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Full Length Research Paper

Acute toxicity and anti inflammatory effects of supercritical extracts of *Ilex paraguariensis*

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This work reports the *in vivo* evaluation of acute toxicity and anti-inflammatory activity of supercritical carbon dioxide (CO_2) extracts of *Ilex paraguariensis*. For the acute toxicity study, the extracts diluted in sunflower oil were administered to adult male wistar rats in single doses of 250 mg/kg by the intraperitoneal route. Adverse effects and mortality were monitored along 14 days. In the case of anti-inflammatory activity, the animals were submitted to acute induced-peritonitis assays by injection of oyster glycogen and a single dose of 500 mg/kg of supercritical *I. paraguariensis* extract diluted in sunflower oil administered by subcutaneous injection. Results obtained in this work suggest the incidence of acute toxicity due to the presence of cells with hyperchromatism, vacuolization and tumefaction in the livers and the presence of cells with tumefaction in the cortical region of the kidneys of the treated group. It was also experimentally observed that treatment inhibited the neutrophil recruitment in the circulating blood, hence demonstrating the anti-inflammatory effect of the *I. paraguariensis* extracts. The results obtained may be very promising since they open new perspectives for the therapeutic use of supercritical extracts of *I. paraguariensis* or its preparations as anti-inflammatory agents.

Key words: Acute toxicity, Anti-inflammatory, Supercritical fluid extraction, *Ilex paraguariensis*.

INTRODUCTION

Mate (*Ilex paraguariensis* St. Hill., Aquifoliaceae) a world-famous tea consumed in Brazil, Paraguay, Uruguay and Argentina, was used by the Indians of South America before the European colonization. The mate tea leaves has remarkable importance in the economy and in the cultural life of South America, with an average of 300,000 t produced each year (Alikaridis, 1987; Tormen, 1995). In the southern region of Brazil, for example, one can find more than 40 mates processing industries and about 180,000 medium and small rural properties dedicated almost exclusively to cultivation of this raw material (Mosele, 2002).

The mate raw material, as leaves and green stalks, is processed as dye herb for the classical “Chimarrão”, “Mate” or “Tereré”, as fine or soluble powder for teas, or as essences used for different industrial purposes. “Chimarrão” or “mate” is prepared by steeping dry leaves and twigs of mate in hot water, while “tereré” is prepared with cold water (Cansian et al., 2008a).

Mate is considered as a stimulant drink that eliminates lassitude through increasing physical and mental activities.

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Abbreviations: CO2, Carbon dioxide; **PBS,** phosphate-buffered saline; **NH_4Cl,** ammonium chloride; **ANOVA,** analysis of variance; **G,** glomerullus; **TCD,** distal convoluted tubules; **TCP,** proximal convoluted tubules; **CV,** centrolobular vein; **C,** control; **T,** treated.

Biological and therapeutical activities on the cardiovascular, respiratory, muscular, gastrointestinal, renal and neurological systems have been attributed to the presence of xanthines, caffeine, theobromine, tannic substances, flavonoids, vitamins, and other substances present in mate extracts (Heck and Mejia, 2007; Cansian et al., 2008b). In folk medicine, mate infusion has been used for the treatment of arthritis, rheumatism and other inflammatory diseases, constipation, hemorrhoids, headache, obesity, fatigue, fluid retention, hypertension, slow digestion and, hepatic and digestive disorders (Cansian et al., 2008b; Silva et al., 2008; Gorzalczany et al., 2001). Recent findings have shown that the aqueous extracts of *I. paraguariensis* can inhibit the progression of atherosclerosis in cholesterol-fed rabbits (Mosiman et al., 2006).

The literature is somewhat vast regarding the extraction and chemical characterization of *I. paraguariensis* (Esmelindro et al., 2004; Esmelindro et al., 2005; Jacques et al., 2006; Jacques et al., 2007a; Jacques et al., 2007b; Jacques et al., 2008) and there are several reports dealing with the major constituents of *Ilex* species, like the presence of saponins (Taketa et al., 2004; Taketa et al., 2000; Ouyang et al., 1998; Pires et al., 1997), the occurrence of flavonoids (Martínez et al., 1997), xanthines (Saldaña et al., 2002; Reginatto et al., 1999; Saldaña et al., 1999), aldehydes (Don-Xu and Zhong-Liang, 1996), hemiterpene glycosides (Fuchino et al., 1997), triterpenes and alkanes (Vangenden and Jaarsma, 1990), anthocyanins (Ishikura, 1975), pentyl esters, hexyl esters, and other lipophylic compounds (Vangederen et al., 1988).

Some phytochemical or pharmacological investigations related to the use of alcoholic or aqueous extracts of *I. paraguariensis* have also been reported in the literature (Prediger et al., 2008; Alves et al., 2008; Silva et al., 2008; Strassmann et al., 2008; Cansian et al., 2008b). Nevertheless, to our knowledge, no reports are available concerning the use of mate extracts obtained from supercritical extraction in pharmacological studies.

The present report is part of a broader project aiming at characterizing and developing new processes and products from mate tea leaves. In this context, the main objective of this work is to evaluate the acute toxicity and the anti-inflammatory activity of the crude mate extracts obtained from supercritical carbon dioxide extraction. The extracts were administered via subcutaneous injections in rats with the aim to obtain information on the safety of extracts of *I. paraguariensis* and provide guidance to the development of new pharmaceutical products from this plant.

MATERIALS AND METHODS

Plant materials and chemicals

Samples of mate tea leaves were collected in an experiment conducted under agronomic control cultivation system, located at Barão de Cotegipe (Rio Grande do Sul State, Brazil, 27° 37° 15°), a typical *I. paraguariensis* open plantation. The leaves were collected from plants of about 7 years old, growing at complete sunlight exposure and without any additional fertilization with age of leaves of 12 months old. For each tree, fractions from the top, middle, and bottom were sampled and homogenized. The samples were immediately dried after collection at room temperature, triturated and sieved, being collected 100 to 200 mesh particles. The final moisture of all samples was around 2%. The samples were then stored at room temperature under nitrogen atmosphere prior to the extraction. The carbon dioxide (CO_2) employed in the extractions (99.9% in the liquid phase) was purchased from White-Martins S.A. (Brazil) and the reagents were all of analytical grade.

Supercritical extraction procedure

The extraction was performed in a laboratory-scale unit, presented in detail elsewhere (Esmelindro et al., 2004; Esmelindro et al., 2005; Dariva et al., 2003; Rodrigues et al., 2004; Jacques et al., 2007a; Jacques et al., 2007b). Basically, the apparatus consists of a CO_2 reservoir, two thermostatic baths, a syringe pump (ISCO 500D – Lincoln, USA), a 100 mL jacketed extraction vessel, an absolute pressure transducer (Smar, LD301 - Brazil) equipped with a portable programmer (Smar, HT 201 - Brazil) with a precision of ± 0.3 bar, a collector vessel with a glass tube, and a cold trap.

Typically, amounts of around 25 g of comminuted mate tea leaves were charged into the extraction vessel. The CO_2 was pumped at a constant mass flow rate of 2 $g.min^{-1}$ into the bed, which was supported by two 300 mesh wire disks at both ends of the extractor. The CO_2 was kept in contact with the herbaceous matrix for one hour to allow the system stabilization. Afterwards, the extract was collected by opening the micrometric valve. The extraction was accomplished until no significant mass was extracted (about 4 h) and at the end of the process the extract was weighed and transferred to an appropriate vessel (“bulk”, stock extract vessel). Based on the results obtained by Jacques et al. (2007a), all the extractions were conducted at constant temperature and pressure of 40°C and 250 bar, respectively. The extraction runs performed led to an overall standard deviation of the extraction yields of about 0.05. A whole experimental run lasted in general 10 h, including all steps involved: sample weighing, temperature stabilization (baths, extractor), depressurization, etc. Extracts obtained were dissolved in dichloromethane prior to gas chromatography/mass spectrometry analysis.

Extract analysis

The extracts were analyzed in a gas-chromatograph coupled with a mass selective detector (GC/MSD, Shimadzu QP5050A – Kyoto, Japan), using a capillary column DB5 (30 m, 0.25 mm, 25 µm). Column temperature was programmed 70°C/3 min, 4°C/min to 260°C, 2.5°C/min to 300°C/25 min. Helium was the carrier gas and the injection port and detector temperatures were 290 and 300°C, respectively. The sample (1 µL of 40.000 $mg.L^{-1}$ in CH_2Cl_2) components were identified by matching their mass spectra with those of Wiley library database. Triplicate measurements were performed for each sample.

Animals

Forty eight adult male wistar rats (3 months old, 180-220 g) were obtained from the breeding colony at the Department of Clinical Pharmacy (URI - Campus de Erechim, Brazil). The animals were kept in a room under controlled humidity (50 ± 5%) and temperature (22 ± 2°C) and subjected to a 12 h light cycle with free access to

food and water. All the procedures used in the present study comply with the guidelines on animal care of the Ethics committee on the use of animals of the University (Register number 187/TCA/07).

Acute toxicity evaluation

For the acute toxicity evaluation of the *I. paraguariensis* extracts obtained from supercritical extraction, the animals were divided into two groups of six animals each. For the treated group, a single dose of 250 mg/kg of the mate extract diluted in sunflower oil was administered by the intraperitoneal route, while the control group received the vehicle only (sunflower oil). The animals were continuously observed for general behavior changes, signs of toxicity, death and latency of death during 1 h after treatment, then, at each 4 h, and thereafter at each 24 h up to 14 days. During the whole period, the weight and the consumption of water and food by the animals were monitored. The tests were carried out in duplicate. To evaluate the statistical significance of the results the signs of toxicity were expressed in terms of semi quantitative analysis as following: 0 - absence, 2 - Low, 3 - moderate, and 4 - Intense.

After the 14^{th} day, the rats were anesthetized with CO_2 and sacrificed with their livers and kidneys carefully dissected out. Small slices of these freshly harvested tissues were fixed in buffered formaldehyde solution (10%), dehydrated by serial ethanol solution, diaphanized with ethanol-benzene and embedded in paraffin. Micrometer sections, cut by a microtome (Leitz 1512), were stained with hematoxylin-eosin and examined under a light microscope, taking photomicrographs of the samples. All the tests concerning acute toxicity evaluation of the *I. paraguariensis* extracts obtained from supercritical extraction were carried out following the resolution of National Agency of Sanitary Vigilance of Brazil, which regulates the pre-clinical studies of toxicity using phytotherapy (ANVISA, 2004).

Anti-inflammatory evaluation

The anti-inflammatory activity of the *I. paraguariensis* extracts obtained from supercritical extraction was evaluated using twenty four animals, which were equally divided into two groups, control and treated. All the animals were submitted to blood collection (orbital plexus) the acute induced-peritonitis assay by the injection of 10 mL of oyster glycogen (1%) dissolved in of phosphate-buffered saline (PBS) 10 mM, 7.4 pH under light Zoletil 50® anesthesia (Oktar et al., 2004). For the treated group, it was administered a single dose of 500 mg/kg of the mate extract diluted in sunflower oil through subcutaneous injection, while the control group received the vehicle only (sunflower oil). 4 h later, rats were re-anesthetized for blood collection (orbital plexus), and then sacrificed in CO_2 chamber, with cells in the peritoneum removed by washing with 30 mL of PBS containing 1000 $U.L^{-1}$ heparin. The peritoneal exudates at 4 h contained >98% neutrophils. The suspension was centrifuged at 2000 rpm for 10 min and the erytrocytes were destroyed by lysis buffer containing 0.15 M ammonium chloride (NH_4Cl). Cells were re-suspended in ice-cold PBS, and the cell counting was performed using a light microscope. None of the treatments altered the cell viability, which was >95%. The assays were carried out in duplicate.

Statistical analysis

The values were expressed as the mean value ± standard error. One-way analysis of variance (ANOVA) followed by Tukey test applied for the statistical evaluations of the results obtained in the anti-inflammatory tests, while for the acute toxicity tests it was applied the ANOVA followed by the Kruskal-Wallis test. Values of $p<0.05$ were regarded as significant.

RESULTS AND DISCUSSION

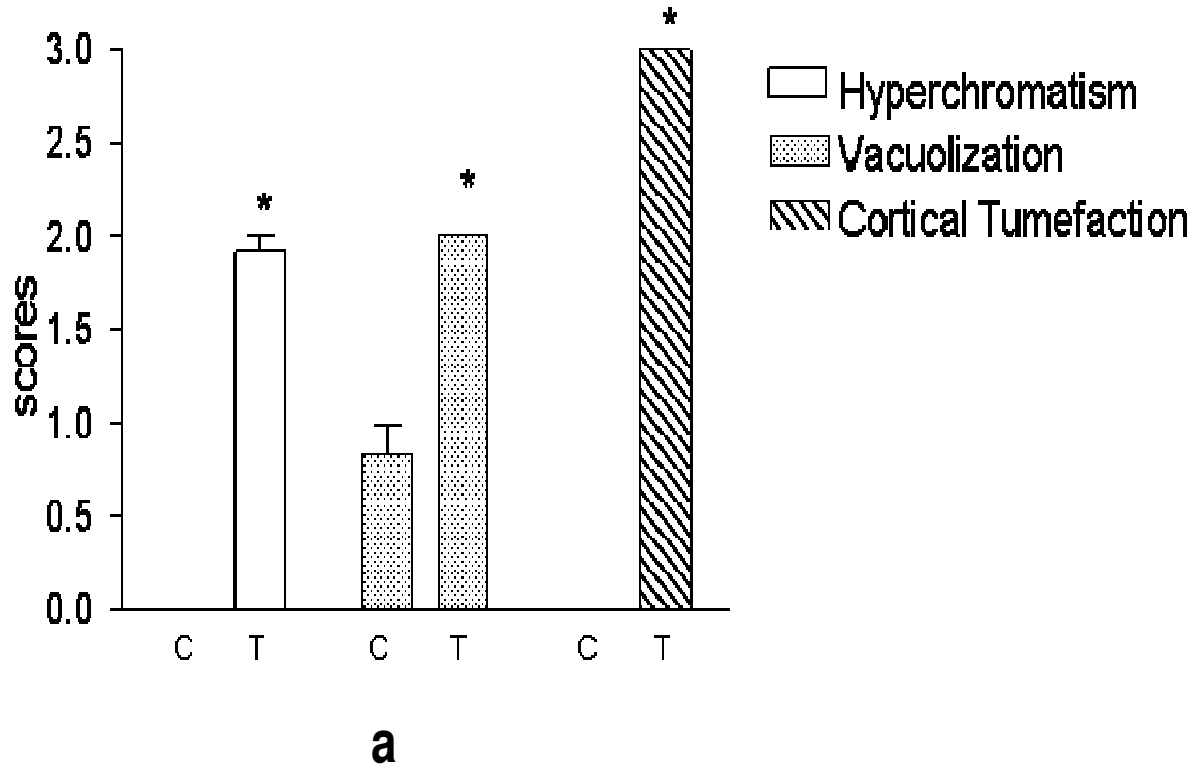
Chemical composition of the extracts

In this work the extraction yield (defined as the weight percentage of the extract obtained with respect to the initial charge of the raw material in the extractor) was about 2.25 wt%, which is of the same order of magnitude to that found by Jacques et al. (2007a). The main compounds identified in the supercritical extracts of *I. paraguariensis* were (as expressed in terms of percent of normalized peak area): caffeine 35.5%, theobromine 0.3%, phytol 1.7%, eicosane 11.9%, squalene 3.9%, pentatriacontane 0.9%, vitamin-E 8.4%, and stigmasterol 1.5%. Other compounds (6 hydrocarbons, 2 aldehydes, 4 alcohols, 1 ketone and 2 aromatic) were also identified by matching their spectra with those of the Wiley library, but attempts to check them were not pursued due to the low confidence level indicated by the chemical analysis. A similar chemical profile of the supercritical mate extracts was also obtained by Cansian et al. (2008a) and Esmelindro et al. (2005). Jacques et al. (2007a) reported the presence of 51 compounds in the mate extracts obtained by supercritical CO_2 extraction at 250 bar and 40°C.

Acute toxicity evaluation

The acute toxicity of the supercritical extracts of *I. paraguariensis* was performed in wistar rats treated with a unique dose of 500 mg/kg. Neither mortalities nor significant changes in the general behavior or other physiological activity were observed during the monitored period of the animals. In addition, the food and water consumption was not altered during the evaluation. These results indicate that the supercritical extracts of *I. paraguariensis* did not produce any toxic symptoms on rats at the concentration level employed in the assays.

After 14 days treatment, the animals were sacrificed and their livers and kidneys were analyzed. Pathological examinations of the tissue indicated the existence of detectable abnormalities. Figure 1 presents the histo-logical changes in the livers (1a) and kidneys (1b) of the animals tested. The liver cells of the treated animals showed considerable increase in the cells with hyper-chromatism, vacuolization and tumefaction in comparison with the control group (Figure 1a). The cellular alteration in the hepatic tissue can be better visualized in Figure 2 that presents the photo-micrographs of the sections of the livers (magnification of 100 times). It is shown that the supercritical extracts of *I. paraguariensis* in a single dose of 250 mg/kg caused several alterations in this organ of the treated animals compared with the control group



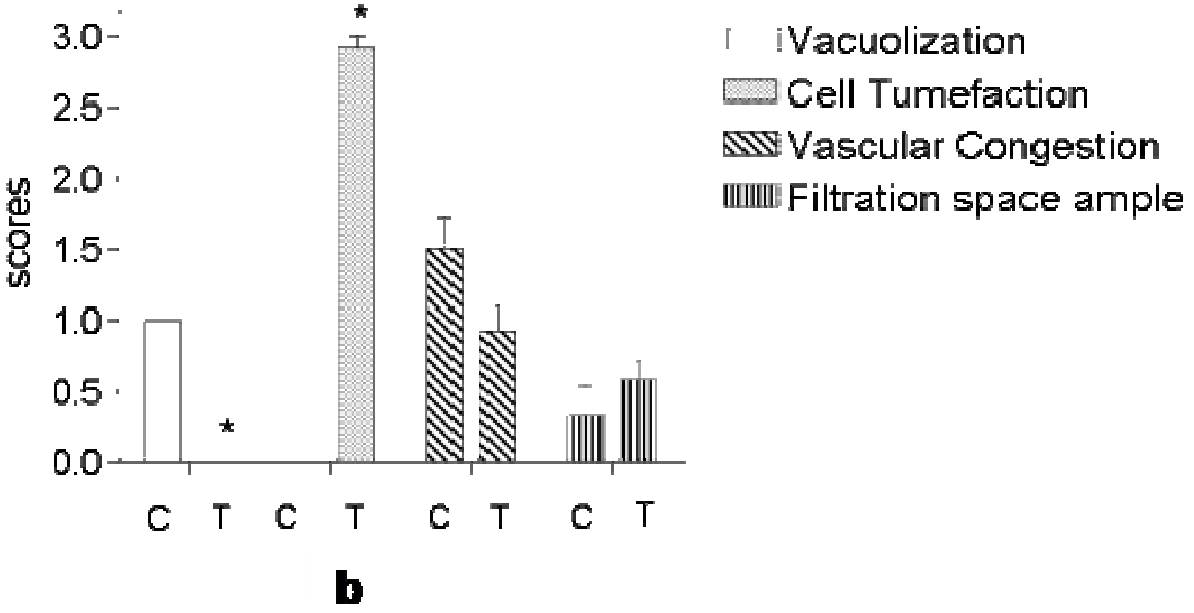
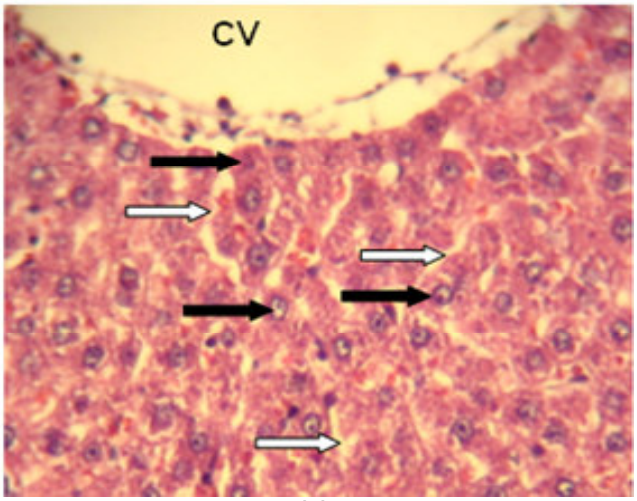


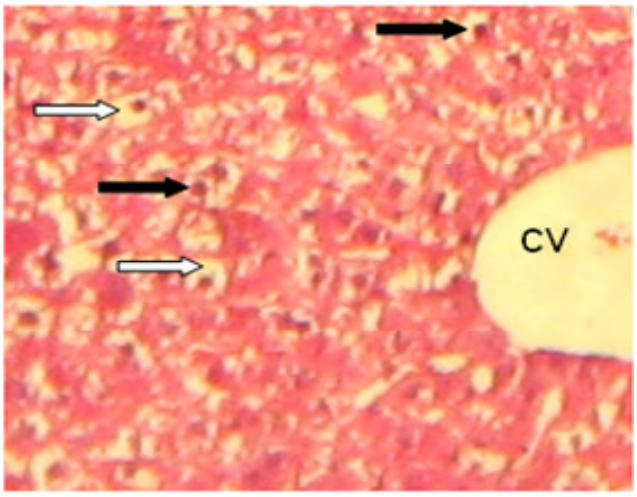
Figure 1. a, Effect of acute toxicity of supercritical *I. paraguariensis* extracts on histopathological parameters of liver; **b,** kidneys tissues of rats. **C,** control; **T,** treated. * $p<0.007$ vs control.

(Figure 2a) as the presence of hyperchromatism and vacuolization (Figure 2b) tumefaction and hepatocytes with hyperchromatism (Figure 2c) suggests the presence of hepatocellular necrosis.

The histological analysis of the renal tissue revealed an increase of cells with tumefaction for the treated group,



(a)



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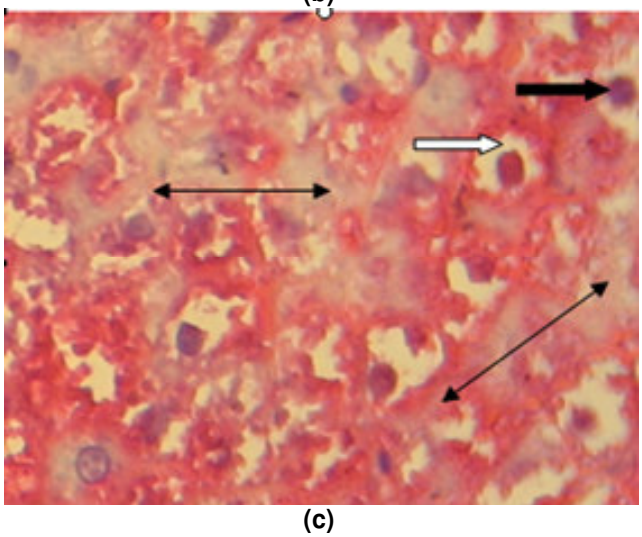

(c)

Figure 2. a, Photomicrographs of the sections of the livers showing normal features in control group; **b,** the liver of animals treated with a single dose of 250 mg/kg of supercritical *I. paraguariensis* extracts after 14 days showing the presence of hyperchromatism (black arrow) and vacuolization (white arrow); **c,** the presence of tumefaction (double black arrow) and hepatocytes with hyperchromatism (single black arrow). **CV,** centrolobular vein. (Hematoxylin-eosin, 100x).

while the cells with vacuolization decreased for this group. There are no signals of cells with hyperchromatism for both control and treated groups (Figure 1b). The cellular alteration in the renal tissue can be better visualized in Figure 3 through photomicrographs of the sections of the kidneys (magnification of 100 times). The supercritical extracts of *I. paraguariensis* caused an increase in the cellular tumefaction in the cortical region of the kidneys of treated group (Figure 3b) when compared with control group (Figure 3a), which suggests the presence of renal necrosis. It was not verified the presence of cells with hyperchromatism and vacuolization, possibly due to the advanced necrosis stage of the renal tissue.

Anti-inflammatory evaluation

Initially, the anti-inflammatory activity of the supercritical extracts of *I. paraguariensis* on acute peritonitis induced by oyster glycogen was investigated in a single dose of 250 mg/kg, but no significant differences were verified in the cell recruitment. However, it was verified the tendency to inhibit the inflammatory process. Then it was decided to inject a single dose of 500 mg/kg. The results obtained are presented at Figure 4.

The animals presented a level of 7.5 leukocytes/mm^3 before inflammatory induction, with no variation verified compared to the treated group after induction with supercritical extracts of *I. paraguariensis*, while the control group decreased the level of leukocytes in the circulating blood. The statistical analysis of the results indicated significant differences ($p<0.001$) among the treated and control groups for the number leukocytes in the circulating blood. The mobilization of leukocytes of the bone marrow to the circulating blood caused a decrease in the leukocytes recruitment, which demonstrates an effective decrease in the acute inflammatory process. The number of total leukocytes observed for the treated group was very similar to that obtained for the animals before the induced-inflammatory process. This result suggests the occurrence of inhibition of the total leukocytes recruitment in the circulating blood after the induced-inflammatory process by the supercritical extracts of *I. paraguariensis* in a single dose of 500 mg/kg.

The induced-inflammation resulted in an increase of approximately 2000 segmented neutrophils/mm^3 in the circulating blood for all the animals. For the treated group with supercritical extracts of *I. paraguariensis* it was verified a reduction in the levels of segmented neutrophils compared with the control group. Such reduction of neutrophils number in the circulating blood demonstrates the anti-inflammatory activity of the supercritical extracts of *I. paraguariensis*, as an increase in the neutrophils in the circulating blood is an indicative of inflammatory process, since these cells are the first defense mechanism of the body (Male, 2003).

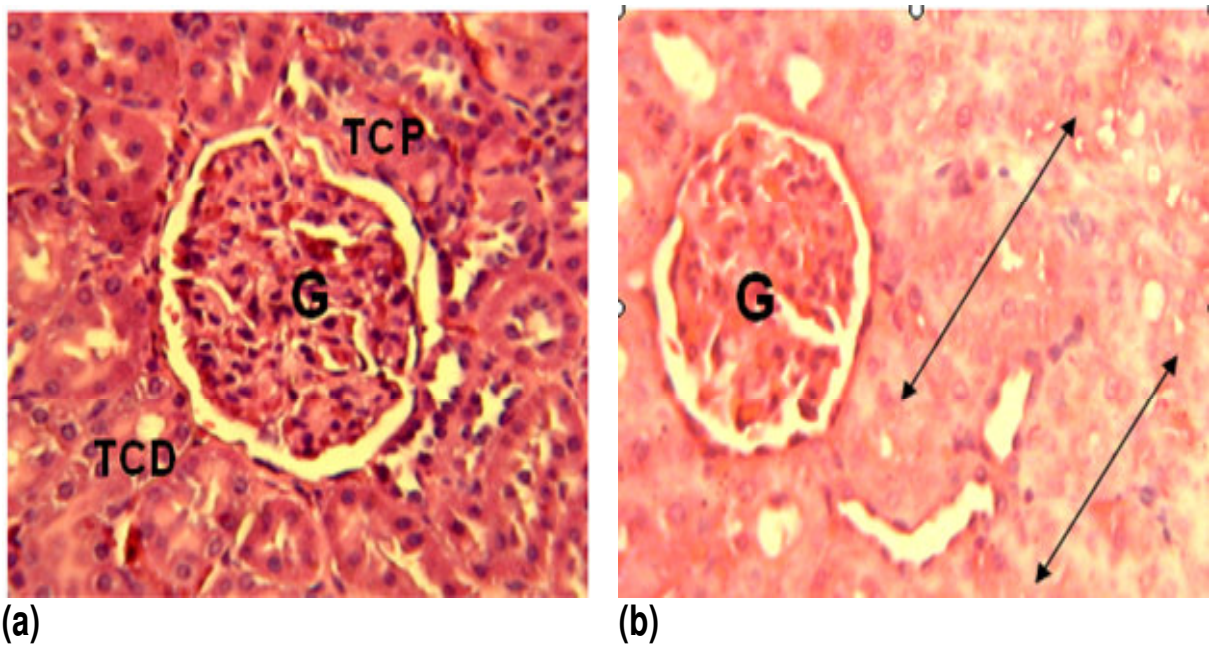

Figure 3. a, Photomicrographs of the sections of the kidneys showing normal features in control group; **b,** the kidneys of animals treated with a single dose of 250 mg/kg of supercritical *I. paraguariensis* extracts after 14 days showing the presence of cellular tumefaction in the cortical region (double black arrow). **G,** glomerullus; **TCD,** distal convoluted tubules; **TCP,** proximal convoluted tubules. (Hematoxylin-eosin, 100x)

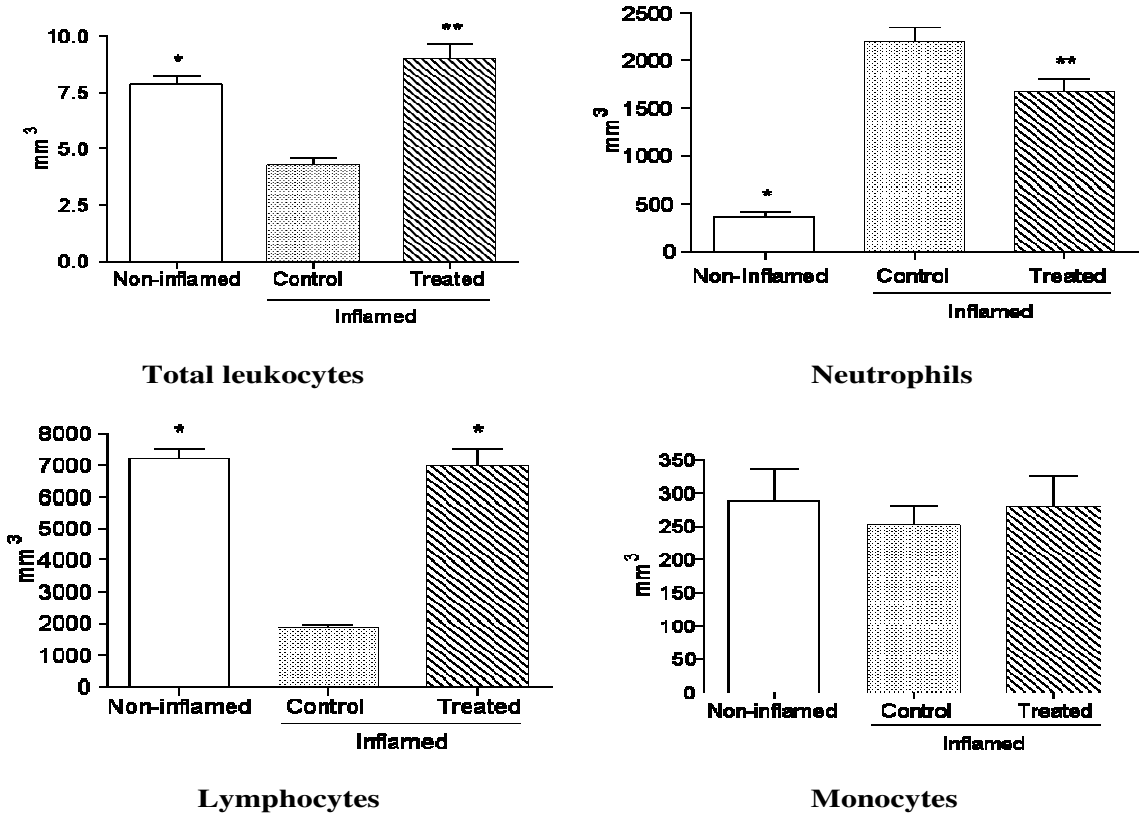

Figure 4. Effect of the supercritical *I. paraguariensis* extracts on the cell recruitment of the circulating blood. $^{*}p<0.001$ and $^{**}p<0.05$ vs control. Non-inflamed refers to the animals before the induction of inflammation; control refers to the inflamed animals that have received only the vehicle; treated refers to inflamed animals that have received the extract.

The mean levels of lymphocytes in the circulating blood was around 7000 lymphocytes/mm^3 for the treated group with supercritical extracts of *I. paraguariensis*, which was very similar to that obtained for the animals before the induced-inflammation. The control group showed around 2000 lymphocytes/mm^3 in the circulating blood after the induced-inflammation process.

The mean levels of monocytes in the circulating blood was around 250 monocytes/mm^3 for both treated and control group, which is similar to the value obtained for the animals before the induced-inflammation. This occurred due to the fact that the blood was collected 4h before the induced-inflammation, where the monocyte level did not change, as previously reported by Male (2003).

In this work it was verified an increase in the number of segmented neutrophils in the control group (this group received sunflower oil only), which indicates that the experimental model used in this study to evaluate the cell recruitment was applicable and effective. The segmented neutrophils are responsible for the initial defense of the body subjected to an inflammatory process, which confirms the existence of an inflammation caused by the oyster glycogen.

The number of cells migrated to the peritoneum of the treated animals was equivalent to that of control animals (8760±3301 cells/mm^3 for treated and 6280±1647 cells/mm^3 for control).

Some of the pharmacological activities of *I. paraguariensis* are attributed to the high content of caffeyol-derivatives and flavonoids (Filip et al., 2001). Among several important biological activities exerted by flavonoids, it may be important to highlight its inhibitory effect on the enzyme systems involved in the initiation and maintenance of the inflammatory and immune response (Gorgen et al., 2005). In fact, it has been shown that *I. paraguariensis* presents a higher content of flavonoids, such as quercetine, when compared to other assayed species (Filip et al., 2001). Although the flavonoids content was not determined in the supercritical extracts of *I. paraguariensis* obtained in this work, based on literature results (Jacques et al., 2007a Jacques et al., 2008, Martínez et al., 1997), it seems reasonable to believe that this class of compounds was also extracted in the process. In this regard, the anti-inflammatory effect of the supercritical extracts of *I. paraguariensis* could be associated with the flavonoids content.

Conclusions

Results obtained in this work suggest the incidence of acute toxicity due to the presence of cells with hyperchromatism, vacuolization and tumefaction in the livers and the presence of cells with tumefaction in the cortical region of the kidneys of the treated group. Toxicity studies in experimental animals may not however be directly extrapolated to humans since a reasonable estimate of the self-administered dose may be difficult to make. Besides, in view of the widespread use of supercritical extracts of *I. paraguariensis*, additional clinical toxicological evaluations need to be performed to determine a safe dose and protect the population from possible toxic effects. Regarding the anti-inflammatory effect of the supercritical extracts of *I. paraguariensis*, it was verified that 500 mg/kg inhibited the neutrophil recruitment in the circulating blood. It is well known that numerous medicinal plants present significant anti-inflammatory activities, evaluated in different models, and several active metabolites which are responsible for these actions have been identified. Therefore, it is believed that results found in the present work may be very promising since they open new perspectives for the therapeutic use of supercritical extracts of *I. paraguariensis* (and other plants) or its preparations as an anti-inflammatory agents. Further studies are under-way within our working group to identify the active compounds responsible for the reported anti-inflammatory activity as well as to elucidate their mechanisms of action.

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