Leaves and seeds of *Syzygium cumini* extracts produce significant attenuation of 2,2 azobis-2-amidinopropane dihydrochloride-induced toxicity via modulation of ectoenzymes and antioxidant activities

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ABSTRACT

Syzygium cumini has shown many pharmacologic properties including anti-inflammatory. Here, we analyzed the antioxidant activity of leaves and seeds from aqueous extracts of *S. cumini* (LASc, SASc, respectively) as well as their effect in a 2,2 azobis-2-amidinopropane dihydrochloride (AAPH) induced model of oxidative damage in human lymphocytes, *in vitro*. It was evaluated the ferric reducing power (FRAP), scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide, hydrogen peroxide radicals and thiol peroxidase-like activity from both extracts. Lymphocytes obtained from blood samples of healthy volunteers were incubated with LASc and SASc (50, 100 and 500 µg/mL) and gallic acid (GA) (100µM) followed or not by incubation with AAPH (1mM). Afterwards, enzymatic activities, oxidative parameters and cytotoxicity were evaluated. This study indicates that extracts of *S.cumini* (i) exhibiting antioxidant activity; (ii) prevented the increase of ectonucleotidase and adenosine deaminase (ADA) activities; (iii) prevented inhibition of acetylcholinesterase (AChE) activity; (iv) protected P-SH groups, decreased lipoperoxidation and NO production; (v) improved the cellular viability in AAPH-induced model of lymphocytes. *S. cumini* extract*s* modulate ectonucleotidase, ADA, AChE activities maintaining levels normal of ATP, adenosine (Ado) and acetylcholine (ACh) and markedly attenuate inflammatory process. In conclusion, *S.cumini* has protective and immunomodulatory effects on AAPH-induced damage in lymphocytes, *in vitro*.

INTRODUCTION

Natural products have been accepted as an important tool to obtain bioactive compounds with therapeutically effective medicines in the prevention and/or treatment of a number of diseases, but the mechanisms involved in it have not been completely understood yet. *Syzygium cumini* (L.) Skeels from Myrtaceae family is a worldwide medicinal popular plant, commonly known as jambolan or jamun, possesses a wide range of medicinal properties such as anti-inflammatory, antioxidant, hypoglycemic and anti-microbial activities, as well as hepatoprotective and cardioprotective effects which have been

attributed to the presence of bioactive molecules in different parts of the plant (Ayyanar and Subash-Babu, 2013; Cargnelutti *et al.,* 2015). In Brazil, the leaves and seeds are the parts of the plant most used by the population in folk medicine (Vizzoto and Pereira, 2008).

The *S. cumini*'s leaves and seeds have been reported to be rich in phenolic compounds, which contribute for the scavenging of free radicals with significant antioxidant activity and protective effect on antioxidant enzymes (Ravi *et al.,* 2004a; Ravi *et al.,* 2004b; Bajpai *et al.,* 2005). Among this class of compounds, gallic acid stands out, a phenolic acid, widely present in extracts from different parts of *S. cumini* (De Bona *et al.,* 2011; Cargnelutti *et al*, 2015) which have already reported antioxidant, anti-inflammatory and antimicrobial activities (Kim *et al.,* 2006; Padma *et al.,* 2011; Borges *et al.,* 2013).

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Oxidative stress is characterized by the imbalance between oxidants and antioxidants in favor of the oxidants which are formed as a result of normal cellular aerobic metabolism but during pathophysiological conditions can be produced at an elevated rate (Roberts *et al.,* 2010). Accumulating evidence suggests that high levels of ROS may cause oxidative changes to DNA, protein and lipids and additionally are involved in several diseases pathogenesis of, including changes in the immune system (Brigelius-Flohe and Flohe, 2011; Ray *et al*., 2012). The immune system changes are critical for its normal functioning, mainly for lymphocytes, the most important cells involved in this system (Delves *et al.,* 2006). In general, exposure to high levels of free radicals has a negative impact on this system and is linked to a loss of T-cell homeostasis and antioxidant defense, lymphocytes activation, cell damage and limited viability (Cope, 2002; Perez de Castro *et al.,* 2004). The inflammatory process involves activation of the immune system and interaction between several components (Rock *et al.,* 2005), including molecules of a purinergic and cholinergic system that contribute to their responses (Bours *et al.,* 2006; Kawashima and Fujii, 2003). Thus, one of the ways in which lymphocyte function may be regulated is by a member of the ectonucleoside triphosphate phosphohydrolase (E-NTPDase) family characterized as marker of lymphocyte activation, the NTPDase (EC 3.6.2.5, CD39). The NTPDase is an ectoenzyme that hydrolyzes extracellular nucleotide (preferably ATP and ADP) (Leal *et al.,* 2005) and the sequential hydrolysis of nucleotide generates ADP, AMP, and adenosine (Ado) that are involved in the regulation of immune defenses (Burch, 2006). The extracellular ATP acts as a pro-inflammatory molecule and stimulates the release of pro-inflammatory cytokines from activated lymphocytes (Langston *et al.,* 2003).

Conversely, the breakdown product of ATP, the Ado, exhibits potent anti-inflammatory activity and has the ability to alter the lymphocyte activation and cytokine production (Gessi *et al.,* 2007). The adenosine levels are regulated by the adenosine deaminase activity (ADA, E.C 3.5.4.4) an enzyme from the purine metabolism that catalyzes the deamination of Ado and deoxyadenosine into inosine and deoxyinosine, respectively (Spychala, 2000). Two ADA's different isoenzymes designated as ADA1 and ADA2 have been reported in mammals. ADA1 isoenzyme is found in higher activity in lymphocytes and monocytes, whereas ADA2 is the predominant isoenzyme in the serum (Ungerer *et al.,* 1992). In fact, in the inflammatory process ADA activity is increased (Schetinger *et al.,* 2007). Also, in this context, nitric oxide (NO), in addition to being a highly reactive radical is also a pro-inflammatory mediator that contributes to the development of a variety of inflammatory diseases (Kim *et al.,* 2010). In addition, another important enzyme in the immune regulation is the acetylcholinesterase (AChE). The lymphocytes possess a complete cholinergic system consisting of acetylcholine (ACh), muscarinic and nicotinic receptors, choline acetyltransferase, and AChE (EC 3.1.1.7). This enzyme is a hydrolase that catalyzes the rapid hydrolysis of ACh (Gabrovska *et al.,* 2008) that is the main circulating molecule involved in

cholinergic functions that may regulate several physiological functions, including immune modulation (Tayebati *et al.,* 2002).

In previous studies of our group we observed that leaves extracts from *S. cumini* protect the lymphocytes against *in vitro* oxidative stress (De Bona *et al.,* 2015) and different parts of *S. cumini* demonstrated beneficial effects against diabetes mellitus *in vitro and in vivo* (De Bona *et al.,* 2014; Bitencourt *et al.,* 2015). Therefore, aqueous seeds and leaves extracts of *S. cumini* are potential candidates to analyze its effects. As a result of the importance of this plant in traditional Brazilian medicine and in order to extend these observations to further investigate its antioxidant properties using 2,2 azobis-2-amidinopropane dihydrochloride (AAPH), a water-soluble azo compound that is thermally decomposed to generate peroxyl radicals at a constant rate, this study was carried out to evaluate the antioxidant activity as well as the effects of LASc and SASc on the cytotoxicity and enzymatic activities on AAPH exposure in human lymphocytes by scientifically validated *in vitro* techniques.

MATERIALS AND METHODS

Chemicals

2,2-diphenyl-1-   picrylhydrazyl   (DPPH), Acetylthiocholine iodide,5'5 dithio-bis-2-nitrobenzoic acid (DTNB), 2,2azobis-2-amidinopropane dihydrochloride (AAPH) 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), the substrates ATP, ADP and adenosine, Gallic acid and Ficoll–Histopaque[TM] plus were obtained from Sigma Chemical Co (St. Louis, MO). All other reagents used in the experiments were of analytical grade and highest purity.

Plant material and aqueous extracts of *S. cumini* preparation

S. cumini seeds and leaves were collected (29°43'22''S and 53°43'47''W) in Santa Maria, Rio Grande do Sul, Brazil) and identified by the Laboratory of Botanic and Pharmacognosy of the Federal University of Santa Maria (voucher number SMDB 14.001). For the preparation of the aqueous seeds extract (SASc), eighty grams of the seed powder were extracted with 400 mL of distilled water during 1h under reflux (Prince *et al.,* 1998). For the preparation of the aqueous leaves extract (LASc), they were dried in a greenhouse with air circulation at 40 ºC for approximately 48h. Then, they were ground in a knife mill and submitted to extraction until exhaustion (Eidi *et al.,* 2006). The presence of 11 antioxidant compounds in both extracts, namely gallic, caffeic and ellagic acids, chlorogenic, catechin, quercetin, epicatechin, quercitrin, isoquercitrin, kaempferol and rutin was investigated by high-performance liquid chromatography with diode array detector (HPLC-DAD). The chemical characterization of the extracts was determined in recent studies as described in Bitencourt *et al*. (2016) and Cargnelutti *et al*. (2015). The HPLC fingerprinting of both extracts revealed that the gallic acid (GA) is the major component of the extracts. Then, the antioxidant activities of the LASc and SASc were compared with the antioxidant activity of GA used as a positive standard in the *in vitro* tests.

Antioxidant activities of extracts

Free radical scavenging ability by the use of a stable DPPH radical

DPPH free radical scavenging activity was determined by reported earlier (Joshi *et al.,* 2011). LASc, SASc and GA at the concentrations of 25, 50, 100, 200, 400 and 800 μg/mL, were added to an ethanolic DPPH solution (6 mM). The mixture was incubated at room temperature for 30 min and determined the absorbance at 518 nm. The percent inhibition was calculated. The dark color of the DPPH radical solution becomes lighter when it is incubated with an antioxidant and the decrease in color indicated the scavenger potential of the antioxidant compounds in relation to ability to donate a hydrogen or electron.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was used to measure the reducing antioxidant power of LASc, SASc and GA at the concentrations 25, 50, 100, 200, 400 e 800μg/mL (Benzie and Strain, 1996). The results were presented as μM Fe^{2+}/mL of extract.

Thiol Peroxidase-Like Activity of Extracts

The catalytic effect of LASc, SASc and GA on the reduction of hydrogen peroxide (H_2O_2) by reduced glutathione (GSH) was assessed using the rate of GSH oxidation. Different concentrations of extracts were incubated in the medium containing GSH (1mM) with and without H_2O_2 (0.3 mM). At 120 min, aliquots of the reaction mixture (200 μL) were checked for the amount SH groups according to Ellman (1959) and the values were expressed in percentage of control (Souza *et al.,* 2009).

Nitric Oxide-Scavenging Assay

The scavenging effect of aqueous extracts on nitric oxide (NO) was measured according to the method of Sreejayan and Rao (1997). For the assay, sodium nitroprusside (10mM), was mixed with LASc, SASc and GA (50, 100, 200, 400 and 800μg/mL), incubated for 150 min and then mixed with 0.5 mL of Griess reagent and measured at 546 nm. In the control, sample extract was substituted by PBS. The capability of scavenging NO was calculated using the following equation: Scavenging effect (%) = [1 − (A sample/A control)] × 100.

Hydrogen peroxide-Scavenging Assay

The ability of the extracts and GA to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.* (1989). LASc, SASc (5, 10, 25, 50 and 100 μg/mL) were added to a hydrogen peroxide solution (0.6 mL, 40mM). The absorbance of hydrogen peroxide at 230 nm was determined 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging of the extracts were calculated: % Scavenged [H_2O_2] = [(A_0− A_1)/A_0] x 100, where A_0 was the control absorbance and A_1 the absorbance in the presence of extract or standard.

Sample collection and isolation of lymphocytes from human blood

Lymphocytes were isolated from blood taken from healthy volunteers, after informed consent was obtained, and collected in Vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ) containing lithium–heparin or EDTA (only NTPDase activity) as anticoagulant and separated on Ficoll–HistopaqueTM plus density gradients, according to the technique described by Böyum (1968). Cell number and viability were determined by Trypan blue exclusion. More than 95% of the cells were found to be viable. The final cell suspensions were performed in phosphate-buffered saline (PBS, pH 7.4) or in saline solution (for NTPDase activity) and 3x106 cells/mL were used for each analysis. All experiments were performed at least three times using lymphocytes obtained at different occasions and a total of eight samples were used for every incubation performed. The study was approved by the Ethics Committee of the Federal University of Santa Maria (experimental protocol 54224316.1.0000.5346.).

In vitro experimental design

Primarily, lymphocytes suspensions were preincubated with different concentrations of LASc, SASc (50, 100 and 500 μg/mL) and GA (100 μM) at 37 ºC for 30 min, followed by incubation in oxidative stress condition with AAPH (1 mM) at 37 ºC for 2h. Also, were observed the *per se* effect of LASc, SASc and GA. The choice of the concentrations of the extracts and GA for the experiments was made based on the development of a concentration curve (data not shown) and also earlier studies (De Bona *et al.,* 2015). All the procedures for enzymatic assays, oxidative stress parameters and viability assays were the same as described below.

Enzymatic assays in lymphocytes suspensions

NTPDase activity

After obtaining of lymphocytes suspension, NTPDase activity was determined according to the method of Leal *et al.* (2005). Protein content of all samples were adjusted to 0.1-0.2 mg/mL and 20 μL of intact cells suspended in saline solution were added to a reaction medium containing 5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose, and 50 mM Tris-HCl buffer, pH 8.0 at a final volume of 200 μL and preincubated for 10 min at 37ºC. The reaction was started by the addition of ATP or ADP substrate at a final concentration of 2 mM and incubation proceeded for 70 min. After the incubation time, 200 μL of 10% trichloroacetic acid (TCA) was added to the medium to stop the reaction.

The samples were chilled on ice for 10 min before testing for the release of inorganic phosphate (Pi) as described by Chan *et al.* (1986) using malachite green as colorimetric reagent and KH_2PO_4 as standard. Controls with the addition of the enzyme preparation (intact lymphocytes) after the addition of TCA were used to correct for non-enzymatic hydrolysis of the substrate. The results were expressed as nmol Pi released/min/mg of protein.

ADA activity

ADA activity

ADA activity in lymphocytes suspension was estimated spectrophotometrically using the method as described by Giusti and Gakis (1984) which is based on the direct measurement of the formation of ammonia produced when ADA acts in excess of adenosine. The activities of total ADA in the presence and absence of erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) were measured. Lymphocytes were treated with 100 μM EHNA, a potent ADA1 inhibitor. ADA1 activities were calculated by subtracting the activity of ADA2 from that of total ADA. The protein content used for the assay was adjusted to between 0.7 and 0.9 mg/mL. The results were expressed as units per liter (U/L).

AChE activity

AChE activity

AChE activity in lymphocytes was performed by the colorimetric method described by Ellman *et al*. (1961). Two hundred microliters of intact cells was added to a reaction mixture composed of 1 mM acetylthiocholine (AcSCh), 0.1 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), and 0.1 M phosphate buffer (pH 8.0). Immediately, the absorbance was read on a spectrophotometer at 412 nm, before and after incubation for 30 min at 27ºC. The results are expressed as μmol AcSCh/h/mg of protein. Physostigmine, a classic inhibitor, in the concentration of 100μg/mL was used as a positive control of inhibition enzymatic activity (Hassan *et al.,* 2013).

Oxidative stress parameters

*Thiobarbituric acid reactive substance**s (TBARS)**

Lipoperoxidation was estimated in lymphocytes by measurement of TBARS according to the method of Ohkawa *et al*. (1979). Lymphocytes were mixed with 8% SDS solution and incubated for 5 min at room temperature. Glacial acetic acid (20%) was added to the reaction mixture and incubated for 2–5 min at room temperature. Finally, 0.8% TBA solution was added to the reaction mixture followed by incubation for 1 h in a boiling water bath. The reaction mixture was cooled, centrifuged and the absorbance of the supernatant was measured spectro-photometrically at 532 nm. The results were expressed in nmol MDA/mL.

Protein thiol (P-SH) groups

P-SH groups in lymphocyte suspensions were determined by the method of Boyne and Ellman (1972), modified by Jacques-Silva *et al*. (2001), which consisted of the reduction of DTNB in 0.3 M phosphate buffer (pH 7.0) measured at 37 ℃ and at 412 nm. A standard curve using glutathione was constructed in order to calculate P-SH group. The results were expressed as nmol P-SH/mg protein.

Oxide nitric (NO) measurement

NO was determined indirectly by quantifying in lymphocytes. NO was measured by the modified Griess method using the Cobas Mira™ automated analyzer as described by Tatsch *et al*. (2011). The results were expressed as μM/L.

Cell viability

Tetrazolium salt method (MTT)

The viability assay was performed by the colorimetric MTT method. 1.2 mM of MTT was added to the cell suspension following by incubation at 25 °C for 60 min. The formazan product obtained during the incubation was solubilized in dimethyl sulfoxide (DMSO) and quantified by spectrophotometer at 560 nm (Moretto *et al.,* 2007). Only viable cells are able to reduce MTT, therefore, each value obtained is proportional to the percentage of cell viability in relation to control considered as 100 % of viability.

Protein determination

Protein concentration was measured according to the method of Lowry *et al*. (1951) using serum albumin as standard.

Statistical Analysis

The analyses were performed using STATISTICA for Windows, version 6.0 (StatSoft. Inc., Tulsa, OK, USA). All data were analyzed using one-way ANOVA, followed by Duncan's multiple range test. All data were expressed as mean ± standard error of the mean (SEM). A value of $p<0.05$ was considered statistically significant for all analyses.

RESULTS

Antioxidant activities of extracts

DPPH

Radical scavenging activity of *S. cumini* extracts and GA against stable DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) demonstrated high radical scavenging activity ($p < 0.001$) and LASc (400μg/mL) had a greater effect than SASc (Fig. 1a).

Hydrogen peroxide-Scavenging Assay

The free radical scavenging activity of LASc, SASc and GA was also examined by using hydrogen peroxide free radical (Fig. 1b). LASc, at lower concentrations (5 and 10 μg/mL), exhibited greater H_2O_2 scavenging activity than SASc. But, this scavenging ability became similar at high concentrations (25, 50 and 100 μg/mL). Even, at the elevated concentration tested (100 μg/mL), the LASc and SASc scavenging capacity presented equally to GA.

FRAP

The GA and the extracts' reducing ability to convert ferric ions to ferrous showed that LASc and SASc at 25, 50, 100 and 200 μg/mL had a similar reduction power in the FRAP assay (Fig. 1c) and LASc (400μg/mL) presented higher reducing power than SASc ($p<0.001$).
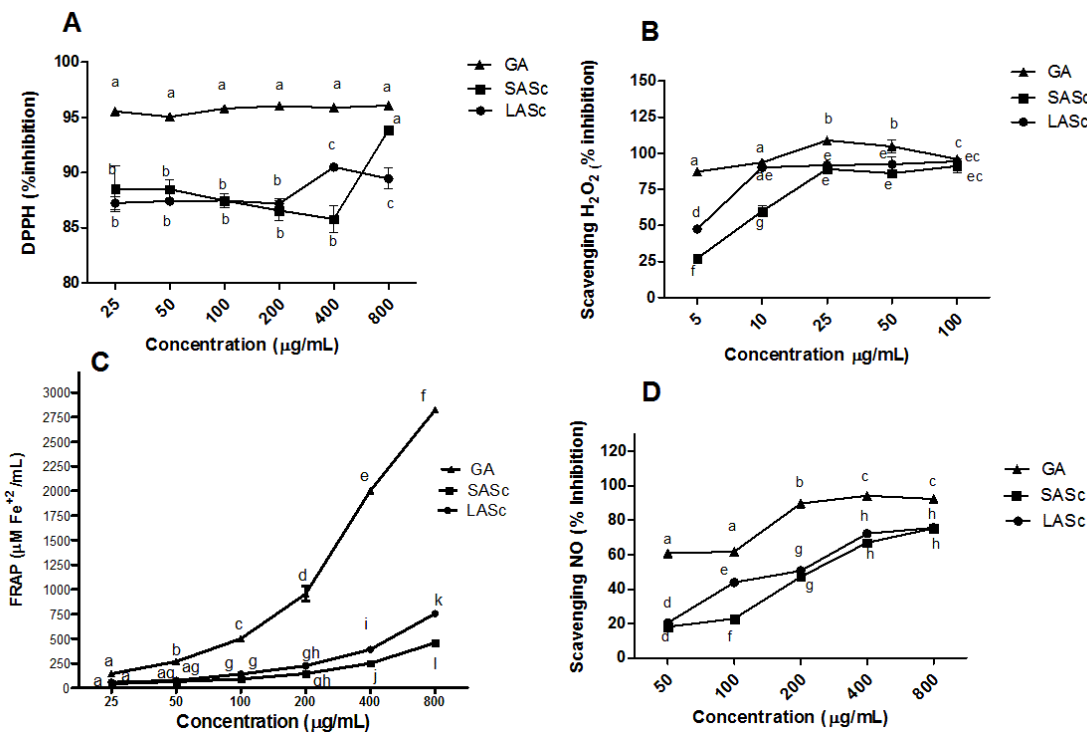
Fig. 1: Antioxidant activities of LASc, SASc and GA.
(a) DPPH assay (b) Hydrogen peroxide-Scavenging activity, (c) Ferric reducing antioxidant power, (d) NO scavenging.
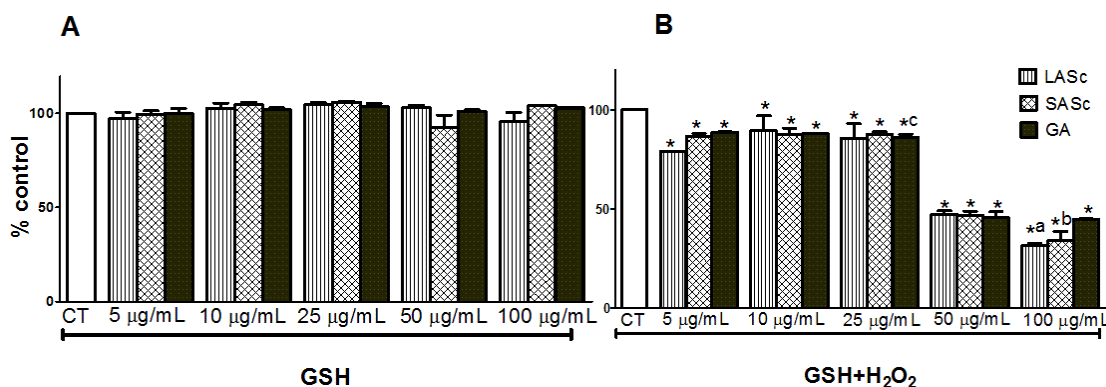

Fig2: (a) Thiol peroxidase-like activity of LASc, SASc and GA in the absence or (**b**) presence of H_2O_2.
.Each value is expressed as mean±SEM, n=3. Data were analyzed by one-way ANOVA followed by Duncan's multiple comparison post hoc test.[*] denotes $p<0.001$ when compared to controls (CT); [a] denotes $p<0.0001$ different from LASc 5, 10, 25 and 50 µg/mL; [b] denotes $p<0.0001$ when compared to SASc 5,10, 25 and 50 µg/mL and [c]denotes difference from GA 50 and 100 µg/mL. LASc, aqueous leaves extract of S. cumini; SASc, aqueous seeds extract of S. cumini; GA, gallic acid.

Nitric Oxide-Scavenging Assay

The NO radical scavenging property of the extracts and GA is represented in the Fig.1d. The extracts had a similar scavenging ability in the most of the concentrations tested, except at the concentration 100 µg/mL, where LASc exhibited higher ($p<0.001$) scavenging potential than SASc.

Thiol Peroxidase-Like Activity

Thiol peroxidase-like activities from both extracts and GA are shown in fig. 2a. None of the extracts were able to promote the consumption of GSH in the absence of H_2O_2 in the

concentrations tested. However, we can observe a decrease in SH groups when LASc, SASc and GA were incubated in the presence of H_2O at all concentrations tested (Fig. 2b).

In vitro effect of LASc, SASc and GA in AAPH-induced oxidative stress in the peripheral lymphocytes
Enzymatic activities in the peripheral lymphocytes
NTPDase, ADA activities assays

Fig. 3 and 4 shows that the in vitro effect of NTPDase on ATP (a) and ADP (b) hydrolysis and ADA activities after AAPH exposure in lymphocytes was significantly increased compared

with the control (p<0.01). LASc and SASc at all concentrations tested prevented the increase in NTPDase activity (ADP and ATP substrates) induced by AAPH (p<0.0001; p<0.01; p<0.0001). Similarly, results were found concerning ADA activity for both the extracts tested.

Otherwise, LASc and SASc (500 µg/mL), *per se,* reduced ATP and ADP hydrolysis compared to control (p<0.0001). The *per se* effect was also observed in relation to ADA activity (Fig. 4a) with LASc (100 and 500 µg/mL, p<0.05), SASc (50 and 100 µg/mL, p<0.0001; p<0.05).

Furthermore, when only the lymphocytic suspension was incubated with EHNA (100 µM) a markedly reduction of ADA1

activity *in vitro* was observed (77, 83%). This reveals that the isoform 1 is predominant in the tested lymphocytes, and therefore, this isoform is the main responsible for the enzymatic activity observed in all assays (Fig. 4b).

AChE activity

LASc (50 and 100µg/mL) and SASc (50 µg/mL) were able to prevent the strong inhibition of enzymatic activity promoted by AAPH (p<0.05). The seeds and leaves effects were different as shown in Fig. 5, according to the concentration analyzed. However, LASc at 500 µg/mL exhibited a synergistic effect with AAPH.
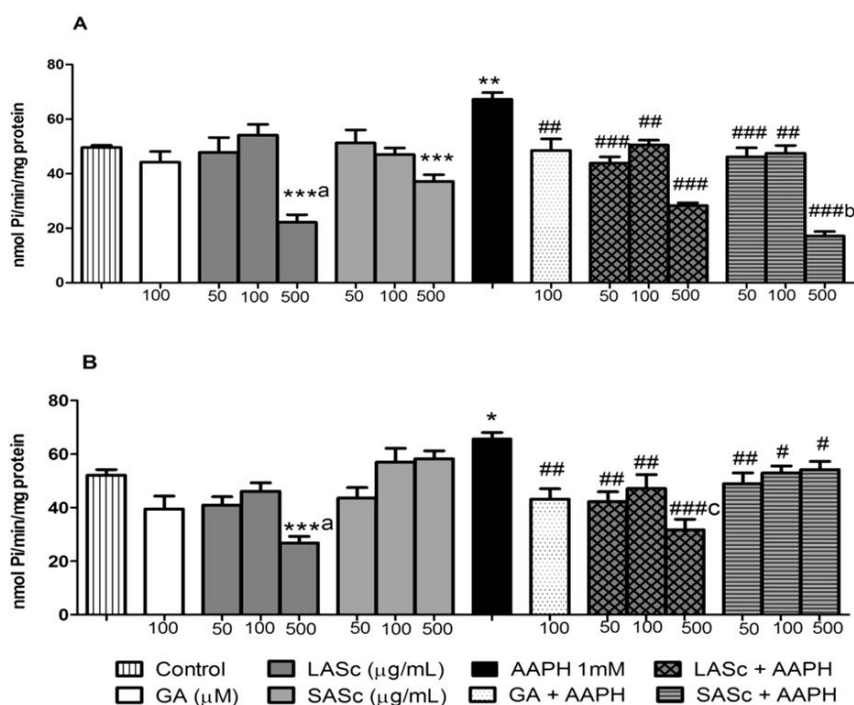

Fig. 3: Effect of GA, LASc and SASc in (a) ATP and (b) ADP hydrolysis in lymphocytes suspensions after 2 h of incubation with AAPH, *in vitro*. Data are reported as mean ± S.E.M. (n=8). Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple range test(* p<0.05, ** p<0.01 and *** p<0.0001 indicates difference from control group; # p<0.05, ## p<0.01 and ###p<0.0001 compared to AAPH 1mM; ᵃp<0.0001 compared to SASc 500 µg/mL; ᵇp<0.05 compared to LASc 500 µg/mL+ AAPH; ᶜp<0.01 compared to SASc 500 µg/mL+AAPH.
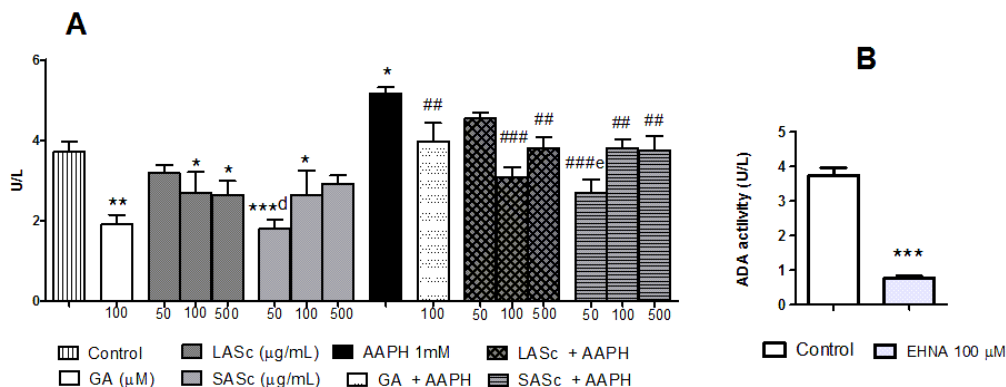

Fig. 4: Effect of GA, LASc and SASc in (a) ADA activities in lymphocytes suspensions after 2 h of incubation with AAPH, *in vitro* (b) Lymphocytes suspensions after incubation with EHNA. Data are reported as mean ± S.E.M. (n=8). Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple range test(* p<0.05, ** p<0.01 and *** p<0.0001 indicates difference from control group; ## p<0.01 and ###p<0.0001 compared to AAPH 1mM; ᵈ p<0.0001 compared to LASc 50 µg/mL and ᵉp<0.01 compared to LASc 50 µg/mL+AAPH).
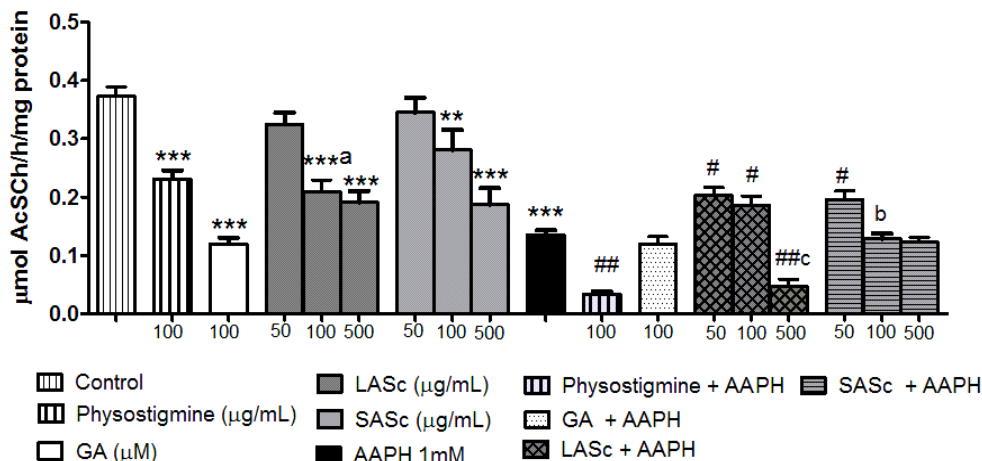
Fig.5: In vitro effect of GA, LASc and SASc on AChE activity in lymphocytes suspensions after 2 h of incubation with AAPH. Data are reported as mean ± S.E.M. (n=8). Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple range test (** $p<0.01$ and *** $p<0.0001$ compared to control group; # $p<0.05$, ## $p<0.01$ compared to AAPH 1mM; [a] $p<0.01$ compared to SASc 100 μg/mL; [b] $p<0.05$ compared to LASc 100 μg/mL+ AAPH; [c] $p<0.01$ compared to SASc 500 μg/mL + AAPH.
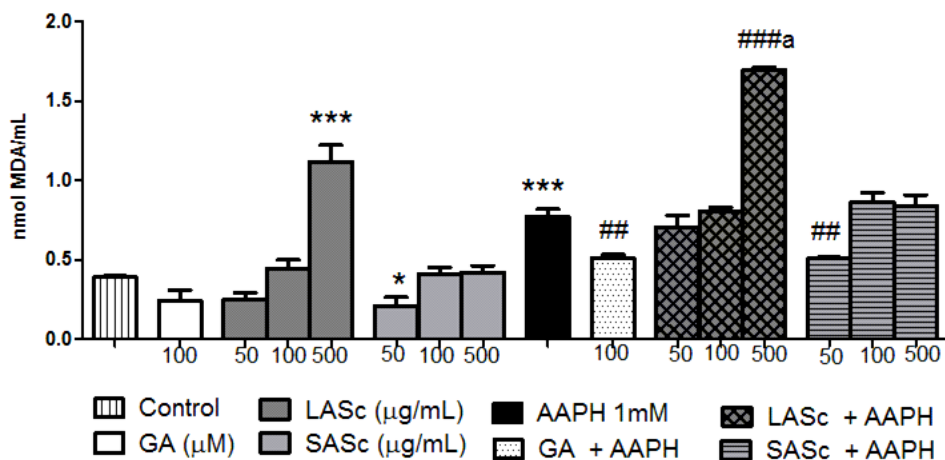

Fig. 6: In vitro effect of LASc, SASc and GA on TBARS levels in lymphocytes suspensions after 2 h of incubation with AAPH. Values are expressed as mean ± SEM (n=8). Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple range test (* $p<0.05$ and *** $p<0.0001$ compared to control group; ## $p<0.01$ and ### $p<0.0001$ compared to AAPH 1mM; [a] $p<0.0001$ compared to SASc 500μg/mL + AAPH).

Oxidative stress parameters

TBARS levels

As shown in Fig. 6, the incubation of the lymphocytes in the presence of AAPH promoted a significant increase in lipoperoxidation levels compared with the control ($p<0.0001$). Only GA and the SASc at 50 μg/mL were able to protect the cells by reducing TBARS levels generated by AAPH ($p<0.01$). Unexpectedly, we observed that LASc (500 μg/mL) *per se* and in the presence of AAPH caused a lipoperoxidation increase ($p<0.0001$).

P-SH groups

As expected, a decrease in P-SH groups was observed when the lymphocytes suspensions were incubated with AAPH ($p<0.0001$, Fig. 7). LASc and SASc tested were able to protect the lymphocytes by increasing P-SH groups levels in presence of

AAPH, wherein LASc had a greater ability than SASc at all concentrations tested (Fig. 6).

NOx production

The AAPH and aqueous extracts' effects on NOx production by lymphocytes are summarized in Fig. 8 and it reveals that the LASc (50 and 100 μg/mL) and SASc at all concentrations tested protected the cells by decreasing NOx levels promoted by AAPH.

Cell viability

In vitro exposure of lymphocytic suspension to AAPH caused a marked reduction of cell viability (41%) and LASc at all concentrations tested and SASc (100 and 500 μg/mL) were able to protect cell viability. Besides, LASc revealed better effect than SASc at all concentrations (Table 1).
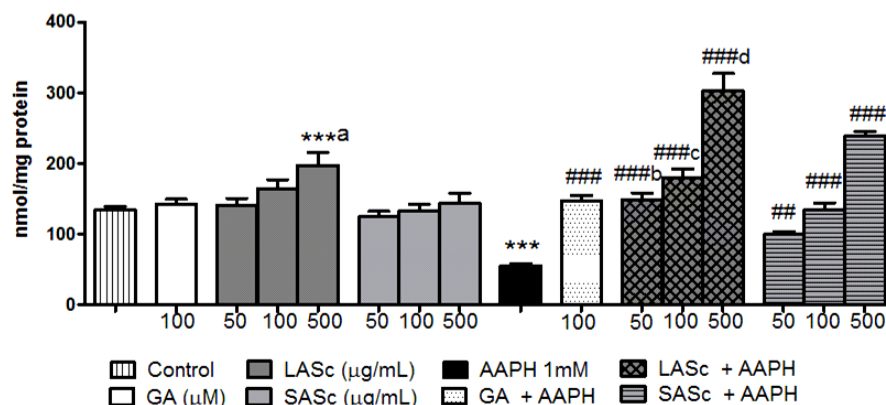
Fig. 7: *In vitro* effect of LASc, SASc and GA on P-SH levels in lymphocytes suspensions after 2 h of incubation with AAPH. Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple range test (*** $p<0.0001$ compared to control; ## $p<0.01$ and ### $p<0.0001$ compared AAPH 1mM; [a]$p<0.01$ compared to SASc 500 µg/mL; [b] $p<0.01$ compared to SASc 50+ AAPH; [c] $p<0.05$ compared to SASc 100+AAPH; [d]$p<0.0001$ compared to SASc 500+AAPH.
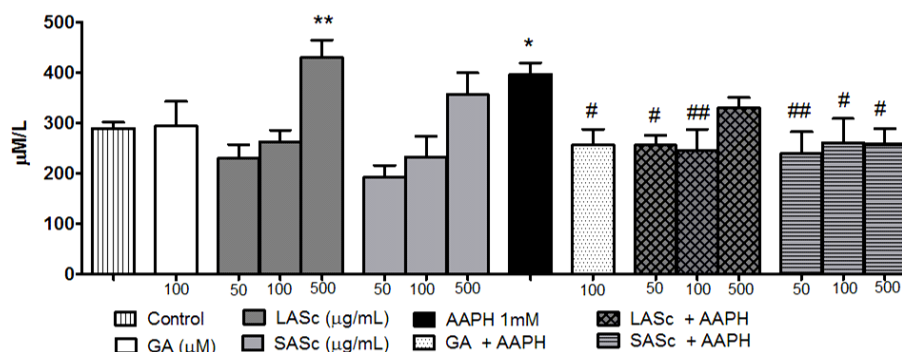

Fig. 8: *In vitro* effect of LASc, SASc and GA on NOx production in lymphocytes suspensions after 2 h of incubation with AAPH. Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple range test (* $p<0.05$; ** $p<0.01$ compared to control; # $p<0.05$; ## $p<0.01$ compared to AAPH 1mM.

Table 1: *In vitro* effect of LASc, SASc and GA on MTT assay in lymphocytes suspensions after 2 h of incubation with AAPH.

	Cell viability (%)
Control	100
GA 100 µM	153 ± 16*
LASc 50 µg/mL	143±13
LASc 100 µg/mL	224±18***[a]
LASc 500 µg/mL	353±10***[b]
SASc 50 µg/mL	120 ± 5.2
SASc 100 µg/mL	123±5.3
SASc 500 µg/mL	245±23***
AAPH 1mM	59±1.5*
GA 100 µM + AAPH	127±9.5[##]
LASc 50 µg/mL + AAPH	131±6.6[##c]
LASc 100 µg/mL + AAPH	194±20[###d]
LASc 500 µg/mL + AAPH	334 ± 13[####e]
SASc 50 µg/mL + AAPH	73 ± 4.7
SASc 100 µg/mL+ AAPH	117 ± 7.1[##]
SASc 500 µg/mL + AAPH	281 ± 8.4[###]

Table 1. Data are reported as mean±SEM (n=8). Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple range test (* $p<0.05$; ** $p<0.01$; ***$p<0.0001$ compared with control; ## $p<0.01$; ### $p<0.0001$ compared with AAPH 1 mM; [a] $p<0.0001$ compared with SASc 100 µg/mL; [b]$p<0.0001$ compared with SASc 500 µg/mL; [c] $p<0.01$ compared with SASc 50 µg/mL + AAPH; [d] $p<0.01$ compared with SASc 100 µg/mL + AAPH and [e] $p<0.05$ compared with SASc 500 µg/mL + AAPH

DISCUSSION

Different approaches have been developed to analyze anti-inflammatory and antioxidant activities of medicinal plants (Madhulika *et al.*, 2016; Mireille, 2001). In the same manner, with

the current and growing interest in oxidative damage mechanisms, azo compounds have frequently been used as convenient free radical initiators. The results of this study clearly indicate the high antioxidant activities and free radical scavenging effect of *S. cumini* extracts that are linked to their phytochemical composition

marked by the presence of polyphenolic compounds that are known to have several pharmacological activities (Ushida *et al.,* 2008).

The LASc, SASc and GA have potent antioxidant activity against *in vitro* oxidative systems*,* since they showed effectiveness as scavengers of DPPH, NO and H_2O_2, reductive ability Fe^{+3} to Fe^{+2}, thiol peroxidase-like activity that mimic the properties of glutathione peroxidase (GPx). These *in vitro* assays indicate LASc and SASc as a significant source of natural antioxidant, possibly due to their ability to scavenge the free radicals and to reduce the intracellular ROS generation which might be helpful in preventing the oxidative stress progress.

Also, the exposure of lymphocytes to AAPH was capable of increasing the activities of the ADA and NTPDases, lipoperoxidation and NO production and reduces AChE activity, P-SH and MTT levels. These results can be assigned on the ability of the hydrophilic radical generator of this azo compound to generate peroxyl radicals, thereby oxidizing lymphocytic suspension (Frei and Gaziano, 1993). Likewise, concerning ectoenzymes, AAPH exposure causes a raise in lymphocytic NTPDases (hydrolyzing ATP and ADP), increasing the release of ATP from stressed cells to extracellular space during inflammation (Virgilio *et al.,* 2009) and ADP may have been formed as consequence of inflammatory process (Bours *et al.,* 2006). Sequentially, the increase in ADA activity could be attributed to an attempt of the organism to compensate the high levels of ATP released, which may affect adenosine levels available for adenosine receptors stimulation expressed on the T-cell surface, leading to impairment of immune regulation and inflammatory process (Mandapathil *et al.,* 2010). Subsequently, the reduction in Ado levels contributes to release of pro-inflammatory cytokines and NO production (Haskó and Cronstein, 2004). Taking into account our results, they suggest that an increase of nucleotide hydrolysis and adenosine deamination in AAPH-induced can lead to inflammatory and immune alterations in lymphocytes. Moreover, it indicates that when ROS levels are generated in excess, cells redox homeostasis is disturbed affecting the redox-sensitive signal transduction cascades, leading to an unbalanced purinergic and immunologic systems and an immune cells activation (Smale, 2011). These immunobiochemical pathways may potentially be altered in the pathological state including infections, autoimmune syndromes, malignant disease, neurodegeneration and cardiovascular disorders (Schetinger *et al.,* 2007; Becker *et al.,* 2014).

Importantly in this study was that both LASc and SASc protected the lymphocytes against the NTPDase and ADA activities increase by preventing AAPH-induced toxicity that may result in an increase of ATP in order to enhance Ado levels production, which has anti-inflammatory effects. Thus, Ado becomes available to exert its anti-inflammatory function by activating receptors present on the cells surfaces, regulating the release of molecules involved in the immune system and also inhibiting the release of NO (Haskó and Cronstein, 2004). Another important aspect to be discussed here is the AAPH effect

on the cholinergic system in lymphocytes. For the first time to our knowledge, this peroxyl radicals initiator was tested in lymphocytes AChE activity causing strong inhibition which affect the acetylcholine levels acting as a compensatory mechanism in order to attenuate the intensity of the progression of inflammatory processes since ACh exhibits the anti-inflammatory effect. Notwithstanding, the pretreatment with LASc (100 μg/mL) and SASc (50μg/mL) prevented this strong inhibition reestablishing the AChE activity. This prevention would minimize the effects generated by exposure to the peroxyl radical initiator, thus, it would decrease the load on the AChE activity.

Although SASc (50μg/mL) and GA have protected lymphocytes against lipoperoxidation, LASc was not able to do it, corroborating with previous study our group (De Bona *et al.,* 2015). Nevertheless, LASc and SASc in all concentrations tested were able to protect levels of P-SH groups indicating that antioxidants compound work against ROS in order to protect the sulfhydryl groups cells from the deleterious effects of oxidative stress (Pandey and Rizvi, 2011) favoring to the proper function of lymphocytes (Knight, 2000).

Furthermore, the pretreatment with LASc and SASc improved cell viability, a cytoprotective effect, and suggested its potential application against ROS-mediated cell damage (Veigas *et al.,* 2008). In fact, AAPH easily penetrates the lymphocytes cellular membrane and accumulated inside these cells, changing the biomembrane composition, potentially leading to an interference with cell-signaling pathways and even to the loss of cell function and viability (Hu *et al.,* 2005), as it can be observed in MTT assay.

We suggest that the differential protective effect exerted by LASc and SASc in some parameters can be linked to the bioactive molecules present in them, since phytochemical characterization previously published (Cargnelutti *et al.,* 2015, Bitencourt *et al.,* 2016) revealed that GA is present in high quantities in both extracts but, LASc exhibits also high concentration of quercetin and caffeic acid. In fact, quercetin has already been able to modulate AChE activity in rats' lymphocytes orally exposed to Cadmium (Abdalla *et al.,* 2014) and an extract containing significant amounts of caffeic acid increased the viability of lymphocytes exposed to H_2O_2 (Sagrillo *et al.,* 2015) also, caffeic acid contributed to the antioxidant defense system of HepG2 cells (Razali *et al.,* 2015). Regarding SASc, which presented high concentrations of chlorogenic acid and rutin, it has recently been shown that the *Scutia buxifolia* Reissek extract with a similar composition was able to inhibit NTPDase in rats lymphocytes (Boligon *et al.,* 2015) while rutin significantly reduced ADA activity in rats induced by carrageenan (Arruda-Silva *et al.,* 2014). Moreover, chlorogenic acid brought TBARS levels in diabetic rats down to normal values (Stefanello *et al.,* 2014). The basis for these distinct effects may be attributed to their structure-dependent physicochemical interactions with biologic membranes which would explain these differential results to the extracts (Böhm *et al.,* 2012). The results of our study still deserve some considerations. When exposure *per se* and/or in the presence

of AAPH, LASc (500 μg/mL) increased lipoperoxidation. In the same way, it had a synergistic effect with AAPH that led to strong inhibition of AChE activity. These effects may be attributed to the ability of the flavonoids to act as antioxidants/prooxidants under *in vitro* system to be dependent on a number of factors such as concentration, structure, the test system (Procházková *et al.,* 2011) and also, it is thought to be directly proportional to the total number of hydroxyl groups (Cao *et al.,* 1997). This suggests that a molecule with the same chemical structure may optimize antioxidant capacity and it can also exacerbate oxidative stress and damage the cellular molecules (Heim *et al.,* 2002).

CONCLUSION

In conclusion, the results of the present investigation indicate that the exposure to AAPH caused changes in the purinergic and cholinergic system's enzymes, as well in parameters of oxidative stress that possibly contributes to decrease viability cellular and to the inflammation process in lymphocytes. Mainly, the ADA1 was affected in the model tested. Of particular importance, our findings indicated that the *S. cumini*'s leaves and seeds extract were able to generate significant effects, preventing these changes across modulation of the ectonucleotidases activities, probably preserving the ATP and Ado levels, which could lead to an anti-inflammatory status. Besides, the extracts can regulate the AChE activity leading to immune system control carried out by the lymphocytes. Such effects may be a consequence of the compounds in higher concentration present in the medicinal extract preparations investigated.

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