± 3	0.39 ± 0.13[a]
5.0	14 ± 1	13 ± 1	16 ± 1	59 ± 2	8 ± 1	0.86 ± 0.11[b]
10.0	8 ± 2	18 ± 2	20 ± 1	39 ± 3	11 ± 2	0.88 ± 0.08[b]
20.0	13 ± 1	10 ± 1	16 ± 3	51 ± 4	10 ± 1	0.87 ± 0.02[b]

SE = standard error; damage index: Σ (1, 2, 3, 4 comet class)/100. Different letters mean statistical differences among control group, 10 μM SNP group and 10 μM SNP plus guaraná treatments using Anova one-way analysis of variance followed by Duncan test. 0 = nucleus without DNA damage.

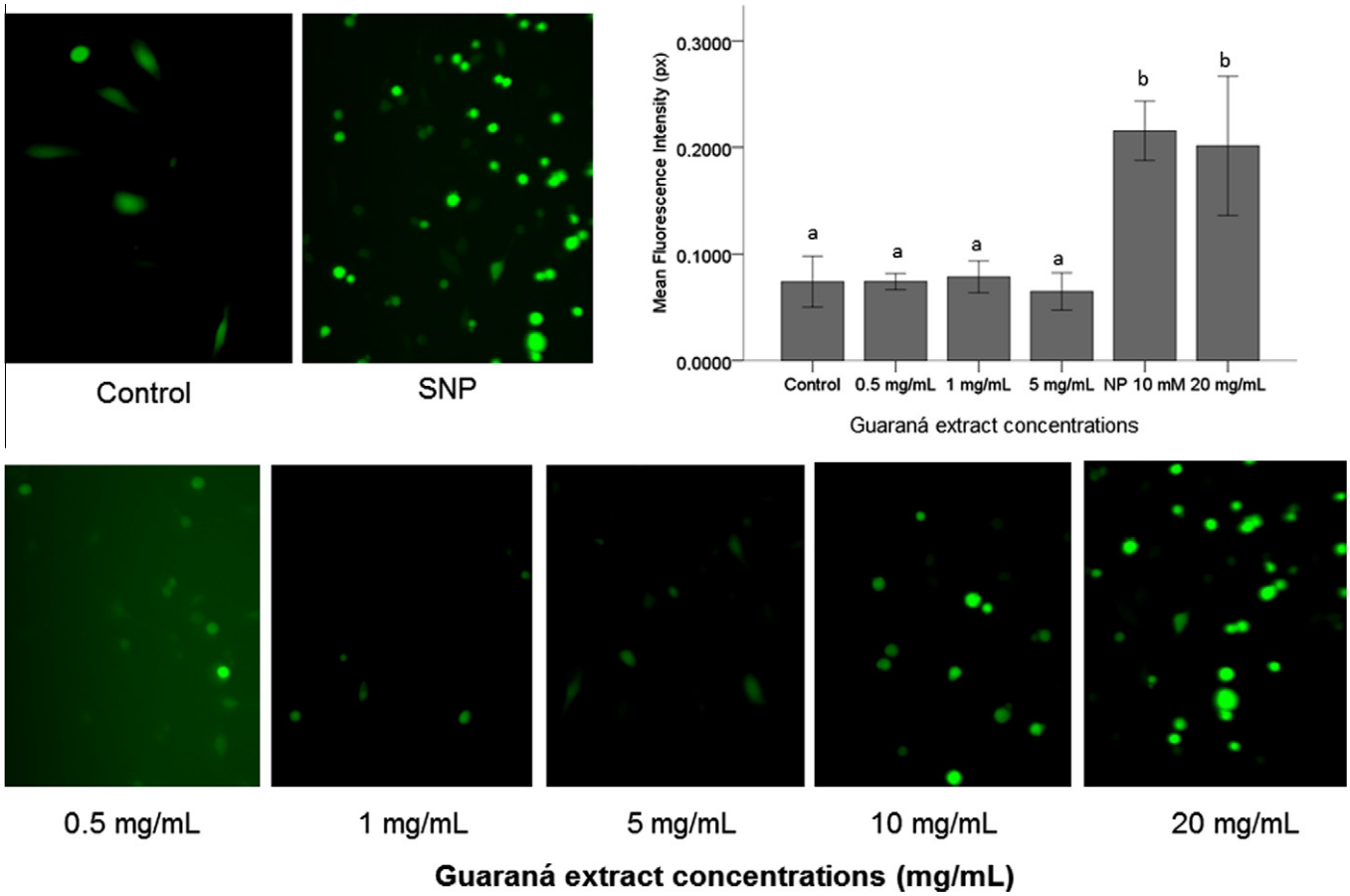

Fig. 3. Effect of different guaraná concentrations plus 10 μM SNP on ROS production by measuring DCF-DA fluorescence intensity in NIH 3T3 living cells in comparison to control cultures (a) and 10 μM SNP cultures without (b) and with guaraná powder extracts at different concentrations. The graphical shows means ± SE values of fluorescence intensity for at least 30 cells exposed to guaraná powder at different concentrations. Different letters indicate statistical differences among cells exposed to guaraná powder extract at different concentrations, SNP and control groups by ANOVA one-way analyses followed by Tukey's *post hoc* test at $p < 0.05$.

of SNP exposure. However, in the presence of guaraná supplementation, the SOD levels were higher than the levels observed in the SNP and control cell groups. These data suggest that guaraná in the presence of SNP can modulate this important enzyme that is directly related to superoxide radical.

However, a question that need to be addressed is the fact that at the highest concentration Guaraná shows a 10% negative effect by measuring MTT - but the positive effects on MDA and SOD and other parameters are seen at the highest concentration. These results need to be considered in more detail since the guaraná in lower concentrations restored completely the cell viability whereas from 10 mg/mL the viability was partially restored. An explanation for these results is the potential occurrence of U-shaped dose–response relationships (often termed hormesis) that have been documented in numerous biological, toxicological, and pharmacological investigations as shown in review performed by Calabrese and Baldwin (2003). Dose–response relationships conforming to the hormetic curve are affected by several factors as the magnitude of the low-dose stimulatory response, the number of doses establishing the reliability to the hormetic curve. Therefore, we cannot discard that the increase in the concentration of bioactive compounds present in guaraná extract exert opposite effects than observed in lower doses. This hypothesis is corroborated by SOD levels since this enzyme maintained similar concentration in cells treated with SNP and SNP plus 10 mg/mL guaraná.

However, the MDA levels found from 10 mg/mL guaraná concentrations were lower than in the guaraná doses that present beneficial effect on cells exposed to SNP. This result is hard to explain but we can suppose that the increase in the cell mortality could to affect the peroxidation levels. In these terms, complementary investigations need to be performed to clarify this question.

Because a higher level of NO generated from SNP can increase the peroxynitrite production from the reaction of NO with superoxide ion, the increase of SOD concentration in the cells treated only with SNP indicates a cellular response to reduce the availability of superoxide and thereby decrease the production of peroxynitrite. A previous study performed by Lushchak and Lushchak (2009) that used yeast as an experimental model described that NO spontaneously generated at SNP decomposition increased the SOD activity. These results agree with our current findings. However, the authors also found an increase in catalase levels from yeast exposed to SNP, which is contrary to what we observed in our study.

SNP produce some levels of cyanide molecules. This compound is a selective inhibitor of the catalase enzyme as well as Cu/Zn SOD enzymes. Therefore, the catalase inhibition could to be explained by the presence of cyanide. Additionally, the results indicate that an increase in mitochondrial SOD2 enzyme activity that is manganese dependent occurred and did not display sensitivity to cyanide. In this case, guaraná cannot reverse this inhibitory reaction in the cells.

ROS and other free radicals as NO are produced at a low level by the cell metabolism and play a physiologically important role in the regulation of cell signaling, proliferation, and differentiation. Due to highly reactive properties the cell presents antioxidant systems to regulate these molecules (Boveris and Cadenas, 1997).

When an imbalance occurs between ROS and NO production and degradation the cells present a oxidative or nitrosative stress state that have dangerous consequences that have been related to several human physiological dysfunction and diseases (Boveris and Cadenas, 1997; Moncada, 1999). Therefore, the persistence of both oxidative and nitrosative stress cause damage to cells although the biological consequences can be differentiated. However, generally when the cell is submitted to nitrosative stress occurs an oxidative stress as consequence of NO imbalance.

In the cellular basal conditions occurs a continuous superoxide anion production mainly as result of mitochondrial biochemical reactions. The superoxide accumulation is limited by the presence of SODs which rapidly convert superoxide into H_2O_2 and oxygen (Halliwell, 2008). This modulation is very important since the superoxide has a high affinity to NO radical that is produced by virtually all cells. When the superoxide anion reacts to molecules of NO, peroxynitrite (PN, OONO–) is formed by the diffusion rate-limited combination. In turn, the PN has been proposed to be a key contributor to oxidative damage, mainly because its highly reactive decomposition produces nitrogen dioxide, hydroxyl radical, and carbonate radical (Alvarez et al., 2002). The PN-derived radicals can oxidise proteins, nitrate tyrosine residues and induce cell membrane lipid peroxidation (Denicola and Radi, 2005). Therefore, when an NO imbalance takes place for any adverse environmental circumstances, some of these molecules can cause direct or indirect damage at the cellular or molecular level, promoting a phenomenon of nitrosative stress (Moncada, 1999).

In our study, we induced a nitrosative stress state when we exposed the NIH 3T3 cells to SNP that is a NO-donor. The exposition caused increase in lipoperoxidation levels as can see in Fig. 3, but also a significant increase in SOD level. The first results was expected and probably caused by increase of PN levels into the cells. However, the possible explanation for the increase on SOD levels is related an effort of cells in decrease the superoxide and NO reaction from increase of the SOD levels that, potentially would reduce the superoxide available to react with NO and generate PN. This compensatory effect appears to be enhanced by exposition to guaraná among 0.5–5 mg/mL.

The antioxidant protective effect of guaraná on embryonic fibroblast cells was also observed from DCF-DA fluorescence analysis and genotoxicity analysis using DNA Comet assay. These analyses confirm that guaraná can restore some oxidative cell damage after SNP exposure. However, the guaraná protective effect on oxidative stress observed from DCF-DA fluorescence occurred in guaraná concentrations $\leqslant$10 mg/mL, whereas genotoxicity protection from SNP exposure observed only in the lower guaraná concentrations <5 mg/mL. Taking into account these results, we estimate that the guaraná doses that are most effective in protecting the cell against the oxidative effects of SNP range from 1 to 5 mg/mL.

The antioxidant guaraná is most likely related to bioactive compounds present in this food. Guaraná is rich in methylxanthines, such as caffeine, theobromine, and theophylline, and it contains tannins, saponins, catechins, epicatechins, proanthocyanidins, and trace concentrations of many other compounds (Bellinardo et al., 1985). A study of the guaraná transcriptome revealed the presence of important secondary compounds in this plant, including transcript sequences related to flavonoid metabolism (Angelo et al., 2008). The results described in this study suggest that guaraná exhibits similar properties to *C. sinensis* (i.e., green and black tea) and coffee. These foods have important antioxidant properties previously described (Singh et al., 2011). It has been found that green and black teas are able to protect against NO toxicity in several ways, including NO and peroxynitrite scavenging (Steffen et al., 2005).

Finally, it is important to comment on the potential methodological limitations of the study design. In fact studies evaluating the potential protective effect of plant extracts on cell exposed to oxidative stress can be performed considering three types of protocol design: investigations about preventive effects of some extract of molecule against an oxidant compound that can be perform from pre-incubation and co-incubation protocols and investigations about therapeutic effects to evaluate potential reversion of some cellular damage caused by an oxidant compound (pos-incubation protocol). However, the choice of the study design that needs to consider the nature of oxidative stressor that the cells will be exposed. The SNP used in our experiment as oxidative stressor is a donor of NO and cyanide ions that causes fast morphological and functional effect that can leads quickly to death cell. In these terms, the pre and post incubation of cells with guaraná would not able to present some positive influence. However, this is an open question that needs to be clarified from additional studies.

4. Conclusions

In conclusion, data supports that guaraná has antioxidant effects on NO metabolism, mainly in situations where increases NO levels occur. This biological effect may be a possible explanation for the lower prevalence of diseases in the elderly who habitually consume guaraná, and it may also serve to explain why the elderly who have not consumed this food are more frequently obese and report metabolic syndromes (Costa Krewer et al., 2011).

Conflict of interest

The authors declare that there are no conflicts of interest.

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