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Scutia buxifolia Reiss inhibit platelet aggregation and alters the activities of enzymes that hydrolyze adenine nucleotides in lymphocytes and platelets

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ABSTRACT

Platelets plays a central role in hemostatic processes and consequently are similarly involved in pathological processes, such as arterial thrombosis and atherosclerosis. In this study we investigate the effect of aqueous crude extracts of *Scutia buxifolia* on NTPDase and 5′-nucleotidase activity on platelets and lymphocytes as well as the profile of the platelet aggregation. The effect of the aqueous crude extract obtained from *S. buxifolia* leaves (SbL) and stem bark (SbS) on enzymatic activities and platelet aggregation was investigated by *in vitro* tests. The platelets and lymphocytes were exposed to aqueous extracts of *S. buxifolia* at concentrations 1–200 μg/mL in the presence of ATP, ADP, AMP as substrates. The results showed that SbS and SbL potentially inhibited the NTPDase and 5′-nucleotidase in platelets and lymphocytes. Moreover, we found that ADP-induced aggregation was only inhibited by the SbS. This data suggest the existence of compounds in *S. buxifolia* that may decrease the NTPDase and 5′-nucleotidase activity, causing peripheral alterations in adenine nucleotide levels and protection against ADP-induced platelet aggregation.

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Introduction

New research directions in the last decade have led to major developments in the uses of plant in bioscience and biomedicine (Quave et al., 2012). Medical ethnobotanical field studies can provide useful information to the allopathic medical community as they seek to reconcile existing and emerging cultural alternative medical therapies with conventional biomedicine. This is of great importance for phytopharmacovigilance and managing risk of herb–drug interactions in mainstream patients that use alternative therapies. Herbal compounds such as phenolics, polyphenols, alkaloids, quinones, terpenoids, lectines and polypeptides have been shown to be very effective alternatives to antibiotics and other synthetic compounds (Habib et al., 2010; Berger, 2011). However, there is still a lack of knowledge of the clinical efficacy and safe used of many natural products use in the alternative medicine. Many plants are used in Brazil in the form of crude extracts, infusions or plasters to treat common diseases without any scientific evidence of efficacy (Silva et al., 2009). Brazil has a great diversity of plants, which increases the chances of identification of substances with pharmacological activities (Boligon et al., 2009).

ATP, ADP, AMP, and nucleoside adenosine are important signaling molecules that mediate diverse biological and pathological processes (Ralevic and Burnstock, 2003). Nucleotides are messengers that modulate the exocrine and endocrine systems, the vasculature and hemostatic mechanisms, as well as musculoskeletal, immune and inflammatory cells (Burnstock and Knight, 2004). Their levels are controlled by a complex cell surface-located group of enzymes called ecto-nucleotidases (Zimmermann, 2006). The cascade of ecto-enzymes for this purpose is formed by NTPDase (ATP diphosphohydrolase, EC 3.6.1.5) that can hydrolyze ATP and ADP to AMP, and by 5′-nucleotidase (EC 3.1.3.5) that hydrolyzes AMP to the end product, adenosine (Schetinger et al., 2007). These ecto-enzymes may be found anchored in the plasmatic membrane of platelets and lymphocytes (Pilla et al., 1996; Koziak et al., 1999; Leal et al., 2005).

The NTPDase1, also known as CD39, had its activity was newly characterized in peripheral lymphocytes by Leal et al. (2005), and its presence has been confirmed by flow cytometry. The physiologic functions of NTPDase1 have shown its involvement in several important cellular events, such as platelet aggregation, neurotransmission, and inflammatory and immune response. The ecto-5′-nucleotidase, also known as CD73, is a GPI-anchored enzyme that represents the major enzyme responsible for the formation of extracellular adenosine from AMP and the subsequent activation of P1 adenosine receptors (Vollmayer et al., 2001; Zimmermann, 2001). Thus, these enzymes (NTPDase1 and 5′-nucleotidase) are responsible for regulating purine-mediated signaling, controlling the rate, amount and timing of nucleotide degradation, and ultimately, the nucleoside formation (Yegutkin et al., 2002; Stefan et al., 2005; Robson et al., 2006).

Scutia buxifolia Reissek belongs to the Rhamnaceae family and is popularly known as ''coronilha''. It is a native plant from South America with a dispersion area that includes Brazil (mainly Rio Grande do Sul state), Argentina and Uruguay. Infusions of the root bark are popularly used as a cardiotonic, antihypertensive and diuretic agent (Wasicky et al., 1964; Da Silva et al., 2012). In spite of the popular use, little is known about its secondary metabolites, with the exception of some cyclopeptide alkaloids and flavonoids isolated by Morel et al. (2005) and Boligon et al. (2012), respectively, which demonstrated some antimicrobial and antiviral activities (Boligon et al., 2013). Moreover, the results obtained by Boligon et al. (2009) showed that *S. buxifolia* has a great potential to prevent diseases caused by the overproduction of radicals free as well as to be used as a potential source of natural antioxidant agents.

Taking into account some aspects such as the limitation of the database on the action of the crude extract of *S. buxifolia* and the crucial role that nucleotidases plays in several physiological events through controlling nucleotides in the extracellular environment, the aims of this study were characterize by HPLC–DAD the aqueous crude extract obtained from *S. buxifolia* leaves (SbL) and stem bark (SbS); and investigate the *in vitro* effect of this extract on enzymes that hydrolyze adenine nucleotides into lymphocytes and platelets, as well as the profile of the platelet aggregation in rats.

Materials and methods

Reagents

Nucleotides, sodium azide, HEPES and Trizma base were purchased from Sigma (St. Louis, MO, USA). Ficoll-Hypaque (LymphoprepTM) was purchased from Nycomed Pharma (Oslo, Norway). All other reagents used in the experiments were of analytical grade and of the high purity.

Chemical, apparatus and general procedures

All chemical were of analytical grade. Methanol, acetic acid, gallic acid (standard compound, purity 98.0%), chlorogenic acid (≥95.0%) and caffeic acid (≥98.0%) purchased from Merck (Darmstadt, Germany). Quercetin (≥98.0%), rutin (≥95%) and kaempferol (≥97.0%) were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC–DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

Plant collection and extractions

Stem bark and leaves of *S. buxifolia* were collected in Dom Pedrito (Rio Grande do Sul State of the Brazil) in October of 2007 (coordinates 30°59′09″ S and 54°27′44″ W). A dried voucher specimen is preserved in the herbarium of the Department of Biology at University by register number SMBD 10919. Nelci Rolim Basto Zacchia identified the species (biologist master).

The parts of the plant were dried at room temperature and powdered in a knife mill. The powder of stem bark (651.52 g) and leaves (372.34 g) were macerated separately at room

temperature with ethanol 70% for a week with daily shake-up. After filtration, the two extracts were evaporated under reduced pressure to remove the ethanol. Each extract was suspended in water.

Quantification of phenolics and flavonoids compounds by HPLC–DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm × 250 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% (B) for 2 min; 25% (B) until 10 min; 40, 50, 60, 70 and 80% (B) every 10 min; following the method described by Sabir et al. (2012) with slight modifications. All the samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.031–0.250 mg/mL for kaempferol, quercetin and rutin, and 0.006–0.250 mg/mL for gallic, chlorogenic and caffeic acids. Quantification was carried out by integration of the peaks using the external standard method, at 254 nm for gallic acid, 325 nm for caffeic and chlorogenic acids, and 365 nm for quercetin, rutin and kaempferol. The flow rate was 0.8 mL/min and the injection volume was 40 μL. The chromatography peaks were confirmed by comparing their retention time and Diode-Array-UV spectra with those of the reference standards. All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves (Sabir et al., 2012). LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

Animals

Adult male Wistar rats (150–250 g) obtained from the Central Animal Facility from this University were utilized from experiments. These animals were maintained under standard diurnal conditions (12 h light, and 12 h dark), at a constant temperature of 22 ± 2 °C with free access to water and food. All animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources from University (project number 02439). The animals were submitted to anesthesia (halothane), then the blood was collected by cardiac puncture for platelet and lymphocytes preparation.

Platelet separation

Platelet-rich plasma (PRP) was prepared by the method of Pilla et al. (1996) modified by Lunkes et al. (2003). Total blood was collected into 0.120 M sodium citrate as anticoagulant and centrifuged at 160 × *g* for 10 min. After this, the PRP was centrifuged at 1400 × *g* for 30 min and washed twice with 3.5 mM HEPES buffer, pH 7.0. The platelet pellets were resuspended in HEPES buffer and used to determine enzymatic activities.

Lymphocytes preparation

The peripheral lymphocytes were isolated using Ficoll-Hypaque density gradients as described by Böyum (1968).

Enzyme assay for lymphocytes

After the isolation of mononuclear cells, NTPDase activity was determined by colorimetric assay in compliance with Leal et al. (2005). The reaction medium contained 0.5 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. Twenty microliters of intact mononuclear cells suspended on saline solution were added to the reaction medium (2–4 μg protein) and preincubated for 10 min at 37 °C. The reaction was started by the addition of substrate (ATP or ADP) at a final concentration of 2 mM and stopped with 200 μL 10% trichloroacetic acid (TCA). The samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (Pi) as described for Chan et al. (1986).

Enzymatic assay for platelets

NTPDase activity was determined by measuring the amount of inorganic phosphate released, using a colorimetric assay. Twenty microliters PRP preparation, 10–15 μg protein, were added to either the NTPDase or 5′-nucleotidase reaction mixture, and the resulting reaction mixture was preincubated for 10 min at 37 °C, until a final volume of 200 μL was reached. NTPDase activity was determined by the method of Pilla et al. (1996) in a reaction medium, containing 5 mM $CaCl_2$, 100 mM NaCl, 4 mM KCl, 6 mM glucose, and 50 mM Tris–HCl buffer, at pH 7.4. The reaction was started by the addition of ATP or ADP as substrate and its final concentration was 1 mM. 5′-nucleotidase activity for AMP hydrolysis was carried out as previously described, except that the 5 mM $CaCl_2$ was replaced by 10 mM $CaCl_2$ and 2 mM AMP was added. The incubation time for both enzymatic assays was 1 h. The NTPDase and 5′-nucleotidase reactions were stopped by the addition of 200 μL 10% TCA. The inorganic phosphate (Pi) released by ATP, ADP and AMP hydrolysis was measured by the method of Chan et al. (1986).

The extract of SbL or SbS (1; 10; 50; 100; 200 μg/mL) was added to the reaction (37 °C) at the same time of the addition of lymphocytes or platelets. All determinations were performed in triplicate. Enzymatic activities are reported in nmol Pi released/min/mg protein.

Effects of propranolol and furosemide on nucleotide hydrolysis

In vitro effects of propranolol and furosemide on NTPDase and 5′-nucleotidase activities were evaluated. Isolated platelets and lymphocytes from rats were incubated with different concentrations of these drugs in the medium reaction as previously described. All concentrations of propranolol (15, 30 and 40.5 μmol/L) and furosemide (10, 15 and 25 μmol/L) used *in vitro* represent approximately the mean plasma values of the medications.

Platelet aggregation

To evaluate the platelet aggregation, the test was performed by turbidimetric measurement with a Chrono-log optical aggregometer, with AGGRO/LINK Model 810-CA software for Windows version 5.1 (Born and Cross, 1963). The baseline value was set using platelet-rich plasma (PRP) and maximal transmission using platelet-poor plasma (PPP). Maximal aggregation was obtained stimulating platelet with ADP 10 μM.

Determination of cytotoxicity

To verify the possible effect of cytotoxicity of *S. buxifolia* on platelets and lymphocytes, we analyzed the leakage of lactate dehydrogenase (LDH) from platelets and lymphocytes of rats. After incubation with test drugs at 37 °C for 3 min, aliquots were collected and centrifuged at room temperature for 2 min at 12,000 × *g*. The level of urea was determined using commercial kit purchased from BioClin (BioClin diagnostics (I) Quibasa Química Básica Ltd., Belo Horizonte, Brazil). The extent of LDH leakage was expressed as % of total enzyme activity measured in platelets completely lysed with 10% sodium dodecyl sulphate (SDS final concentration was 0.05%).

Protein determination

Protein was measured by the Coomassie Blue method using bovine serum albumin as standard as described by Bradford (1976).

Statistical analysis

Data were analyzed by analysis of variance (one-way ANOVA). *Pos hoc* analysis was carried out by Duncan multiple range test. Results were expressed as the mean ± standard deviation (SD) and evaluated at the significance level $2\alpha = 0.05$.

Results

Chemical characterization of the extract

The HPLC analysis of the SbS (a) and SbL (b) is shown in Fig. 1. The crude extracts of *S. buxifolia* contain other minor compounds in addition to gallic acid (retention time, $t_R = 12.4$ min, peak 1), chlorogenic acid ($t_R = 23.1$ min, peak 2), caffeic acid ($t_R = 27.6$ min, peak 3), rutin ($t_R = 38.2$ min, peak 4), quercetin ($t_R = 47.5$ min, peak 5) and kaempferol ($t_R = 54.9$ min, peak 6).

The HPLC profile of the crude extracts was acquired as well the quantification of quercetin, rutin, kaempferol, gallic, chlorogenic, and caffeic acids by HPLC–DAD based on references of standard calibration curves (Table 1). Calibration curve for gallic acid: $Y = 53985x + 1020.6$ ($r = 0.9999$); chlorogenic acid: $Y = 52548x + 1082.3$ ($r = 0.9981$); caffeic acid: $Y = 87846x + 1093.7$ ($r = 0.9998$); rutin: $Y = 39861x - 1235.8$ ($r = 0.9921$); quercetin: $Y = 50833x - 4741.5$ ($r = 0.9995$) and kaempferol: $Y = 30745x - 1897.9$ ($r = 0.9996$). HPLC analysis revealed flavonoids (rutin, quercetin and kaempferol) and phenolics acids (gallic, caffeic and chlorogenic acids) are the major components of the extract. The results are similar to other studies (Boligon et al., 2009; 2012).

Cytotoxic effect of SbS and SbL on platelets and lymphocytes

Firstly, to examine the cytotoxicity of *S. buxifolia* extracts, we measured the LDH release from platelet and lymphocytes for the determination of cell lysis. None of the tested concentrations of *S. buxifolia* extracts induced LDH release. Data not showed.

Effect of SbS and SbL on NTPDase and 5′-nucleotidase activities in platelets and lymphocytes

The SbS showed higher inhibition than SbL on NTPDase and 5′-nucleotidase activity. In experiments using platelets from adult rats, ATP hydrolysis was inhibited *in vitro* by SbS at final concentrations of 10, 50, 100 and 200 μg/mL ($p = 0.0056$, $p = 0.0025$, $p = 0.0019$ and $p = 0.0028$, respectively), and by SbL at final concentrations 100 and 200 μg/mL ($p = 0.0037$ and $p = 0.0045$, respectively) when compared with the control enzyme activity (Fig. 2A). Hydrolysis of ADP (Fig. 2B) presented a similar profile when compared with ATP (Fig. 2A), however, when ADP was used as substrate, the inhibitory effect was observed in all concentrations tested.

Experimental data clearly illustrated inhibition of NTPDase (Fig. 2B) in platelets and the biphasic effect of SbS on ADP hydrolysis. Treatment with SbS at a dose of 50 μg/mL predominantly inhibited the NTPDase activity followed by a

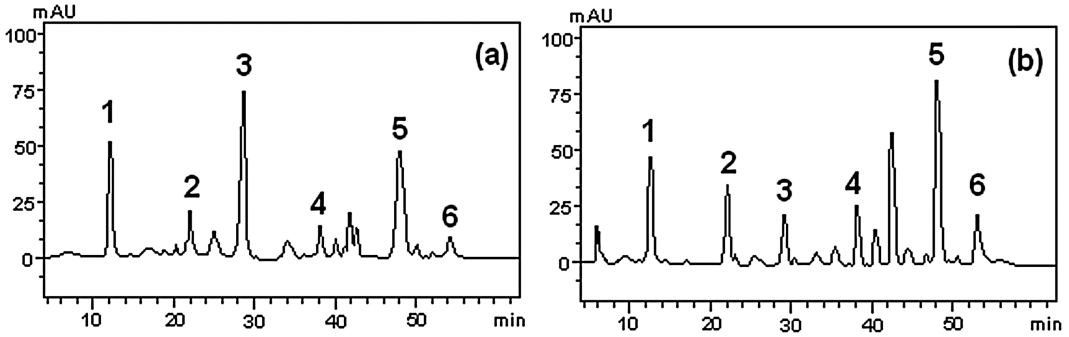

Fig. 1 – High performance liquid chromatography phenolics and flavonoid profile of crude extract from the SbS (a) and SbL (b). Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4), quercetin (peak 5) and kaempferol (peak 6).

Table 1 – Phenolics and flavonoids composition of *Scutia buxifolia* stem bark and leaves. Results are expressed as mean ± SEM of three determinations.

Compounds	*Scutia buxifolia*					
	Stem bark		Leaves		LOD	LOQ
	mg/g	Percent	mg/g	Percent	μg/mL	μg/mL
Gallic acid	41.3 ± 0.22	4.13	30.2 ± 0.09	3.02	0.034	0.112
Chlorogenic acid	19.2 ± 0.19	1.92	21.1 ± 0.27	2.11	0.015	0.049
Caffeic acid	77.5 ± 0.03	7.75	19.4 ± 0.16	1.94	0.009	0.027
Rutin	8.9 ± 0.34	0.89	20.8 ± 0.04	2.08	0.026	0.085
Quercetin	90.3 ± 0.05	9.03	107.6 ± 0.21	10.76	0.010	0.034
Kaempferol	5.4 ± 0.15	0.54	12.2 ± 0.17	1.22	0.043	0.141

small increase in the activity at the concentrations of 100 μg/mL and 200 μg/mL.

The effect of the *S. buxifolia* extract on 5′-nucleotidase (hydrolysis of AMP) is also demonstrated in Fig. 2C. The inhibition of 5′-nucleotidase was showed only with SbS at the concentrations of 100 and 200 μg/mL ($p = 0.041$ and $p = 0.035$, respectively). The experimental findings showed that SbL did not change the activity of platelet 5′-nucleotidase (Fig. 2C).

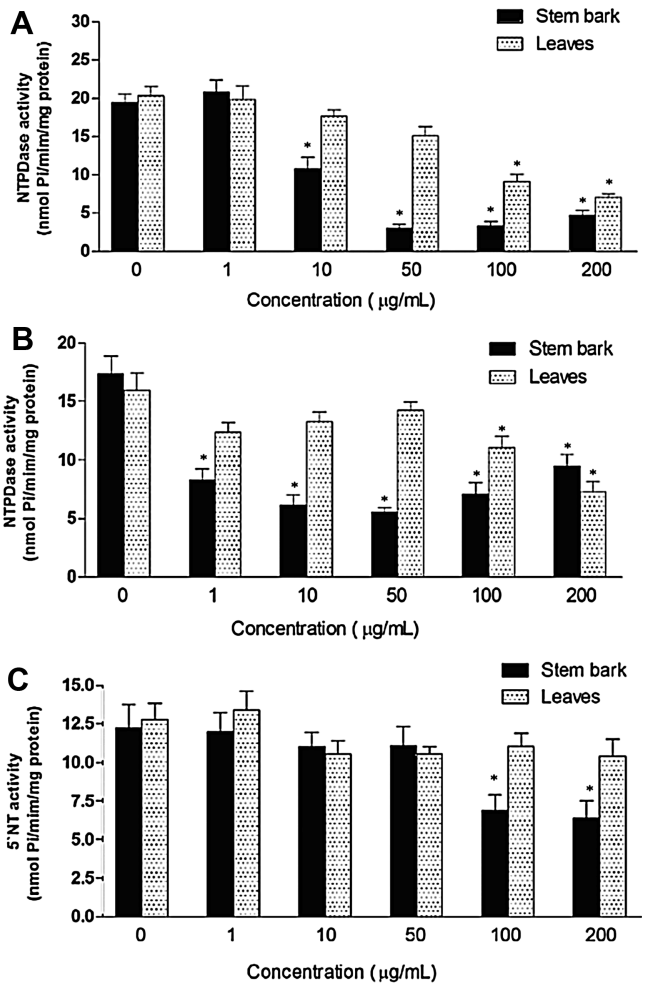

Fig. 2 – Effect of the crude extract fractions from leaves and stem bark (1–200 μg/mL) of *S. buxifolia* on NTPDase (A and B) and 5′-nucleotidase (C) activities in platelets. Data are presented as mean ± SEM. Hydrolysis is expressed as nmol Pi/min/mg protein. Data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range test. *Statistically significant versus controls.

In experiments using lymphocytes from adult rats, the SbL showed higher inhibition than SbS on NTPDase and 5′-nucleotidase activity. ATP hydrolysis was inhibited *in vitro* by SbL at final concentrations of 10 ($p = 0.0093$), 50, 100 and 200 μg/mL ($p = 0.0024$, $p = 0.0017$ and $p = 0.0029$, respectively), and SbS at 10, 50, 100 ($p = 0.0046$, $p = 0.0015$ and $p = 0.0023$, respectively) and 200 μg/mL ($p = 0.0087$), respectively, when compared with the control enzyme activity (Fig. 3A).

Hydrolysis of ADP (Fig. 3B) presented a similar profile when compared with ATP. However, when ADP was used as substrate, the inhibitory effect was observed at concentrations of 1, 10, 50, 100 and 200 μg/mL ($p = 0.0086$, $p = 0.0053$, $p = 0.0017$, $p = 0.0041$ and $p = 0.0034$, respectively) for SbS, and 10, 50 ($p = 0.0093$ and 0.0067, respectively), 100 and 200 μg/mL ($p = 0.0065$ and $p = 0.0049$, respectively) for SbL.

Effects of propranolol and furosemide on NTPDase and 5′-nucleotidase activities

Table 2 summarizes the effect on platelets. The results showed that propranolol at 30 and 40.5 μM concentrations and furosemide at 10, 15 and 25 μM decrease the ATP hydrolysis by platelets ($p = 0.0065$ and $p = 0.0420$, respectively). The AMP hydrolysis was affect only propranolol at 15, 30 and 40.5 concentrations ($p = 0.0001$). In lymphocytes only ATP hydrolysis was altered when incubated with furosemide at 30 and 40.5 μM concentrations ($p = 0.0394$).

Effects of SbS and SbL on platelets aggregations in vitro

Fig. 4 presents the results obtained for platelet aggregation. As can be observed, a significant decrease was found in platelet aggregation when the SbS was utilized at 100 and 200 μg/mL concentrations ($p = 0.0167$). The SbL did not show any effect on platelet aggregation.

Discussion

HPLC analysis of *S. buxifolia* crude extracts revealed that its major component was quercetin. In addition to quercetin, the stem bark is rich in phenolic compounds (gallic, caffeic and

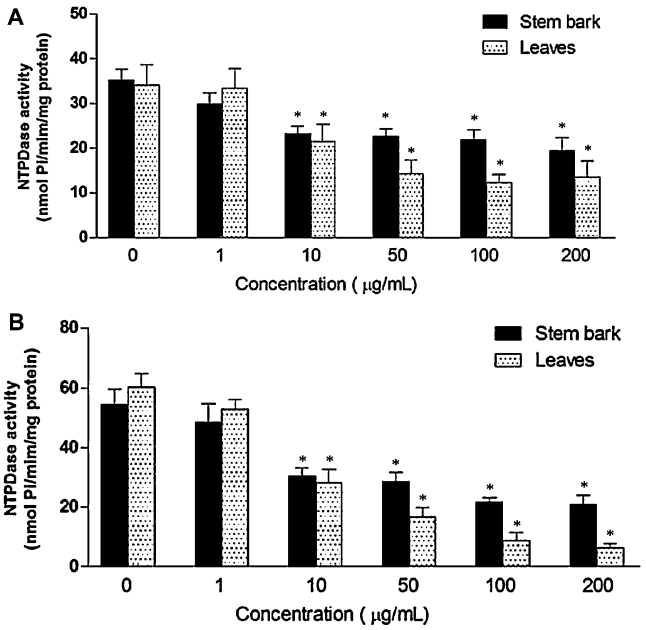

Fig. 3 – Effect of the crude extract fractions from leaves and stem bark (1–200 μg/mL) of *S. buxifolia* on NTPDase (A and B) and 5′-nucleotidase (C) activities in lymphocytes. Data are presented as mean ± SEM. Hydrolysis is expressed as nmol Pi/min/mg protein. Data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range test. *Statistically significant versus controls.

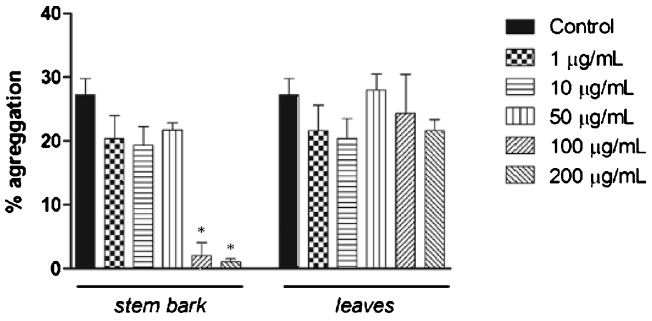

Fig. 4 – Platelet aggregation was evaluated by using ADP at concentration of 10 μM as agonist. Results are expressed as percentage of aggregation. Data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range test. *Statistically significant versus controls.

chlorogenic acids). In leaves, flavonoids (quercetin, rutin and kaempferol) are present in higher concentrations, when compared with stem bark (Table 1).

Numerous studies have shown the inhibition of a variety of enzymes by flavonoids (Havsteen, 1983; Letan, 1996; Gülçin, 2006). Da Silva et al. (2006) suggest that chelating property of flavonoids can diminish NTPDase-like, 5′-nucleotidase and Na^+/K^+-ATPases activities in cortical membrane preparation.

Table 2 – Effect of drugs on NTPDase and 5′-nucleotidase activities from platelets. Values represent mean ± SEM enzyme specific activities are reported as nmol/min/mg of protein. *Statistically significant as compared with controls. Data were analyzed statistically by one-way ANOVA.

Drug (mM)	NTPDase ATP	NTPDase ADP	5′-Nucleotidase AMP
Control	25.47 ± 2.21	18.28 ± 1.98	10.34 ± 0.45
Propranolol			
15	18.88 ± 2.22	25.32 ± 2.58	36.73 ± 2.79*
30	12.86 ± 1.27*	23.20 ± 2.89	35.55 ± 2.34*
40.5	36.48 ± 3.12*	26.60 ± 3.18	40.45 ± 2.52*
Furosemide			
10	12.26 ± 2.57*	19.19 ± 1.67	14.63 ± 6.52
15	11.21 ± 0.30*	20.21 ± 2.77	16.19 ± 4.13
25	9.56 ± 1.43*	18.44 ± 1.96	13.64 ± 3.36

In this way, we propose that the decrease of NTPDase and 5′-nucleotidase activity of both platelets and lymphocytes was due to the action of chelating flavonoids found in both extracts of *S. buxifolia* studied. These results reinforce evidences that compounds derived from plants could affect purinergic system and may modulate the nucleotide levels present in the circulation.

Circulating nucleotides are known to be important signaling molecules that render a variety of physiological responses. The role of adenine nucleotides (ATP, ADP, AMP) and their derivative nucleoside, adenosine, as compounds with effects is well established (Burnstock, 2009). Extracellular ATP in the circulation is rapidly degraded into ADP, AMP, and adenosine by ectonucleotidases (Zimmermann, 2006). Vasoconstriction produced by ATP released along with noradrenaline from perivascular sympathetic nerves has been recognized earlier (Burnstock, 1988). However, following the seminal discovery of endothelium-dependent vasodilatation, it was shown that ATP acts on endothelial cells to release endothelial derived relaxing factor resulting in vasodilatation and dual purinergic neural and endothelial control of vascular tone established (Folkow, 1949; Burnstock, 1990; Rongen et al., 1994; Ellsworth, 2004).

ATP may also regulate blood pressure via renal mechanisms or brain stem regulation or by binding to receptors on the endothelium stimulating vasodilatation (Hashimoto et al., 1998; Wihlborg et al., 2003). This vasodilation is regulated by $P2Y_{13}$ receptors on the lumenal membrane of endothelial cells, which in turn leads to the production of nitric oxide, prostaglandins, and endothelium-derived hyperpolarizing factor (EDHF). In view of our findings that the extract had inhibitory effect on the activities of ectonucleotidases of both platelets and lymphocytes, we suggest that *S. buxifolia* can enhances extracellular ATP levels, thus the extract infusion could modulate the ATP levels in favor of vasodilation leading to reduction in blood pressure. Accordingly, this might be the underlying reason because this plant is used to treat hypertension in traditional Brazilian medicine.

Several studies from our laboratory have demonstrated that ectonucleotidases are enzymes that play an important role in thromboregulation mechanisms. Alterations in their activities have been verified in various diseases, suggesting

that they could be important physiological and pathological parameters (Schetinger et al., 2007). Platelets are the most important blood components that participate in the regulate thrombus formation by releasing active substances such as ADP (Bakker et al., 1994; Pinsky et al., 2002). This process is relevant in a variety of cardiovascular and cerebrovascular disorders. One of the novel findings of the present work was that only the SbS reduced percentage of ADP-induced aggregation. Our results suggest that the SbS can interfere with the direct interaction between ADP and platelets, leading to a decrease of aggregation induced by an agonist acting on ADP specific receptor, this indicates that *S. buxifolia* contains one or more compounds with antiaggregant action.

Moreover, several studies have showed that while competition is the major mechanism of inhibition of ADP-induced platelet responses, an additional mechanism antagonizes other agonist responses via ATP binding to a second platelet P_2 receptor (Soslau and Parker, 1989; Soslau et al., 1995; Soslau and Youngprapakorn, 1997). Thus, *S. buxifolia* extract could lead to the extracellular increase of ATP levels *in vivo* by inhibition of nucleotidase, favoring the antiplatelet aggregation effect of this nucleotide.

To further compare the effect of drugs commonly used in the treatment of hypertension and the effect obtained when the extracts were used, we investigated the influence of propranolol and furosemide (two medicaments with antihypertensive mechanism well described in the literature) on the NTPDase and 5′-nucleotidase from platelets and lymphocytes (Davidov et al., 1967; Bhagat, 1979; Jackson and Campbell, 1981; Leenen et al., 1983). All concentrations studied of propranolol increased significantly the AMP hydrolysis from platelets. On the other hand, when incubated with furosemide, we observed a significant decrease in the ATP hydrolysis in platelets and lymphocytes. Here, we demonstrated these medicaments can alter the NTPDase and 5′-nucleotidase activities in platelets and lymphocytes. Several lines of evidence support the proposition that ATP and adenosine are an important factor in the genesis of hypertension (Fukunaga et al., 1982; Taneyama et al., 1991; Konduri, 1994; Duncker et al., 1996; Stepp et al., 1996; Erga et al., 2000). Consequently, these effects could favor the vasodilation and decreases arterial blood pressure mediated by an increase in ATP and adenosine levels in the circulation.

Conclusion

This is the first study to investigate the effect of the *S. buxifolia* on the activity of NTPDase and 5′-nucleotidase in platelets and lymphocytes from rats. Thus, we suggest that one possible way by which the extract *S. buxifolia* plays its hypotensive effect reported in popular medicine may be due to changes in the ectonucleotidase activities of platelets and lymphocytes, increasing concentrations of ATP circulating, lowering blood pressure through endothelial vasodilation, in order to help in the maintenance of the vascular homeostasis in hypertensive patients. Moreover, our study showed for the first time that SbS may inhibit ADP-induced platelet aggregation indicating that *S. buxifolia* has promising compounds to be tested as potential anti-platelet aggregation drugs.

Conflict of interests

All the authors deny any conflict of interest.

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