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Hypercholesterolemia and Ecto-enzymes of Purinergic System: Effects of *Paullinia cupana*

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Hypercholesterolemia is a metabolic disorder characterized by high levels of low-density lipoprotein and blood cholesterol, causing inflammatory lesion. Purinergic signaling modulates the inflammatory and immune responses through adenine nucleotides and nucleoside. Guaraná has hypocholesterolemic and antiinflammatory properties. Considering that there are few studies demonstrating the effects of guaraná powder on the metabolism of adenine nucleotides, we investigated its effects on the activity of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-adenosine deaminase activity in lymphocytes of rats with diet-induced hypercholesterolemia. The rats were divided into hypercholesterolemic and normal diet groups. Each group was subdivided by treatment: saline, guaraná powder 12.5, 25, or 50 mg/kg/day and caffeine concentration equivalent to highest dose of guaraná, fed orally for 30 days. An increase in adenosine triphosphate hydrolysis was observed in the lymphocytes of rats with hypercholesterolemia and treated with 25 or 50 mg/kg/day when compared with the other groups. The hypercholesterolemic group treated with the highest concentration of guaraná powder showed decreased ecto-adenosine deaminase activity compared with the normal diet groups. Guaraná was able to reduce the total cholesterol and low-density lipoprotein cholesterol to basal levels in hypercholesterolemic rats. High concentrations of guaraná associated with a hypercholesterolemic diet are likely to have contributed to the reduction of the inflammatory process.
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Keywords: ecto-enzymes; guaraná; hypercholesterolemia; lymphocytes; purinergic system.

INTRODUCTION

Hypercholesterolemia is a metabolic disorder characterized by high levels of serum, low-density lipoprotein (LDL), and blood cholesterol (Otunola *et al*., 2010). Hypercholesterolemia indicates a strong risk factor for the development of ischemic heart diseases. These include angina, myocardial infarction, and atherosclerosis (Tailor and Granger, 2003) and all chronic inflammatory conditions, resulting in a series of highly specific molecular and cellular responses characterized by the presence of lipid abnormalities, endothelial dysfunction, inflammation, and accumulation of platelets on the artery walls (Rocha and Lybby, 2009).

In atherosclerosis, LDL becomes entrapped in the subendothelial space and is subject to oxidative modification. Oxidized LDL stimulates the migration of monocytes and foam cell formation. Once formed, oxidized LDL results in injury or endothelial dysfunction (Riwanto and Landmesser, 2013). This activates T lymphocytes to proliferate and secrete proinflammatory cytokines that cause greater activation of macrophages, vascular activation, and inflammation (Charakida *et al*., 2009). In hypercholesterolemic patients, the influx of these cells is preceded by the extracellular deposition of amorphous and membranous lipids through the internalization of oxidized LDL by macrophages. This promotes the activation of T lymphocytes and the secretion of cytokines (Torzewski and Bhakdi, 2013).

The purinergic signaling system plays an essential role in modulating the inflammatory and immune responses by extracellular biomolecules such as adenine nucleotides [adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP)] and their derived nucleoside adenosine (Ralevic and Burnstock, 2003). Evidence indicates that high extracellular ATP levels act through specific cell surface receptors as a proinflammatory agent that potentiates the release of proinflammatory cytokines from activated lymphocytes (Bours *et al*., 2006). However, low extracellular ATP levels play an additional role as a negative modulator of immunity (Di Virgilio *et al*., 2009). ADP is an important signaling molecule of platelet regulation, activation, and recruitment (Hechler and Gachet, 2011). Generated from the hydrolysis of ATP, ADP has no defined role in lymphocytes (Di Virgilio *et al*., 2001). Adenosine is

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formed from the ATP precursor in the intracellular and extracellular spaces, playing an important role as an endogenous antiinflammatory (Kinsey *et al.*, 2012) and immunosuppressive agent (Yegutkin, 2008).

Levels of extracellular ATP, ADP, AMP, and adenosine are dynamically controlled during inflammation by the action of enzymes expressed in immune cells (Bours *et al.*, 2006). E-NTPDase (CD39) is the membrane-bound enzyme involved in the breakdown of ATP and ADP to AMP, which is sequentially hydrolyzed by E-5′-nucleotidase to adenosine (Zimmermann *et al.*, 2007; Yegutkin, 2008). Furthermore, ecto-adenosine deaminase activity (E-ADA) is another important enzyme that catalyzes the irreversible deamination of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively, therefore contributing to the removal of adenosine from the extracellular compartment (Latini and Pedata, 2001).

In addition, previous experimental investigations have suggested that guaraná (*Paullinia cupana*) has positive effects on lipid metabolism because increased metabolism of adipose tissue in rats was observed with increasing lipolysis because of the activation of adenosine A1 receptors (Lima *et al.*, 2005) as well as body weight loss (Boozer *et al.*, 2001). Guaraná is a rich source of caffeine, which stimulates the metabolism and may enhance thermogenesis, acting as an ergogenic agent and assisting in the degradation of lipids and atherosclerosis prevention (Shimada *et al.*, 2004). All these positive effects contribute to the reduction of risk factors for cardiovascular diseases. It also presents high concentration of polyphenols, such as tannins, flavonoids, and catechins, (Leite *et al.*, 2011) substances with antioxidant action (Razzaghi-Asl *et al.*, 2013). In addition, guaraná presents several other properties, such as hypotensive, hypocholesterolemic, and antiinflammatory activities (Krewer *et al.*, 2011).

Taking into account that hypercholesterolemia is a condition associated with endothelial dysfunction and inflammatory disorders, there is a clinical interest to investigate the therapeutic action of compounds with antiinflammatory properties, such as guaraná. Considering that there are only a few studies demonstrating the effects of guaraná on the metabolism of adenine nucleotides, it is relevant to investigate its effects on the activity of E-NTPDase and E-ADA in lymphocytes, as well as on biochemical parameters of rats with induced hypercholesterolemia.

MATERIALS AND METHODS

Chemicals. The substrates adenosine 5′-triphosphate disodium salt (ATP), adenosine 5′-diphosphate sodium salt (ADP), adenosine, bovine serum albumin, Trizma base, HEPES, Coomassie Brilliant Blue G, and Caffeine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Powdered *P. cupana* seed was produced and supplied by EMBRAPA (Brazilian Corporation of Agricultural Research). The detailed description and determination of the main bioactive compounds present in guaraná used in this study were reported by Bittencourt *et al.* (2013). The main xanthines and catechins present in guaraná powder were analyzed by chromatography, and results showed caffeine = 3.754 mg/g, theobromine = 2.065 mg/g, total catechins = 1.330 mg/g, and condensed tannins = 6.747 mg/g. All chemicals used in this experiment had analytical grade and highest purity.

Animals. Adult male Wistar rats (200–250 g) obtained from the Central Animal House of the Federal University of Santa Maria were used in this experiment. The animals were housed with three/four per cage on a 12/12 h light cycle at a temperature of 20–23°C with free access to water. All animal procedures were approved by the Ethics Committee on Animal Experiments of the Federal University of Santa Maria (protocol number: 102-2012).

Diet. A normal diet was fed according to the Nutrient Requirements of Laboratory Animals (1995), while the hypercholesterolemic diet described by Gutierres *et al.* (2012) was fed with some modification, 38% additional animal fat and decreasing sugar by 18.5%. According to this study, the normal concentration of fat in a diet for rats must lie between 20% and 30% of the total content of macronutrients (carbohydrate, fat, and protein).

The animals were fed for 1 month with normal diet and hypercholesterolemic diet to induce hypercholesterolemia. After induction, they continued receiving the same diet for another month during which the experimental protocol was performed (described later). To certify the induction of hypercholesterolemia, the levels of total cholesterol and its fractions were assessed in a sample of euthanized animals. After determining hypercholesterolemia, the other animals continued with their respective treatments. The measurement of diet given per day per rat was 10% of body weight in accordance with Harkness and Wagner (1993).

Experimental protocol. The rats used in this study were randomly divided into ten groups: N + S (normal diet + saline), N + 12.5 (normal diet + guaraná 12.5 mg/kg), N + 25 (normal diet + guaraná 25 mg/kg), N + 50 (normal diet + guaraná 50 mg/kg), N + C (normal diet + caffeine 0.2 mg/kg), H + S (hypercholesterolemic diet + saline), H + 12.5 (hypercholesterolemic diet + guaraná 12.5 mg/kg), H + 25 (hypercholesterolemic diet + guaraná 25 mg/kg), H + 50 (hypercholesterolemic diet + guaraná 50 mg/kg), and H + C (hypercholesterolemic diet + caffeine 0.2 mg/kg). Guaraná powder and caffeine were fed by gavage once a day, 7 days a week, for a period of 1 month. It was diluted in saline (0.9% NaCl) at doses of 12.5, 25, and 50 mg/kg/day and fed after the detection of hypercholesterolemia, as well as caffeine. These doses of guaraná were based on the study of Otobone *et al.* (2005). The caffeine concentration is equivalent to the highest dose of guaraná.

To better view the effects of the experimental animal model, the equivalent dose of guaraná powder and caffeine, between rats and humans, was calculated according to Reigner and Blesch (2002). Equation 1 was used to transform milligram per kilogram into milligram per square meter to determine animal surface area:

$$\mathrm{BSA}\left(\mathrm{m}^2\right) = 1.85(\mathrm{W}/70)^{2/3}, \qquad (1)$$

where BSA is the body surface area and W is the body weight in kilograms. As human, body surface

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was considered the average value ($1.8\,m^2$) to a human 70 kg. Substituting into the equation, one rat (0.250 kg) consists of one body area $0.0432\,m^2$, there converting milligram per kilogram to milligram per square meter. Next, the dose in milligram per square meter was multiplied by human surface area to find the human dose. For doses of 12.5, 25, and 50 mg/kg of guaraná powder, equivalent doses to human of 520.83, 1041.66, and 2083.34 mg were obtained, respectively. For dose of 0.2 mg/kg of caffeine, equivalent dose to human was 8.33 mg.

Separation of blood serum. Rats were anesthetized with isoflurane, and blood was collected with cardiac puncture. Blood samples were collected in tubes with no anticoagulant, and after clot formation, they were centrifuged at 1400 g for 15 min at room temperature. The resultant serum samples were aliquoted in microtubes and kept on ice for subsequent measurements.

Biochemical parameters. Serum levels of glucose, total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and albumin were evaluated in a semi-automatic analyzer (TP Analyzer Plus®, Thermoplate) using commercial kits (Labtest® Diagnóstica S.A.). Tests were carried out in duplicate. The low-density lipoprotein cholesterol (LDL-C) level was calculated with the formula of Friedewald *et al.* (1972).

Isolation of lymphocytes from blood. Rats were anesthetized with isoflurane, and blood was collected by cardiac puncture. Blood was collected with 7.2 mg dipotassium EDTA as anticoagulant, and lymphocyte-rich mononuclear cells were isolated and separated using a Ficoll–Histopaque gradient density as previously described by Böyum (1968). The percentage of lymphocytes exceeded 93%, as previously described by Jaques *et al.* (2011). The integrity of the lymphocyte preparation was confirmed when the lactate dehydrogenase activity was determined in intact and disrupted lymphocytes using the kinetic method of the Labquest apparatus (Diagnostics Gold Analyzer). The procedure was repeated before and after the incubation period. The protocol was carried out according to manufacturer instructions. Triton X-100 (1%, final concentration) was used to disrupt the lymphocyte preparation. The enzymatic activity is expressed as units per liter, with one unit (1U) corresponding to 1 μmol of NADH formed per minute per liter.

Protein determination. Protein was measured by the Coomassie Blue method according to Bradford (1976) using serum albumin as standard.

Ecto-NTPDase activity. After lymphocyte isolation, E-NTPDase activity was determined as previously described by Leal *et al.* (2005). The reaction was initiated by the addition of substrate (ATP or ADP) at a final concentration of 2.0 mM. The released inorganic phosphate (Pi) was assayed by a method previously described by Chan *et al.* (1986). All samples were run in triplicate, and the specific activity is reported as nanomole of Pi released per minute per milligram of protein.

Ecto-adenosine deaminase activity. Ecto-adenosine deaminase activity was spectrophotometrically measured in lymphocytes by the method of Giusti and Galanti (1984), which is based on direct measurement of the ammonia produced when E-ADA acts in excess of adenosine. All the experiments were performed in triplicate, and the values were expressed in unit per milligram of protein for E-ADA activity. One unit (1U) of E-ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

Statistical analysis. The statistical analysis was performed using the Student's *t*-test to demonstrate the induction of hypercholesterolemia and the other data before treatment with guaraná. Two-way analysis of variance Newman–Keuls multiple comparison test was used to evaluate the data after treatment with guaraná. $p < 0.05$ was considered to represent a significant difference. All data were expressed as mean ± standard error of the mean.

RESULTS

Hypercholesterolemia induction

To confirm hypercholesterolemia induction, glucose, total cholesterol, triglycerides, HDL-C, LDL-C, albumin levels, and ALT, AST, and ALP activity were analyzed. A significant increase (83.13%, $p < 0.001$; $n = 7$ and 122.59%, $p < 0.01$; $n = 7$) in total cholesterol and LDL-C, respectively, was observed in hypercholesterolemic rats when compared with controls. These data confirm the induction of hypercholesterolemia after 1 month of

Table 1. Biochemical parameters after 1 month of supply with hypercholesterolemic or normal diet

Parameters	Control rats	Hypercholesterolemic rats
Glucose	135.5 ± 17.91	145.30 ± 6.17
TC	64.60 ± 2.30	118.30 ± 2.30**
Triglycerides	76.84 ± 8.48	91.21 ± 11.41
HDL-C	71.48 ± 7.31	50.36 ± 5.28
LDL-C	29.04 ± 3.30	64.64 ± 10.46**
AST	121.5 ± 9.45	121.30 ± 6.89
ALT	24.40 ± 3.76	40.50 ± 7.85
ALP	74.49 ± 10.76	113.60 ± 22.77
Albumin	2.70 ± 0.10	2.73 ± 0.04

Glucose (mg/dL); TC, total cholesterol (mg/dL); triglycerides (mg/dL); HDL-C, high-density lipoprotein cholesterol (U/L); LDL-C, low-density lipoprotein cholesterol (mg/dL); AST, aspartate transaminase (U/L); ALT, alanine transaminase (U/L); ALP, alkaline phosphatase (U/L); albumin (g/dL).
The results were analyzed using Student's *t*-test and expressed as mean ± standard error of the mean.
**The value is significantly different from control group ($p < 0.01$, $n = 7$).

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hypercholesterolemic diet. Glucose, triglycerides, HDL-C, ALT, AST, ALP, and albumin measurements showed no significant difference in hypercholesterolemic rats when compared with controls, as shown in Table 1.

Food consumption and body weight

Results obtained for the average daily food intake of animals fed with hypercholesterolemic or normal diet, untreated and treated with guaraná, and their body weights after 2 months are presented in Tables 2 and 3, respectively. As it can be observed, the average daily food intake was decreased by 39.47% in the hypercholesterolemic groups when compared with normal groups ($p < 0.001$; $n = 7$). However, the body weights and body weight gain showed no significant difference between hypercholesterolemic and normal diet groups before and after treatment.

Table 2. Mean daily food consumption of the animals fed with hypercholesterolemic or normal diet and/or treated with guaraná (*Paullinia cupana*)

Groups	Food intake/day (g)
N + S	21.46 ± 0.71
N + 12.5	19.61 ± 0.63
N + 25	21.31 ± 0.71
N + 50	23.07 ± 0.74
N + C	22.58 ± 0.70
H + S	12.99 ± 0.40***
H + 12.5	12.75 ± 0.33***
H + 25	13.61 ± 0.41***
H + 50	12.93 ± 0.44***
H + C	12.54 ± 0.42***

N + S, normal food + saline; N + 12.5, normal food + guaraná 12.5 mg/kg; N + 25, normal food + guaraná 25 mg/kg; N + 50, normal food + guaraná 50 mg/kg; N + C, normal food + caffeine 0.2 mg/kg; H + S, hypercholesterolemic food + saline; H + 12.5, hypercholesterolemic food + guaraná 12.5 mg/kg; H + 25, hypercholesterolemic food + guaraná 25 mg/kg; H + 50, hypercholesterolemic food + guaraná 50 mg/kg; H + C, hypercholesterolemic food + caffeine 0.2 mg/kg.
The results were analyzed using two-way analysis of variance Newman–Keuls multiple comparison test and expressed as mean ± standard error of the mean.
***The value is significantly different from normal diet plus saline group ($p < 0.001$, $n = 7$).

Biochemical parameters after treatment with guaraná

Determinations of glucose, total cholesterol, triglycerides, HDL-C, LDL-C, AST, ALT, ALP, and albumin from both normal and hypercholesterolemic rats that either received or did not receive the oral administration of guaraná are shown in Table 4. A significant increase of total cholesterol (72.71%, $p < 0.001$; $n = 7$) and LDL-C (159.41%, $p < 0.001$; $n = 7$) was observed only in the H + S group when compared with all the other groups.

Activities of hepatic enzymes AST and ALP in serum did not differ between hypercholesterolemic and normal diet groups after treatment. However, the activity of ALT in serum increased significantly (82.06%) in the hypercholesterolemic group when compared with the normal diet group ($p < 0.001$; $n = 7$). Other biochemical parameters such as glucose, HDL-C, and albumin in serum did not differ for hypercholesterolemic and normal diet groups after treatment.

Cellular integrity

The lactate dehydrogenase activity measurement showed that approximately 5% of lymphocytes of both groups were disrupted, indicating that the preparation was predominantly intact after the isolation procedure (data not shown).

Table 3. Body weight and body weight gain of the animals fed with hypercholesterolemic or normal diet and/or treated with guaraná (*Paullinia cupana*)

	Weight (g)			
	At onset of experiment	Before guaraná treatment	After guaraná treatment	Body weight gain
N + S	311.5 ± 14.15	393.4 ± 11.64	397.6 ± 14.22	86.06 ± 20.11
N + 12.5	281.7 ± 10.92	368.9 ± 9.65	389.1 ± 8.82	107.4 ± 14.56
N + 25	300.1 ± 12.19	380.6 ± 13.33	409.1 ± 18.32	109.0 ± 21.27
N + 50	313.1 ± 16.28	409.0 ± 15.02	419.1 ± 14.15	106.0 ± 21.89
N + C	301.8 ± 13.39	380.7 ± 10.02	402.3 ± 12.21	100.5 ± 15.82
H + S	295.8 ± 14.55	375.9 ± 13.63	409.0 ± 11.07	113.3 ± 18.71
H + 12.5	308.6 ± 9.17	370.3 ± 11.47	399.0 ± 13.71	90.38 ± 16.12
H + 25	308.5 ± 9.05	379.6 ± 11.36	393.2 ± 9.59	84.70 ± 13.78
H + 50	315.9 ± 12.17	391.8 ± 11.07	400.0 ± 10.04	84.13 ± 16.58
H + C	305.6 ± 11.12	378.1 ± 12.02	391.4 ± 11.23	85.8 ± 13.21

N + S, normal food + saline; N + 12.5, normal food + guaraná 12.5 mg/kg; N + 25, normal food + guaraná 25 mg/kg; N + 50, normal food + guaraná 50 mg/kg; N + C, normal food + caffeine 0.2 mg/kg; H + S, hypercholesterolemic food + saline; H + 12.5, hypercholesterolemic food + guaraná 12.5 mg/kg; H + 25, hypercholesterolemic food + guaraná 25 mg/kg; H + 50, hypercholesterolemic food + guaraná 50 mg/kg; H + C, hypercholesterolemic food + caffeine 0.2 mg/kg.
The results were analyzed using two-way analysis of variance Newman–Keuls multiple comparison test and expressed as mean ± standard mean of the error ($p > 0.05$, $n = 7$).

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Table 4. Biochemical parameters of the animals fed with hypercholesterolemic or normal diet and/or treated with guaraná (*Paullinia cupana*)

Groups	N + S	N + 12.5	N + 25	N + 50	N + C	H + S	H + 12.5	H + 25	H + 50	H + C
Glucose	146.3 ± 0.88	141.7 ± 15.49	135.4 ± 11.84	123.8 ± 9.53	136.8 ± 8.25	137.7 ± 12.11	128.6 ± 8.96	125.0 ± 16.26	138.2 ± 8.85	135.2 ± 11.85
TC	79.21 ± 4.53	81.95 ± 4.59	86.72 ± 2.18	72.32 ± 6.37	76.56 ± 3.65	136.8 ± 12.95***	68.64 ± 3.47	75.91 ± 7.82	82.35 ± 8.81	132.4 ± 9.59***
Tri	64.23 ± 8.97	71.86 ± 5.92	66.56 ± 15.94	76.60 ± 6.49	68.73 ± 7.78	64.18 ± 5.97	64.84 ± 13.34	56.79 ± 10.44	47.49 ± 6.84	62.31 ± 8.21
HDL-C	62.33 ± 3.52	63.38 ± 3.93	67.63 ± 2.10	56.43 ± 4.65	61.34 ± 2.34	59.86 ± 2.99	58.14 ± 5.29	63.80 ± 6.29	61.33 ± 7.04	62.40 ± 4.38
LDL-C	26.73 ± 5.43	13.32 ± 1.07	15.84 ± 4.01	20.22 ± 0.03	17.93 ± 2.02	69.34 ± 7.18***	29.30 ± 0.30	17.95 ± 0.57	18.29 ± 2.39	64.96 ± 5.23***
AST	144.0 ± 5.53	142.1 ± 9.59	168.5 ± 4.44	174.6 ± 5.93	157.8 ± 3.98	151.7 ± 7.67	146.2 ± 10.93	161.6 ± 22.79	166.5 ± 20.94	168.4 ± 15.68
ALT	29.71 ± 1.28	30.90 ± 1.86	30.32 ± 1.63	28.91 ± 3.10	29.59 ± 1.26	54.09 ± 6.90***	44.60 ± 2.57***	44.90 ± 3.93***	44.91 ± 6.18***	51.42 ± 2.43***
ALP	76.85 ± 3.14	76.92 ± 7.87	72.76 ± 8.66	65.97 ± 7.77	75.58 ± 6.25	107.4 ± 11.23	89.00 ± 15.10	89.49 ± 8.58	83.25 ± 12.37	92.17 ± 6.43
Albumin	2.54 ± 0.17	2.75 ± 0.15	2.95 ± 0.08	2.63 ± 0.16	2.68 ± 0.12	2.62 ± 0.12	2.50 ± 0.23	2.76 ± 0.13	2.53 ± 0.12	2.63 ± 0.11

N + S, normal food + saline; N + 12.5, normal food + guaraná 12.5 mg/kg; N + 25, normal food + guaraná 25 mg/kg; N + 50, normal food + guaraná 50 mg/kg; N + C, normal food + caffeine 0.2 mg/kg; H + S, hypercholesterolemic food + saline; H + 12.5, hypercholesterolemic food + guaraná 12.5 mg/kg; H + 25, hypercholesterolemic food + guaraná 25 mg/kg; H + 50, hypercholesterolemic food + guaraná 50 mg/kg; H + C, hypercholesterolemic food + caffeine 0.2 mg/kg; glucose (mg/dL); TC, total cholesterol (mg/dL); Tri, triglycerides (mg/dL); HDL-C, high-density lipoprotein cholesterol (mg/dL); LDL-C, low-density lipoprotein cholesterol (mg/dL); AST, aspartate transaminase (U/L); ALT, alanine transaminase (U/L); ALP, alkaline phosphatase (U/L); albumin (g/dL).

The results were analyzed using two-way analysis of variance Newman–Keuls multiple comparison test and expressed as mean ± standard error of the mean.

***The value is significantly different from normal diet plus saline group ($p < 0.001$, $n = 7$).

Ecto-NTPDase activity

Figure 1 shows the effect of oral administration of guaraná and caffeine on ATP and ADP hydrolysis by E-NTPDase activity in lymphocytes of rats submitted to an experimental model of hypercholesterolemia. As it can be seen in Fig. 1A, the hydrolysis of ATP was increased by 53.56% and 77.34% in H + 25 and H + 50 groups, respectively, when compared with the control (N + S).

In addition, results obtained for E-NTPDase activity in lymphocytes with ADP as substrate are shown in Fig. 1B, where ADP hydrolysis was similar for all groups.

Ecto-adenosine deaminase activity

Results obtained for adenosine deamination by E-ADA activity in lymphocytes are shown in Fig. 2. As it can be observed, E-ADA activity was significantly decreased (35.13%, 39.86%, 43.83%, 69.69%, and 35.64% in H + S, H + 12.5, H + 25, H + 50, and H + C groups, respectively) when compared with the control (N + S). Moreover, the H + 50 group presented a decrease of 46.73% in lymphocyte ADA activity when compared with the H + S group.

DISCUSSION

Hypercholesterolemia is widely accepted as one of the major risk factors for the development of ischemic heart diseases and atherosclerosis (Tailor and Granger, 2003). At the beginning of the atherosclerotic process, LDL-C acts as a carrier of cholesterol in blood circulation, playing an important role in atherogenesis if it undergoes oxidative modification by endothelial cells, vascular smooth muscle, or macrophages within the arterial wall (Lusis, 2000).

Changes in the activity of ecto-enzymes, such as E-NTPDase, were observed in some diseases such as multiple sclerosis (Spanevello *et al*., 2010), ischemic heart disease (Bagatini *et al*., 2011), and lung cancer (Zanini *et al*., 2012), indicating that this enzyme could be involved in the pathogenesis of many diseases, including hypercholesterolemia (Duarte *et al*., 2007). Guaraná has antiinflammatory, hypotensive, and hypocholesterolemic properties (Krewer *et al*., 2011). Literature data show that a high-fat diet is associated with cardiovascular disease, endothelial dysfunction, and inflammation. In line with these findings, the present study investigated the effects of treatment with guaraná powder and caffeine, as one of its principal components, on the activities of purinergic system enzymes in lymphocytes and biochemical parameters in hypercholesterolemic rats.

At first, hypercholesterolemia was induced in animals by supplying a high-fat diet and then confirmed through the measurement of increased serum levels of total cholesterol and LDL-C, in accordance with Otunola *et al*. (2010). After that, the ability of guaraná powder and caffeine to reverse this process was analyzed. After 30 days of treatment with guaraná, hypercholesterolemic rats presented total cholesterol and LDL-C levels that had returned to baseline levels for all doses tested. This result corroborates with data

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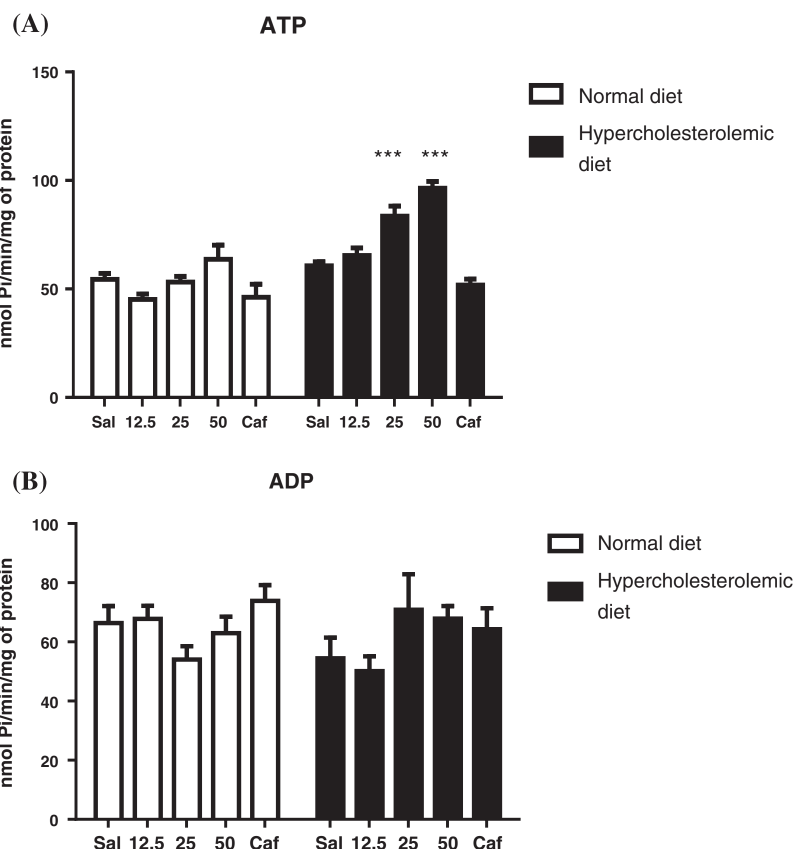

Figure 1. E-NTPDase activity with adenosine triphosphate (ATP) (A) and adenosine diphosphate (ADP) (B) as substrate in lymphocytes of rats submitted to an experimental model of hypercholesterolemia treated with guaraná (*Paullinia cupana*) at doses of 12.5, 25, and 50 mg/kg/day or caffeine 0.2 mg/kg/day for a period of 30 days. The results were analyzed using two-way analysis of variance Newman–Keuls multiple comparison test and expressed as mean ± standard error of the mean. ***The value is significantly different from normal diet plus saline group ($p < 0.001$, $n = 7$).

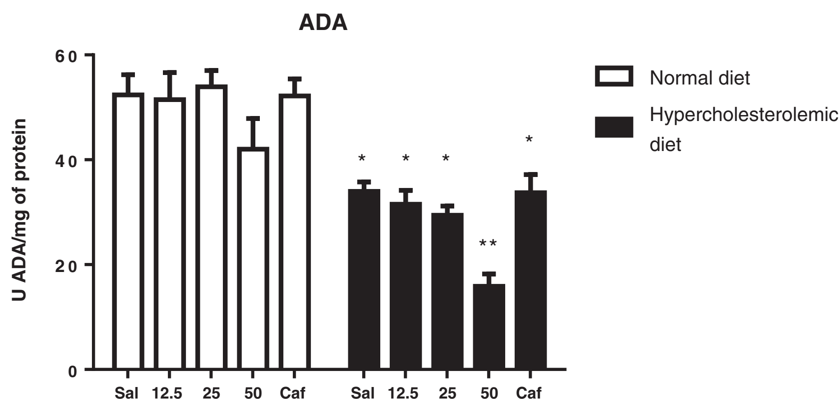

Figure 2. Ecto-adenosine deaminase activity (ADA) in lymphocytes of rats submitted to an experimental model of hypercholesterolemia treated with guaraná (*Paullinia cupana*) at doses of 12.5, 25, and 50 mg/kg/day or caffeine 0.2 mg/kg/day for a period of 30 days. The results were analyzed using two-way analysis of variance Newman–Keuls multiple comparison test and expressed as mean ± standard error of the mean. *The value is significantly different from normal diet plus saline group ($p < 0.05$, $n = 7$), and **the value is significantly different from normal diet plus saline group ($p < 0.01$, $n = 7$).

from the literature, which demonstrate the action of guaraná powder in lowering cholesterol and LDL-C levels in humans (Krewer *et al.*, 2011). Caffeine in the tested dose was not able to reduce these levels. This result disagrees with data obtained in hypercholesterolemic rats treated with caffeine 400 mg/kg, where LDL and total cholesterol levels are decreased (Xu *et al.*, 2015). This is justified, possibly because the concentration used is much lower than that found in the literature.

Based on an average daily food intake of animals fed with the hypercholesterolemic or normal diets, both untreated and treated with guarana or caffeine, and body weight measurements after 2 months, we observed

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that the hypercholesterolemic groups intake the least amount of food, but their body weights were not altered when compared with the normal diet groups. This may be due to the higher energy value of the hypercholesterolemic diet when compared with the normal diet (Moura *et al*., 2012).

In relation to aminotransferase activity, we found increased ALT activity in all of the hypercholesterolemic groups, while AST activity was not altered. Nevertheless, approximately 80% of AST in hepatocytes appears to be located in the mitochondria, whereas ALT is thought to be predominantly non-mitochondrial and has been indicated in 'mild' hepatocellular injury, in which the hepatocytes are plasmatic but the mitochondrial membrane is not damaged (Jiang *et al*., 2014).

Previous studies show that the hypercholesterolemic state generates an inflammatory process, because of the presence of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α (Pereira *et al*., 2013). Taking this into account, this study suggests that the hypercholesterolemic diet increases the levels of LDL-C and total cholesterol in the serum of rats, which could generate an inflammatory process.

Literature data indicate that adenine nucleotides and adenosine nucleoside are important modulators of atherosclerosis and immune response (Seye *et al*., 2002). In fact, the inflammatory process generated by hypercholesterolemia may lead to significant increase in the levels of purine and pyrimidine nucleotides on the sites involved, probably contributing to the enhancement of inflammatory reaction (Miyara and Sakaguchi, 2007). Extracellular ATP can act as damage-associated molecular patterns given that it is normally confined to intracellular sites but can be released at high local levels following cell lysis, infection, or via regulated efflux. ATP released into the extracellular space can modulate the immune response through its capacity to bind and activate multiple nucleotide receptor family members (Kvist *et al*., 2014).

The results of this present study show an increase in the rate of ATP hydrolysis in rats supplied with a hypercholesterolemic diet and treated with guaraná at doses of 25 and 50 mg/kg/day, when compared with all the other groups. This may be explained by an increased degradation of ATP as a compensatory organic response because the ATP is released into the extracellular environment at high concentrations, activating the proinflammatory purinergic P2X7 receptors and, thereby, stimulating the Th1 immune response and contributing to tissue damage and inflammation (Di Virgilio, 1995).

For this reason, we suggest that guarana powder, at doses of 25 and 50 mg/kg, modulates the E-NTPDase activity to decrease the excess extracellular ATP possibly caused by a hypercholesterolemic state and thereby decreases the activation of the P2X7 receptor by increasing the rate of ATP hydrolysis. Extracellular ATP at low concentrations has an affinity for the P2Y receptor subtype on the surface of lymphocytes that stimulates the Th2 immune response, leading to the production of antiinflammatory cytokines (Bours *et al*., 2006).

In this study, no alteration in the E-NTPDase activity in lymphocytes of rats with hypercholesterolemia, untreated or treated with caffeine, was observed. This result disagrees with data obtained in platelets from humans with hypercholesterolemia, where the activity and expression of E-NTPDase are increased (Duarte *et al*., 2007). We believe that alterations in E-NTPDase activity were not observed in our study possibly because of the short time of high-fat dietary supplementation. Caffeine dose equivalent to the highest concentration of guarana did not show alteration in the enzyme activity, possibly because the concentration used is much lower than that found in the literature.

On the other hand, when hypercholesterolemic rats were treated with guaraná or caffeine, neither guaraná or caffeine nor the hypercholesterolemia, *per se* or in combination, promoted significant alterations in E-NTPDase activity using ADP as a substrate, keeping this nucleotide at basal levels. Because of the fact that these enzymes act in cascade, extracellular ATP may be associated with the nucleoside adenosine (Yegutkin, 2008). For this reason, E-NTPDase and E-ADA control the extracellular concentration of ATP and adenosine (Bours *et al*., 2006). The immunosuppressive actions of adenosine are triggered by activation of four receptor subtypes: A1, A2A, A2B, and A3. These receptors are transmembrane glycoproteins coupled to protein G (Sitkovsky, 2003).

As large amounts of ATP are released from injured cells, the rapid hydrolysis of ATP and ADP (by E-NTPDase) favors the production of adenosine, which possesses antiinflammatory and analgesic properties (Fredholm *et al*., 1994). In this study, the hypercholesterolemia reduced the E-ADA activity when compared with the normal diet group, possibly as a compensatory mechanism to increase levels of extracellular adenosine, resulting in antiinflammatory action. These data corroborate with studies showing that the inflammatory activity in a hypercholesterolemic state depends on the balance between Th1 proinflammatory response and Th2 antiinflammatory response (Nilsson *et al*., 2009). Adenosine in the extracellular environment can bind to specific receptors expressed on the cell surface, exerting its antiinflammatory function including inhibition of proinflammatory cytokine release, inhibition of adhesion of immune cells, and inhibition of a proliferation of T cells through the activation of A2A receptors (Gessi *et al*., 2007).

The results of this present work have shown that greater reduction in E-ADA activity was observed in the hypercholesterolemic group treated with the highest concentration of guaraná powder when compared with the other groups. These results suggest that, in addition to the balance of the inflammatory response because of the hypercholesterolemic state, guaraná possibly acts by modulating the activity of this enzyme.

In conclusion, our data demonstrate that guaraná powder was able to modulate the enzymatic activity when associated with a hypercholesterolemic state. Caffeine, in the same concentration present in the highest dose of guaraná, was not able to promote this modulation, bringing scientific prominence to this work. Increased E-NTPDase activity and decreased E-ADA activity in lymphocytes of hypercholesterolemic rats treated with guaraná contribute to the reduction of the inflammatory process by decreasing ATP levels and increasing extracellular adenosine. It was also observed that guaraná decreases total cholesterol and LDL-C to basal levels. Although requiring further study, guaraná powder could be a promising compound to be used as a complementary therapy for the benefit of patients with hypercholesterolemia.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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