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Consumption of mate *Ilex paraguariensis*: a folk beverage with antioxidant power against myocardial ischemic injury

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ABSTRACT. The effects of the aqueous extract of *Ilex paraguariensis* (*Ip*) and the flavonoid quercetin were tested during the induction of *in vivo* myocardial ischemia/ reperfusion in *Rattus norvegicus*. The antioxidant power of the extract and quercetin were chemically determined. The experimental groups were: control, ischemia/reperfusion induction, *Ip* oral treatment, *Ip* oral treatment and ischemia /reperfusion, quercetin oral treatment, and quercetin oral treatment and ischemia/reperfusion. Rats were anesthetized with sodium thiopental and xylazine via intraperitoneal injection and subsequently underwent 15 minutes of ischemia followed by 15 minutes of reperfusion. Ischemia was promoted by tying the left anterior descending coronary artery. Areas of risk and infarction were stained by intravenous Evans blue and triphenyl tetrazolium chloride. Reactive oxygen species (ROS), antioxidant capacity against peroxyl radicals, and lipid peroxidation of the myocardium were quantified. A significant reduction in areas of risk and infarction was detected in the ischemic myocardium treated with *Ip* and quercetin; ROS generation and lipid peroxidation were significantly reduced, and the antioxidant capacity was elevated. Oral administration of *Ip* promoted antioxidant benefits in the myocardium during ischemia and reperfusion, which reduced infarction. We suggest that Mate (a hot drink made from steeped dried leaves of *Ip*) consumption is a potential cardioprotective habit of indigenous people from southern South American countries, which must be better understood scientifically and ethnographically.

Keywords: *Ilex paraguariensis*; infarction; heart; quercetin; antioxidant.

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Introduction

Studies on strategies to generate cardioprotection have been on the rise. The literature shows an inverse association of flavonoid consumption from fruits, vegetables, tea, and wine, with mortality from cardiovascular disease and cancer (Baron-Menguy et al., 2007; Schinella et al., 2009). Reactive oxygen species (ROS) are involved in the etiology of diseases, including damage to the ischemic myocardium. There is evidence that dietary supplementation with antioxidant beverages and foods is able to reduce some oxidative damage in biological systems (Liu et al., 2010). Cardioprotection by vegetable antioxidants during ischemia and reperfusion (IR) has been determined in terms of heart rate stabilization and elevation of myocardial antioxidant activity in rats (Adlam, 2005).

Mate is a traditional hot beverage from South America that is appreciated in Brazil; and is prepared using *Ilex paraguariensis* (*Ip*), as an infusion or decoction from the dried and chopped branches and leaves (Reis et al., 2014). The literature shows biological benefits of the consumption of *Ip*, including improvements in lipid metabolism, anti-inflammatory responses, and antidepressant effects (Gosmann et al., 2012). Its chemical composition has already been characterized; containing vitamins, amino acids, triterpenoid saponins, saccharides, phenolic compounds, chlorogenic acid (3-cafeoil) and its oxidation products, and methylxanthines (Furlong, Colla, Bortolato, Baisch, & Souza-Soares, 2003). The aqueous extract of *Ip* has been identified as possessing elevated antioxidant potential (Schinella, Troiani, Daávila, De Buschiazzo, & Tournier, 2000) and the capability to reduce lipid peroxidation in isolated rat hearts during ischemia and reperfusion induction (Schinella, Fantinelli, & Mosca, 2005). Bixby, Spieler, Menini, and Gugliucci (2005) showed that antioxidant therapy reduces post-ischemic cardiac dysfunction in a study of antioxidant diets with natural compounds and vegetable extracts rich in flavonoids.

Quercetin, one of the most frequent flavonoids found in plants, shows antioxidant effects against many diseases including cardiac infarction, atherosclerosis, hepatic fibrosis, renal lesions, and biliary duct obstruction, when used as a supplement in animal experiments (Liu et al., 2010). In addition to antioxidant effects (Abdel-Raheem, Abdel-Ghany, & Mohamed, 2009), quercetin also exerts anti-inflammatory and anti-apoptotic actions (Liu et al., 2010). Other studies have demonstrated coronary and systemic vasodilation, protection against cell death *in vitro* (Pérez-Vizcaíno et al., 2002), and a reduction in blood pressure, oxidative stress, and myocardial damage in experimental models of hypertension chronically treated with quercetin (Sánchez et al., 2006), including spontaneous hypertensive rats (Romero et al., 2010). Flavonoids are readily absorbed following ingestion, and are known to be cardioprotective as a result of their antioxidant and anti-inflammatory activities (Shashank & Pandey, 2013).

Considering the current relevance of cardiomyopathies and by adding the aspect of ethnopharmacology, the aim of the present study was to compare the cardioprotective effects of acute oral administration of the aqueous extract of *Ip* and one of its compounds, the flavonoid quercetin, during ischemia and reperfusion in *Rattus norvegicus*.

Material and methods

Animals

The present study was approved by the Ethical Research Committee of Animal Care (CEUA number P051/2013) at the Federal University of Rio Grande – FURG, Brazil, and was conducted in accordance with the international guidelines (National Institutes of Health publication No. 88-2959). *Rattus norvegicus* Wistar (60 males), with an average of 2-3 months of age and weighing 312 ± 10 g, were obtained from the Central Animal House of the University. Animals were housed in plastic cages (5 animals/cage) in a temperature-controlled room (21 ± 1ºC) with a 12 h. light-dark cycle, and fed commercial rodent food (25 g animal^{-1} day^{-1}, according to AIN-93) with *ad libitum* access to water.

Administration of the aqueous extract of *I. paraguariensis* (*Ip*) and quercetin

The aqueous extract of Ip was prepared in a Soxhlet from the commercial product using 30 g Erva-Mate in 150 mL distilled water during a 3h. extraction. A 5-mL aliquot was dehumidified (infrared moisture determination – AD–4714) to determine the concentration of the extract (mg mL-^{1}). A 500 mg kg^{-1} dose of *Ip* was chosen based on a previous study Stein et al. (2005) that identified this dose as anti-hypercholesterolemic and vasodilative in rat mesenteric arterial beds.

Quercetin dihydrate (Sigma-Aldrich) was administered orally at 0.23 mg kg^{-1}, which according to Hollman et al. (1996), reflects the average daily human consumption. Quercetin reaches peak plasma levels within 3 hours following oral ingestion (Hollman et al., 1996) thus, 3 hours was the time chosen for the treatments of quercetin and Ip prior to IR induction.

Radical scavenging activity

The effects of Ip and quercetin on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals were measured using a modified version of the method described by Sharma and Bhat (2009). The compounds were diluted to final concentrations of 0.1, 1, 5, and 10 µg mL^{-1}. The reaction mixture was shaken thoroughly and incubated for 30 min at 30ºC in the dark, and the absorbance was measured at 517 nm against a blank. The IC_{50} value (µM) is the effective concentration at which 50% of DPPH radicals were scavenged.

The determination of the radical scavenging effects of *Ip* and quercetin on ABTS (2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) – ABTS^{+}) radicals was performed according to a modified version of the method described in a previous study (Re et al., 1999). Briefly, ABTS radicals were added to medium containing Ip and quercetin (0.1, 1, 5, and 10 µg mL^{-1}) and incubated for 30 min. at 25ºC. The decrease in absorbance was measured at 734 nm, depicting the scavenging activity of compounds against the ABTS radicals. Results are expressed as the percentage of the blank (without compound).

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out as described by Stratil et al. (2006), with slight modifications. The FRAP reagent was prepared by mixing 38 mM sodium acetate (anhydrous) in milli-Q water pH 3.6, 20 mM

$FeCl_3 \cdot 6H_2O$ in milli-Q water, and 10 mM 2,4,6-tri(2-pyridyl)-s-triazine in 40 mM HCl, in proportions of 10:1:1. This reagent was freshly prepared before each experiment. Different concentrations of Ip, quercetin, and FRAP reagent were added to each sample, and the mixtures were incubated at 37ºC for 40 min. in the dark. The absorbance of the resulting solutions was measured at 593 nm using a spectrophotometer. FRAP values are expressed as absorbance.

Total phenolic and flavonoid content

The total phenolic content (TPC) of the extracts was determined following the Folin–Ciocalteu procedure (Sánchez-Rangel et al., 2013). The appropriate dilutions of extracts were oxidized with Folin–Ciocalteu reagent, and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 765 nm. The TPC is expressed as mg gallic acid equivalents (GAE) per 100 g (mg GAE/ 100 g). The total flavonoid content of the extract was determined using a modified colorimetric method employing aluminum chloride (Zhishen, Mengcheng, & Jianming 1999). The absorbance was measured at a wavelength of 510 nm, against a prepared blank (water), using a spectrophotometer. The flavonoid content was determined using a standard curve of catechin (0-250 mg L^{-1}), and the results are expressed as mg catechin equivalents per g (mg CTE g^{-1}).

Phenolic compounds

The identification of the phenolic acids in the extract was performed using Sigma-Aldrich reference standards, named: gallic, protocatechuic, chlorogenic, p-hydroxybenzoic, caffeic, syringic, p-coumaric, ferulic acids and vanillin. The extract was diluted in methanol: water and analyzed by High Performance Liquid Chromatography, according to Scaglioni, Souza, Schmidt, and Badiale-Furlong, (2014) with adaptations for Photodiode Array Detector (PDA). The detection was monitored with photodiode system with wavelength band between 230-340 nm for 25 minutes.

Ischemia and reperfusion (IR) induction

Methodology for IR induction was adapted from Kobayashi, Kokita, and Namiki, (2008). Briefly, rats were anesthetized intraperitoneally (*Ip*) using 40 and 20 mg kg^{-1} sodium thiopental and xylazine, respectively. The trachea was cannulated for artificial respiration, using a mechanical air ventilator pump, and the femoral vein was cannulated for drug administration. Thoracotomy was performed horizontally in the fourth intercostal space, approximately 2 mm to the left of the sternum. Following pericardial incision, the heart was exteriorized. A nylon thread (6-0) was passed around the left anterior descending coronary artery between the pulmonary artery and the left atrial appendage. The ends of the thread were passed through a small plastic bead to form a snare. The bead was threaded through the ligature and placed in contact with the heart. The coronary artery could then be occluded by applying strength to the ligature, and reperfusion could be achieved by releasing tension.

Experimental design

Rats were distributed into 6 experimental groups (n = 10): control (C) - orally treated with distilled water, surgically treated after 3 h, and monitored for 30 min. afterwards; ischemia/reperfusion (IR) - orally treated with distilled water, surgically treated after 3 h., subjected to 15 min. ischemia and 15 min. reperfusion; *I. paraguariensis* (*Ip*) - orally treated with 500 mg kg^{-1} aqueous extract of *Ip*, surgically treated after 3 h., and monitored for 30 minutes afterwards; association of ischemia and reperfusion with Ip (IR-*Ip*) - orally treated with 500 mg kg^{-1} aqueous extract of *Ip*, surgically treated after 3 h., subjected to 15 min. ischemia and 15 min. reperfusion; quercetin (Q) - orally treated with 0.23 mg kg^{-1} quercetin, surgically treated after 3 h., and monitored for 30 min. afterwards; association of ischemia/reperfusion with quercetin (IR-Q) - orally treated with 0.23 mg kg^{-1} quercetin, surgically treated after 3 h., subjected to 15 min. ischemia and 15 min. reperfusion.

Areas of risk and myocardial infarction

Staining of the areas of risk and myocardial infarction followed an adaptation of the method described by Kobayashi et al., (2008). Briefly, at the end of ischemia/reperfusion induction, 5 rats from each group received an intravenous femoral injection of 0.5 mL 10% Evans Blue to stain the myocardial area at risk (ischemic region). Hearts were dissected and sliced. To stain the area of infarction, the slices were incubated with 1%

triphenyl tetrazolium chloride (TTC) at 37ºC for 20 min., and subsequently fixed in 10% formalin solution to enhance the contrast between the viable and non-viable tissue. Following Evans Blue (area of risk) and TCC (infarcted area) incubation, slices were photographed using a stereoscopic microscope (Leica® model DMLS) coupled to a computer. To quantify the areas of risk and infarction, images were analyzed using the Image Tool (mm^2) software. When comparing the areas of risk and infarction among the groups, those in the IR group are considered 100%, and the values in the IR-*Ip* and IR-Q groups are expressed as a percentage.

Oxidative status

At the end of ischemia and reperfusion induction, the hearts were excised from 5 rats in each group, and the ventricles were separated from the atriums. Fresh ventricular tissue was homogenized according to Jacob et al. (2006), and ROS was measured following the methodology described by Ferreira-Cravo et al. (2007). The total protein content was determined by a colorimetric assay (Biuret method LABTEST kits) in triplicate, using a microplate reader (BioTek LX 800) at 550 nm. Each sample was diluted to 2 mg protein mL^{-1} in the homogenization buffer. ROS generation was assayed using the 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) indicator (Invitrogen, Carlsbad, CA, USA). The non-fluorescent compound, H_2DCF-DA, is first de-acetylated and then oxidized by ROS to the fluorescent compound H_2DCF-DA, which was detected at 488 and 525 nm, for excitation and emission, respectively, using a fluorescence microplate reader (Victor 2, Perkin Elmer). Readings were performed every 5 for 90 minutes. Fluorescence data were adjusted to a second order polynomial function and integrated to calculate the area expressing ROS.

The total antioxidant capacity against peroxyl radicals was measured as described by Amado et al. (2009), and the tissue capacity to confront peroxyl radicals produced by the thermal decomposition of the generator, 2,2´-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Sigma-Aldrich) was determined at 37ºC. This methodology was conducted as a ROS assay, but in this case, H_2DCF-DA production was increased by ABAP peroxyl radical generation. The ROS concentration in the presence of ABAP is also expressed as the area calculated from the second order polynomial function, resulting from the adjustment of fluorescence units over time. The total antioxidant capacity is expressed as the relative area calculated by the rate of the difference between the [ROS] area with and without ABAP divided by the [ROS] area without ABAP, as a standardization factor of background ROS production.

To determine the lipid damage, lipid peroxidation was evaluated using 50 mg ventricular tissue homogenized with 1.15% KCL and 35 mM butylated hydroxytoluene (BHT), according to Oakes and Van Der Kraak, (2003). This method involves the reaction of malondialdehyde (MDA), a degradation product of lipid peroxidation, with 2-thiobarbituric acid (TBA) under conditions of high temperature and acidity to generate a fluorescent adduct that can be measured spectrofluorometrically (Victor 2, Perkin Elmer); excitation at 515 nm and emission at 553 nm. The concentration of malondialdehyde (nmols mg^{-1} protein).

Statistical analysis

Data are expressed as the mean ± S.D. ANOVA assumptions of normality and homoscedasticity were tested using Kolmogorv–Smirnof and Levene's tests. Data were analyzed by one-way ANOVA, or by the Kruskal–Wallis test when assumptions were not reached. Significant differences between groups were tested using Tukey's HSD *post-hoc* test. For *in vitro* tests, statistical analyses were performed using one-way ANOVA followed by the Newman–Keuls multiple comparison test, when appropriate. Differences are considered statistically significant at a probability less than 5% ($p < 0.05$).

Results and discussion

The antioxidant activities of the aqueous extract of *lp* and quercetin were evaluated by different *in vitro* methods. Assays based upon the use of DPPH and ABTS radicals are among the most popular spectrophotometric methods for the determination of antioxidant capacity; both chromogens and radical compounds can directly react with antioxidants (Choi, Jeong, & Lee, 2007).

The DPPH radical searching activities of the aqueous extract of Ip and quercetin are presented in Table 1. The extract, at concentrations from 0.1 µg mL^{-1}, was able to scavenge DPPH radicals, with an IC_{50} value (concentration of sample required to scavenge 50% of the free radicals) of 0.16 (0.14-0.18) µg mL^{-1} and an I_{max} of 90.13%. Quercetin presented an IC_{50} value of 0.96 (0.5-1.87) µg mL^{-1} and an I_{max} of 88.49%. Thus, a lower concentration of extract than quercetin was required to scavenge 50% of the free radicals.

Table 1. DPPH radical scavenging activity of the aqueous extract of *I. paraguariensis* and quercetin.

Compound	Concentration (μg mL^{-1})				IC_{50} μg mL^{-1}
	0.1	1	5	10	
Ip	37.29^{b§}	73.43 ± 1.11 ^{b§}	86.45 ± 1.7$^{b\#}$	90.13 ± 1.36^{b}	0.16 (0.14 - 0.18)*
Quercetin	16.59^{a}	48.37 ± 11.8^{b}	56.43 ± 6.3^{b}	88.49 ± 0.32^{b}	0.96 (0.50 - 1.87)

As shown in Table 2, the aqueous extract of Ip demonstrated scavenging activity from 1 μg/mL, presenting an IC_{50} of 1.6 (1.4-1.8) μg mL^{-1}, which was higher than that of quercetin, which showed effective ABTS radical scavenging activity at concentrations equal to or higher than 0.1 μg mL^{-1}, with an IC_{50} of 0.15 (0.13-0.17) μg mL^{-1}. The extract presented an I_{max} of 98.53% and that of quercetin was 98.77%.

Table 2. ABTS radical scavenging activity of the aqueous extract of *I. paraguariensis* and quercetin.

Compounds	Concentration (μg mL^{-1})				
	0.1	1	5	10	IC_{50} μg mL^{-1}
Ip	1,47§	11.37 ± 1.2^{a§}	98.53 ± 0.09^{b}	98.3 ± 0.11^{b}	1.6 (1.4-1.8)*
Quercetin	34,26^{b}	94.00 ± 5.1^{b}	98.77 ± 0.45^{b}	96.01 ± 2.6^{b}	0.15 (0.13-0.17)

Reducing power reflects electron donation capability and is associated with antioxidant activity. In the FRAP assay, the extract of Ip and quercetin showed potential reducing power from a concentration equal to or higher than 1 μg mL^{-1} (Figure 1).

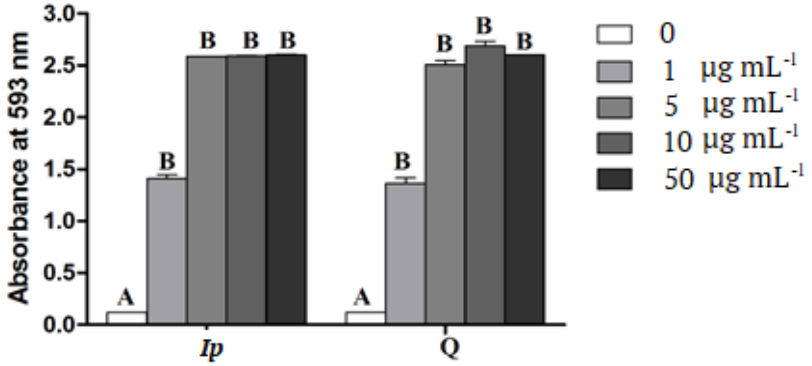

Figure 1. Ferric ion reducing antioxidant power (FRAP) of *I. paraguariensis* aqueous extract and quercetin. Data presented as the mean ± SD of the absorbance values at 593 nm (n = 3). Different letters represent ($p < 0.01$) compared to the 0 group (control without compounds).

Tests were performed at least three times in duplicate. The IC_{50} values (the concentration of sample required to scavenge 50% of the free radicals) were calculated from the graph of the scavenging percentage versus the compound concentration. The values are expressed as a percentage inhibition in relation to the control (without compounds). The mean value of absorbance of the control at 517 nm was 0.770 ± 0.028. Values are expressed as the mean ± SD (n = 3). $^{a}p < 0.01$ and $^{b}p < 0.001$ as compared with the control; $\#p < 0.01$ and $^{\S}p < 0.001$ as compared with quercetin tested at the same concentration as *Ip*; and $*p < 0.001$ as compared with the IC_{50} of quercetin, by one-way ANOVA followed by the Newman–Keuls multiple range test, when appropriate.

Tests were performed at least three times in duplicate. The IC_{50} values (the concentration of sample required to scavenge 50% of the free radicals) were calculated from the graph of the scavenging percentage versus the compound concentration. The values are expressed as the percentage inhibition in relation to the control (without compounds). The mean value of absorbance of the control at 734 nm was 0.87 ± 0.32. Values are expressed as the mean ± SD (n = 3). $^{a}p < 0.01$, $^{b}p < 0.001$ as compared with the control; $^{\S}p < 0.001$ as compared with quercetin tested at the same concentration as Ip; and $*p < 0.001$ as compared with the IC_{50} of quercetin, by one-way ANOVA followed by the Newman–Keuls multiple range test, when appropriate. The total phenolic content in the aqueous extract of *Ip* was 73.50 ± 4.57 mg GAE 100 g^{-1}, and the total flavonoid content was 243.77 ± 0.45 mg catechin CE g^{-1}. The types of phenolic acids present in each fraction were demonstrated by the phenolic acid profile (Table 3).

Table 3. The phenolic acid profile present in the aqueous extract of *I. paraguariensis*.

Phenolic acids	Soluble in methanol Concentration (μg g^{-1})
Galic	n.d
Protocatechic	0.698
Chlorogenic	1261.360
p-hydroxybenzoic	369.241
Caffeic	31.400
Syringic	n.d
Vanillin	n.d
p-coumaric	n.d
Ferulic	n.d

n.d = not detected.

A plethora of natural products have been confirmed as cardioprotective, and among these, the flavonoid quercetin, chosen here, is abundant in many fruits and vegetables, such as *I. paraguariensis*; in free form or conjugated to β-glycosides. Human dietary consumption of quercetin has been estimated to be 16 mg for an adult weighing 70 kg (Thuc et al., 2010), and its half-life plasma distribution has been shown to be approximately 2-3 hours (Hollman et al., 1996).

The total phenolic and flavonoid content of the aqueous extract of *I. paraguariensis* (*Ip*) found in the present study is in accordance with the results obtained in previous studies (Choi et al., 2007; Thuc et al., 2010). The antioxidant activity of *Ip* has long been attributed to the polyphenolic content. Some studies have found that the functional effects of a compound depend on the amount ingested and its bioavailability (Alves & Kubota, 2013; Colpo et al., 2016).

Here, the antioxidant activity of the aqueous extract of Ip was shown in the three different assays employed (DPPH, ABTS, and FRAP). The DPPH and ABTS scavenging assays are commonly used to evaluate the antioxidant activity and neutralization of radicals.

Comparing the IC_{50} values in the DPPH (Table 1) assay with those in the ABTS (Table 2) assay, it is possible to propose that the aqueous extract of Ip was more potent in the ABTS assay, suggesting that the mechanism of its antioxidant activity is based on electron and hydrogen atom transfer.

Considering the radical scavenging activity and the iron-reducing antioxidant power of the aqueous extract of *Ip*, we were able to demonstrate that such a beverage prepared according to the cultural habitats of southern Brazilians (as an aqueous solution at 80ºC) had a higher radical scavenging activity (I% and IC_{50} in the DPPH test) than quercetin, demonstrating greater electron sharing and proton donation proprieties than pure quercetin. The aqueous extract of *Ip* was also capable of greatly reducing ferric iron, another important antioxidant behavior. These characteristics of *Ip* may be attributed to the complex mixture of compounds that constitute Mate. Quercetin, in turn, demonstrated a greater ability to share electrons and reduce ferric iron. Taken together, these results demonstrate the great antioxidant power of the aqueous extract of *Ip*, confirming the popular beliefs regarding Mate usage.

Surgical procedures did not promote myocardial infarction in the control, Ip, or quercetin (Q) groups, as can be seen by the areas of risk (Figure 2) and infarction (Figure 3). Animals in the IR group presented significant areas of risk and infarction as compared with control hearts, representing 44 mm^2 ± 1.96 and 42 mm^2 ± 2.13, respectively, from the total left ventricular area. Treatment with Ip or Q significantly reduced areas of risk and infarction ($p < 0.05$). The areas of risk were 30 mm^2 ± 1.47 (IR-*Ip*) and 29 mm^2 ± 1.87 (IR-Q) (Figure 2), and the areas of infarction were 27 mm^2 ± 1.87 (IR-*Ip*) and 26 mm^2 ± 1.77 (IR-Q) (Figure 3).

To the best of our knowledge, only a few dozen articles have proven the cardioprotective action of natural products on myocardial ischemia and reperfusion damage; in different animals, distinct models of ischemia and reperfusion induction, *in vivo*, *ex vivo*, and *in vitro* (Ahmet et al., 2009; Alves & Kubota, 2013; Cuadrado-Berrocal et al., 2015). Among these, Chan & Tomlinson (2000) reviewed evidence showing that trilinolein, a triacylglycerol from *Panax pseudoginseng* commonly used in traditional Chinese medicine, was able to reduce arrhythmia and areas of infarction in rats subjected to coronary ligation. Similarly, Ahmet et al., (2009) proved that a blueberry-enriched diet protects the heart from ischemic damage, reducing the infarction size by 22% in rats with permanent coronary ligation. Tan et al. (2005) showed a two-fold reduction in the area of infarction in the hearts of rabbits treated with caffeic acid phenethyl ester, a usual component of propolis. Working with *Ginkgo biloba*, (Hao et al., 2009) found a reduction in the area of infarction in the hearts of rats subjected to ischemia and reperfusion. The flavanol (-)-epicatechin, from cacao, has been shown to reduce the area of infarction in rats with

permanent coronary occlusion by 52% after 3 weeks (Yamazaki et al., 2014). A recent study has shown that administration of 5 mg kg^{-1} diterpene labdane prior to reperfusion *in vivo* reduces the area of infarction (Cuadrado-Berrocal et al., 2015). Thuc et al. (2010) was able to reduce the area of infarction by 25% using pravastatin administration immediately prior to ischemia (20 minutes ischemia and 30 minutes reperfusion) in isolated rat hearts.

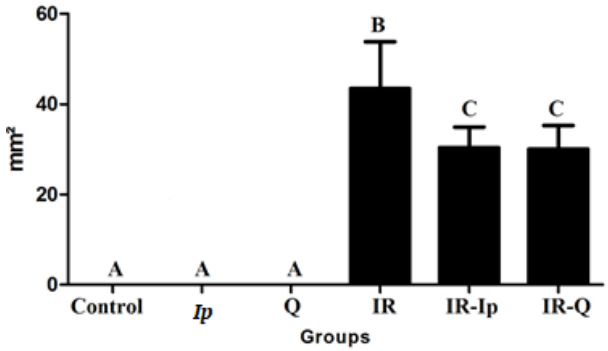

Figure 2. Myocardial areas of risk (mean ± standard deviation, mm^2) in rats with induced ischemia and reperfusion. Control; Ip - treatment with the aqueous extract of *Ilex ischemia* (500 mg kg^{-1}); Q - treatment with quercetin (0.23 mg kg^{-1}); IR - rats subjected to 15 minutes ischemia and 15 minutes reperfusion; IR-*Ip* - treatment with the aqueous extract of *I. paraguariensis* during IR; IR-Q - treatment with quercetin during IR; Different letters represent significant differences between groups, from one-way ANOVA ($p < 0.05$).

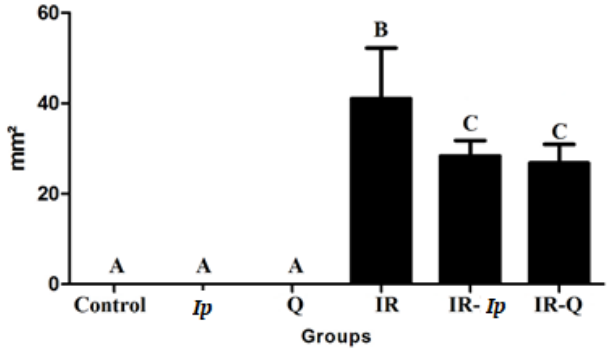

Figure 3. Myocardial areas of infarction (mean ± standard deviation, mm^2) in rats with induced ischemia and reperfusion. Control; *Ip* - treatment with the aqueous extract of *I. ischemia* (500 mg kg^{-1}); Q - treatment with quercetin (0.23 mg kg^{-1}); IR - rats subjected to 15 minutes ischemia and 15 minutes reperfusion; IR-*Ip* - treatment with the aqueous extract of *I. paraguariensis* during IR; IR-Q - treatment with quercetin during IR; Different letters represent significant differences between groups, from one-way ANOVA ($p < 0.05$).

Both *Ip* and quercetin are known for their vasodilatory and antioxidant properties (Hollman et al., 1996). We can suggest that the reduction in the areas of risk and infarction could be a result of less-intense ischemia due to coronary vasodilation prior to (3 h. treatment) and following IR.

The generation of ROS was significantly augmented by IR, and decreased below basal levels when rats were treated with Ip or Q ($p > 0.05$) (Figure 4). Rats with induced IR that were treated with Ip or Q showed statistically diminished ROS generation ($p > 0.05$) (Figure 4). A significant reduction in ACAP was observed in IR rats, while Ip or Q elevated the basal tissue antioxidant capacity ($p < 0.05$) (Figure 5). During IR induction, treatment with *Ip*, but not with Q, statistically increased the antioxidant capacity of the IR myocardium ($p < 0.05$). During IR, lipid peroxidation did not change with respect to the control group ($p > 0.05$); however, treatment with Ip or Q was able to significantly diminish lipid peroxidation in the presence or absence of IR ($p < 0.05$), with no differences between the *Ip* and Q groups (Figure 6).

Oxidative stress is an imbalance between oxidant generation and antioxidant activity (Birben et al., 2012). During reperfusion, myocardial damage is commonly linked to mitochondrial ROS generation, and consequently, to lipid, protein, and DNA oxidation (Akhlaghi & Bandy, 2009). Recuperation from IR depends on mitochondrial ATP production and the level of damage of this organelle (Stein et al., 2005). Quercetin has been suggested to elevate mitochondrial function during IR, and this could be an explanation for its cardioprotective action (Khurana, Venkataraman, Hollingsworth, Piche, & Tai, 2013). Mitochondrial stabilization during IR could also be a mode of action of Ip and quercetin.

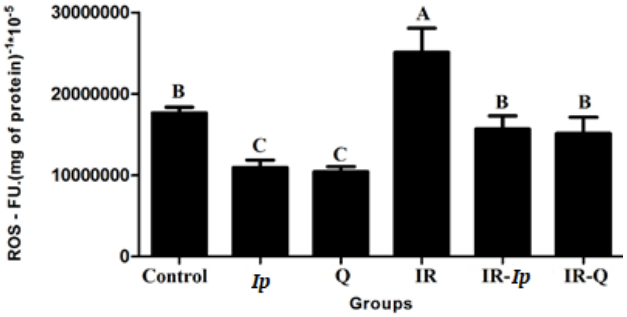

Figure 4. Myocardial reactive oxygen species (ROS - FU mg/protein $*10^{-5}$) in rats with induced ischemia and reperfusion. Control; *Ip* - treatment with the aqueous extract of *I. ischemia* (500 mg kg^{-1}); Q - treatment with quercetin (0.23 mg kg^{-1}); IR - rats subjected to 15 minutes ischemia and 15 minutes reperfusion; IR-*Ip* - treatment with the aqueous extract of *I. paraguariensis* during IR; IR-Q - treatment with quercetin during IR.

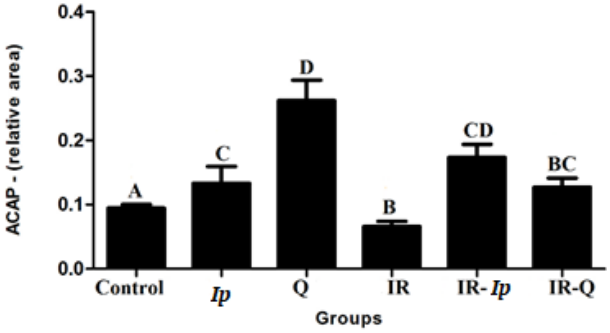

Figure 5. Myocardial antioxidant capacity against peroxyl radicals (ACAP - relative area) in rats with induced ischemia and reperfusion. Control; *Ip* - treatment with the aqueous extract of *I. paraguariensis* (500 mg kg^{-1}); Q - treatment with quercetin (0.23 mg kg^{-1}); IR - rats subjected to 15 minutes ischemia and 15 minutes reperfusion; IR-*Ip* - treatment with the aqueous extract of *I. paraguariensis* during IR; IR-Q - treatment with quercetin during IR; Different letters represent significant differences between groups, from one-way ANOVA ($p < 0.05$).

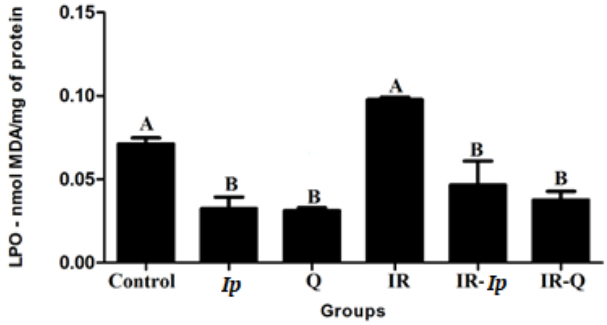

Figure 6. Myocardial lipoperoxidation (LPO - nmol MDA mg^{-1} protein) in rats with induced ischemia and reperfusion. Control; *Ip* - treatment with the aqueous extract of *I. paraguariensis* (500 mg kg^{-1}); Q - treatment with quercetin (0.23 mg kg^{-1}); IR - rats subjected to 15 minutes ischemia and 15 minutes reperfusion; IR-*Ip* - treatment with the aqueous extract of *I. paraguariensis* during IR; IR-Q - treatment with quercetin during IR; Different letters represent significant differences between groups, from one-way ANOVA ($p < 0.05$).

The high antioxidant capacity of Ip is known to reduce lipid peroxidation and ROS generation and quercetin has been shown to possess the same properties (Schinella et al., 2009). Considering the ROS elevation in the IR group and the decrease following *Ip* and Q treatment, in addition to the increase in antioxidant capacity and the reduction in lipid oxidation observed in the IR-*Ip* and IR-Q groups, we can confirm the antioxidant effect of the aqueous extract of lp is similar to that of pure quercetin. Taking into account that the cardioprotective effect of flavonoids is derived from their antioxidant capacity (Rao, Kumar, Viswanath, & Subbaraju, 2005), our results of the DPPH scavenging and FRAP assays confirm the capability of the aqueous extract of *Ip*.

Other articles have found similar results with respect to the reduction in lipid peroxidation during IR, which has been attributed to the antioxidant potential of natural products, such as the plant extract of *Cordifolia tinospora* (Rao et al., 2005) and the fruit extract of *Aristotelia chilensis*, (Céspedes, El-Hafidi, Pavon, & Alarcon, 2008) among others. There is a positive correlation between antioxidant capacity and phenolic

compounds, and *Ip* has a higher correlation than many kinds of red wine or green tea (Schinella et al., 2005). This correlation is evidenced by our findings of significant damage reduction when the aqueous extract of *Ip* was administered prior to IR induction.

Conclusion

Minor ROS generation and low lipid damage, in addition to high antioxidant capacity to direct the scavenging of free radicals by sharing electrons, donating protons, and reducing ferric iron, could be responsible for the minor areas of risk and infarction observed in rats treated with the aqueous extract of *Ip* during IR insult. We suggest that polyphenol compounds in the aqueous extract of *Ip*, when orally administered, had antioxidant potential capable of reducing the size of the areas of risk and infarction.

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