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Original Full Paper

Hepatoprotective and antioxidant activities of dry standardized extract of *Apeiba tibourbou* Aubl, in mice

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Abstract

Due to its chemical composition and use in folk medicine, the dry standardized extract of *Apeiba tibourbou* Aubl. (Tiliaceae) leaves (DSEAT) was tested to assess its hepatoprotective activity against carbon tetrachloride (CCl_4)-induced hepatotoxicity in mice. The animals were treated with DSEAT previously for 7 days, at doses of 25, 50, 100, 200, and 400 mg/kg and 18 mg/kg of rosmarinic acid; the liver damage was induced by administering CCl_4 intraperitoneally (i.p.) at days 3 and 7, and 1 h before treating with DSEAT. The hepatoprotective activity was assessed using various biochemical assays such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), malondialdehyde (MDA) and histopatological studies. DSEAT doses of 400, 200, and 100 mg/kg were not capable of protecting the liver against CCl_4. However, the dose of 50 mg/kg reduced AST by 31.50% the dose of 25 mg/kg reduced GGT by 57.18% compared to the CCl_4 ($p < 0.05$). In the liver, DSEAT dose of 50 mg/kg and rosmarinic acid reduced MDA by 27.45% and 63.61%, respectively, whereas in plasma, MDA was reduced in all the groups treated with DSEAT and rosmarinic acid. In conclusion, DSEAT exhibits hepatoprotective effect only at low doses and antioxidant activity *in vivo* after peroral administration. The experimental protocol was approved by the Animal Research Ethics Committee of UFG (CEUA, no. 177/2011).

Key words: *Apeiba tibourbou*, hepatic lesion, lipid peroxidation, malondialdehyde, rosmarinic acid.

Introduction

Liver diseases can lead to serious complications and are generally associated with lifestyle (e.g. alcoholic beverages), poor dietary habits, environmental pollution, occupational hazards, and prescription drug abuse (5, 20). The process of biotransformation of xenobiotic and/or endogenous substances is one of the functions performed by the liver, which makes this organ an excellent target for toxic compounds, such as drugs, alcohols, pollutants, among others. These aggressors cause liver damage mostly by producing reactive oxygen species (ROS), capable of accelerating even more the pathological process (6, 23).

Searching for new hepatoprotective compounds, researchers were able to reproduce *in vivo* acute hepatic lesions by lipid peroxidation using carbon tetrachloride (CCl_4) as an inducer (17). After being metabolized in the liver, CCl_4 releases trichloromethyl ($\bullet CCl_3$), a radical capable of attacking liver cells and releasing enzymes in the blood stream as well as the dialdehyde malondialdehyde (MDA) as a secondary product (10, 12, 14). Taking into consideration the inductive power of

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CCL_4, the plant species *Apeiba tibourbou* Aubl. (Tiliaceae) was selected to verify whether it exhibits hepatoprotective activity.

Apeiba tibourbou, popularly known as monkey comb, is found in Central America and in the Brazilian Cerrado region (18). The ethnopharmacological uses of this species include treatment of rheumatism, spasms, and respiratory secretions (15). Moreover, the leaves are rich in antioxidant compounds, such as flavonoids, tannins, and mainly rosmarinic acid, a secondary metabolite with anti-inflammatory, astringent, and antioxidant activities (19). Therefore, the present study aimed to identify the potential of the dry standardized extract of *A. tibourbou* leaves (DSEAT) to protect the liver against CCl_4-induced hepatotoxicity in mice.

Material and methods

Materials

The following compounds were used: 1,1,3,3-tetraetoxypropane (TEP – standard for MDA; minimum purity of 96%, Sigma-Aldrich, St. Louis, MO, USA); thiobarbituric acid pa (TBA; J.T. Baker, Mexico City, Mexico); and carbon tetrachloride ACS (Labimpex, Diadema, SP, Brazil); the other chemical products were of analytical grade. Ultrapure water was obtained by distillation, deionization, and additional purification using a reverse osmosis system (Os10 LZ, Gehaka, SP, Brazil). The reagents used in the diagnoses of hepatic enzymes were purchased from Doles Reagentes e Equipamentos para Laboratórios Ltda (Goiânia, GO, Brazil), and the measurements were performed using BioPlus® 2000 equipment (Bioplus Desenvolvimento Biotecnológico Ltda, Porto Alegre, RS, Brazil).

Plant material

The leaves of *A. tibourbou* were collected at the Instituto do Trópico Subúmido, Pontifícia Universidade Católica de Goiás (PUC-Goiás), in the municipality of Goiânia, GO (16º44'10,8"S, 49º12'47,4"W), in February 2008. The material was identified by Prof. Dr. José Realino de Paula, from Universidade Federal de Goiás (UFG), and a voucher specimen was deposited in the herbarium of UFG (no. UFG-40.119). The dry extract of *A. tibourbou* leaves, standardized with 6.5% rosmarinic acid (DSEAT), was prepared in the Laboratório de Pesquisa de Produtos Naturais (LPPN), of UFG, using a spray-drying technique (25).

Animals

In this study, 48 healthy, Swiss Webster female mice (*Mus musculus*), weighing 35 ± 5 g were obtained from the Central Animal House of UFG and acclimated in the Animal Trial Room of the Pharmacy School of UFG, following the recommendations for laboratory animal best practice procedures. They were housed in plastic cages (40 cm × 30 cm × 16 cm) at 25 ± 2°C and 50–70% humidity, with a light-dark natural cycle of 12 h, with ad libitum access to commercial rodent diet (Labina, Ecibra Ltda., Santo Amaro, SP, Brazil) and filtered tap water provided via water bottles.

Experimental design

The experimental protocol was approved by the Animal Research Ethics Committee of UFG (CEUA, no. 177/2011). Animals were treated according to the principles defined by the Colégio Brasileiro de Experimentação Animal (COBEA) and the Brazilian law (no. 11794/2008). The trial was carried out according to the method of Kalantari et al (11) with modifications. The animals were randomly allocated to eight treatment groups of six animals each as follows: Group 1 (negative control) received distilled water [0.25 mL, administered perorally (p.o.)] for 7 days and olive oil [2 mL/kg, administered intraperitoneally (i.p.)] at days 3 and 7; Group 2 (positive control) received CCl_4:olive oil (1:1, 2 mL/kg, i.p.) at days 3 and 7; groups 3, 4, 5, 6, and 7 were treated with DSEAT at the doses of 25, 50, 100, 200, and 400 mg/kg (p.o.), respectively, for 7 days, and received CCl_4:olive oil (1:1, 2 mL/kg, i.p.) at days 3 and 7, 1 h after administration of DSEAT; Group 8 was treated for 7 days with 18 mg/kg of rosmarinic acid dissolved in distilled water and received CCl_4:olive oil (1:1, 2 mL/kg, i.p.) at days 3 and 7. At day 8 all the animals were anesthetized with ketamine:xylazine (87.5%:12.5%, 0.2 mL/100 g, i.p.), 24 h after receiving CCl_4 (13).

Blood samples were collected by cardiac puncture and the animals were euthanized by cervical dislocation. The liver was removed, weighed, and fixed with 10% formalin. Before fixation, the right lobe of the liver was removed, washed with 0.09% normal saline, and stored at -20ºC until analysis. Each blood sample was centrifuged at 860 x g and the plasma was separated to measure MDA and hepatic enzymes.

Parameters assessed

The hepatic enzymes alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and gamma-glutamyltransferase (GGT), used as liver injury biomarkers, were analyzed using commercial kits for biochemical testing suitable for the BioPlus® 2000 equipment (ALT assay kit, lot no. ALTB 11071; AST assay kit, lot no. ASTB 11051; ALP assay kit, lot no. FALC 11041; GGT assay kit, lot no. GLUT 11051). MDA was measured in macerated liver and plasma pool (plasma samples from each group were placed in the same test tube), following the procedures described by Nunes (2008). Aliquotes of 100 mg thawed and lysed liver were transferred to Falcon tubes containing 2 mL 1.15% (w/v)

KCl (prepared using ultrapure water), homogenized, and centrifuged at 860 x g for 3 min. Aliquotes of 500 µL supernatant or plasma pool were transferred to vials containing 1 mL of trichloroacetic acid (TCA)-thiobarbituric acid (TBA)-hydrochloric acid (HCl) solution. This mixture was heated on a boiling water bath for 15 min, cooled to room temperature, centrifuged at 860 x g for 10 min and the supernatant was read in a spectrophotometer at 535 nm. MDA concentration was calculated from a standard curve prepared with TEP and reagent blanks, used to calibrate and zero the spectrophotometer, respectively. The TCA-TBA-HCl solution was prepared according to the description of Oliveira et al. (22).

Preparation of the standard curve with 1,1,3,3-tetraetoxypropane (TEP)

A standard curve for MDA was prepared with TEP using an ethanolic stock solution of 1.0 mg/mL. Aliquots of 0.12, 0.36, 0.72, 1.44, and 2.88 µg/mL diluted in ultrapure water were prepared and 500 µL of each final solution were read in a spectrophotometer at 535 nm, after reaction with TCA-TBA-HCl (as described previously). The analyses were carried out in duplicate and the linear equation y = 0.256x – 0.031 and the correlation coefficient (r = 0.998) were obtained.

Statistical analysis

The results underwent frequency distribution tests ($\chi 2$) to certify the normal distribution, followed by one-way analysis of variance (ANOVA), Tukey's multiple comparison test, and Student's t test, using the GraphPad Prism® software version 5.0. Statistical differences were considered significant at $p < 0.05$ and highly significant at $p < 0.0001$. The results of MDA and hepatic enzymes in liver and plasma are expressed as mean ± standard error of the mean (mean ± SEM).

Results and discussion

CCl_4-induced acute liver injury increases plasma levels of the liver enzymes AST and ALT, as observed in Group 2 (positive control), which presented higher levels of AST (117.82%) and ALT (154.53%) compared to Group 1 (negative control), with $p < 0.0001$ (Figs. 1A and 1B). The increased activity of these enzymes occurs during CCl_4 reductive dehalogenation, catalyzed by the enzyme CYP-2E1 (citochrome P-450 isoform), which releases the radical $\bullet CCl_3$. This radical reacts with the oxygen molecules present in the cells producing another very reactive radical, trichloromethyl peroxide, which in turn can bind to DNA, lipids, proteins, and carbohydrates, causing the oxidation and destruction of citochrome P-450, deposition of collagen, and liver fibrosis. Furthermore, it increases the levels of secondary metabolites, mainly MDA (10, 23), causes liver hypertrophy, enzyme overflow into the blood stream, and liver necrosis (9). The histopathological exams revealed that Group 2 presented alterations in the liver, such as hydropic degeneration and glycogenosis (Fig. 2), but no alterations were found in the kidneys (Fig. 3).

The animals treated with DSEAT presented diversified responses to CCl_4-induced hepatotoxicity (Fig. 1). Compared to the positive control (Group 2), plasma levels of AST and ALT decreased by 31.50% and 20.63% in Group 4 and by 31.17% and 9.12% in Group 5, whereas in groups 3, 6 and 7, treated with higher doses of DSEAT, they had increased statistically significant compared to the Group 1 ($p < 0.05$ and $p < 0.0001$). It seems that high doses of DSEAT induce liver toxicity if administered on a daily basis, taking into account that the liver can be injured in a dose-dependent manner, increasing plasma levels of AST and ALT with high doses of the inducer (1, 4). This fact was confirmed by histopathological exams, which showed alterations in the liver and kidneys identified by inflammatory infiltrate, moderate hydropic degeneration, steatosis com glycogenosis and necrosis (Figs. 2 and 3). The occurrence of inflammatory cells in liver tissue may suggest that DSEAT interacts with proteins and/or enzymes of this organ interfering and/or potentializing the mechanism of antioxidant defense and leading to the generation of ROS, which in turn can mimic an inflammatory response (8). Jarrar and Taib (8) affirmed that hydropic degeneration can be associated with hydrolytic enzyme overflow into the blood stream, cytoplasmic and macromolecular degeneration, and raised levels of transaminases.

The presence of cholestasis can be evidenced by elevated serum levels of ALP and GGT, which can achieve a 10-fold increase in the threshold levels under some circumstances (12, 21). Cholestasis results from certain conditions that affect bile drainage to canaliculi, causing reduction or failure of secretory activity of hepatocytes, leading to accumulation of blood with high levels of ALP and GGT (3). In most cases, cholestasis is caused by tumors of the biliary tract or liver diseases induced by drugs or alcohol (27).

In this study, groups 5, 6 and 7, treated with 100, 200 and 400 mg/kg of DSEAT (Fig. 1D), respectively, exhibited increased GGT ($p < 0.05$) compared to groups 1 and 2 and presented inflammatory infiltrate, steatosis and necrosis (Fig. 2). Steatosis is considered toxic to tissues, since it is normally associated with loss of membrane integrity of the cells that cover bile canaliculi, leading to the formation of lipid micelles and reducting the release of ALP. Bertone et al. (2) tried to elucidate the main function of ALP in the liver of lean and obese animals, but did not come to any final conclusions. However, the authors observed that the presence of fat changes liver functions and can increase or decrease ALP level. Thirumalai et al (26) believe that the presence of ALP can be considered a mixed liver lesion which includes the cytotoxic effect

associated with cholestatic components. GGT is a microsomal enzyme found in the liver, related to the transference of amino acids from the cell membrane and to glutathione (GSH) metabolism, which maintains intracellular integrity during oxidative stress and, given that GGT is highly sensitive, it is the best marker of biliary tract injury. In the present study, occurred a significant increase in GGT level in groups treated with the three highest doses compared to Group 1, and the decrease in this parameter was significant in Group 3 compared to Group 2 (Fig. 1B).

An antioxidant is any substance capable of slowing or preventing the oxidation of other compounds, thus protecting biologic systems against the harmful effects of processes or reactions that can cause excessive oxidation (27). The "ideal" and most important mechanism which shows that a substance or plant exhibits antioxidant activity is the stabilization of free radicals (10; 23). The antioxidant activity of rosmarinic acid (Group 8) was proven by the decreased levels of MDA in the liver ($p < 0.05$) compared to Group 2 (Fig. 4A), as well as by the results of histopathological exams similar to the Group 1 (Fig. 2).

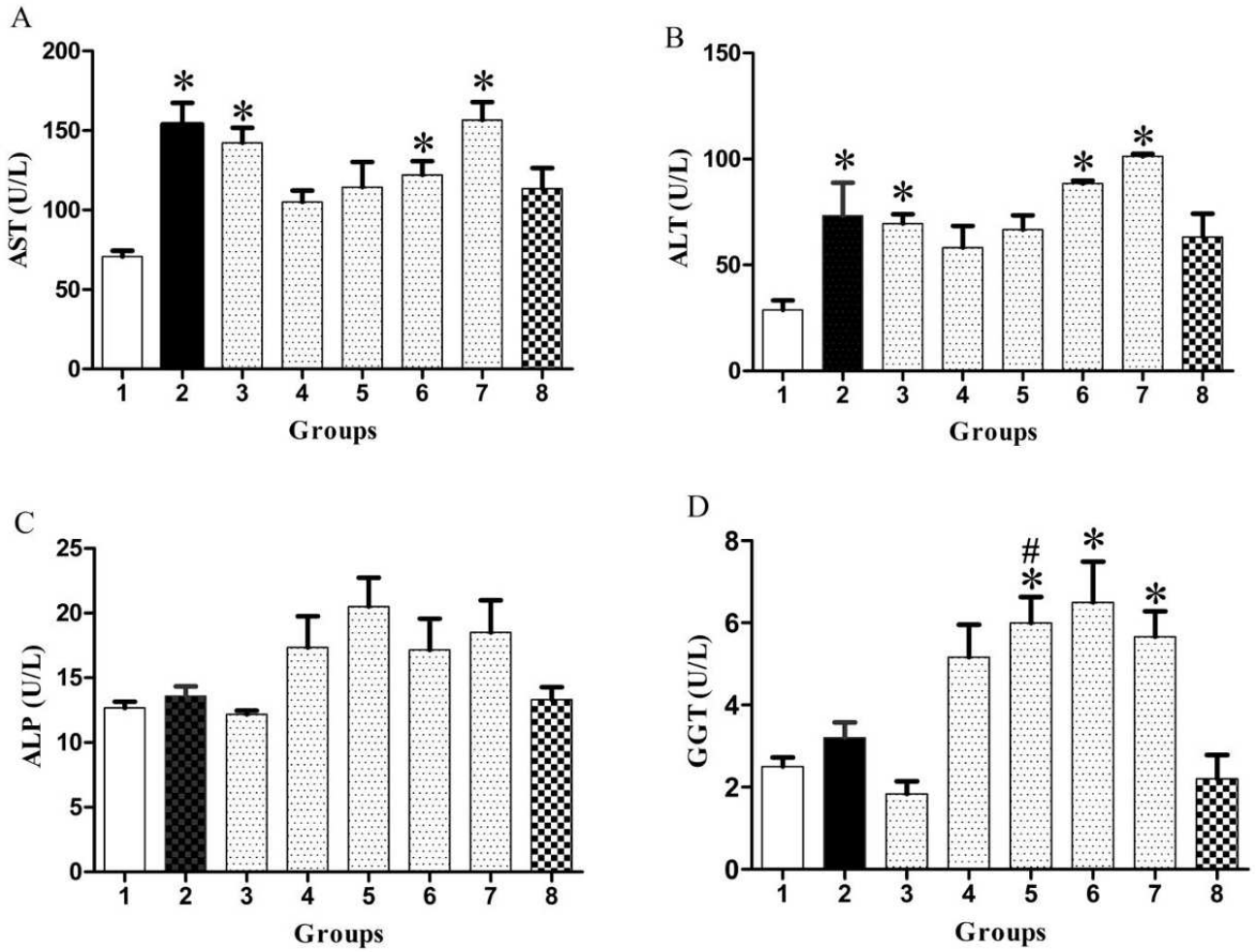


Figure 1. Plasma levels of liver injury biomarkers (U/L) in mice after 7 days of trial. **A.** AST = aspartate transaminase; **B.** ALT = alanine transaminase; **C.** ALP = alkaline phosphatase; **D.** GGT = gamma-glutamyltransferase. Group 1 = negative control; Group 2 = positive control (CCl_4); Group 3 (DSEAT 25 mg/kg + CCl_4); Group 4 (DSEAT 50 mg/kg + CCl_4); Group 5 (DSEAT 100 mg/kg + CCl_4); Group 6 (DSEAT 200 mg/kg + CCl_4); Group 7 (DSEAT 400 mg/kg + CCl_4); Group 8 (rosmarinic acid 18 mg/kg + CCl_4). Values are expressed as the mean ± standard error of the mean (mean ± SEM) (n = 6 mice per experimental Group). $^{*}p < 0.05$ compared to Group 1; $^{\#}p < 0.05$ compared to Group 2; CCl_4 = carbon tetrachlorinde; DSEAT = dry standardized extract of *Apeiba tibourbou* leaves.

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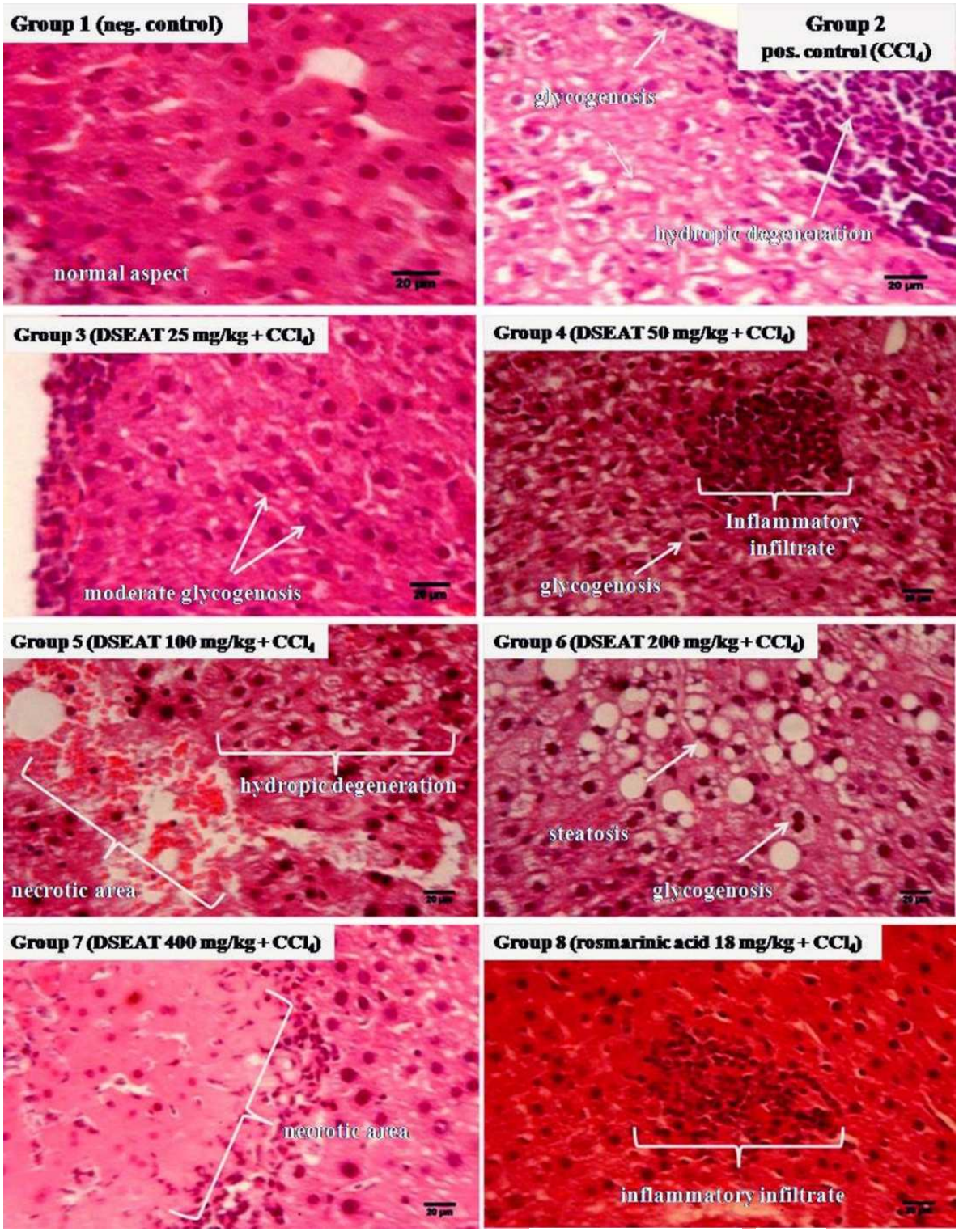


Figure 2. Liver from mice treated with dry standardized extract of *Apeiba tibourbou* leaves (DSEAT) and CCl_4 for 7 days. Arrows indicates the histological alterations (n = 6 mice per experimental group). Hematoxylin and eosin; scale bar = 20 µm.

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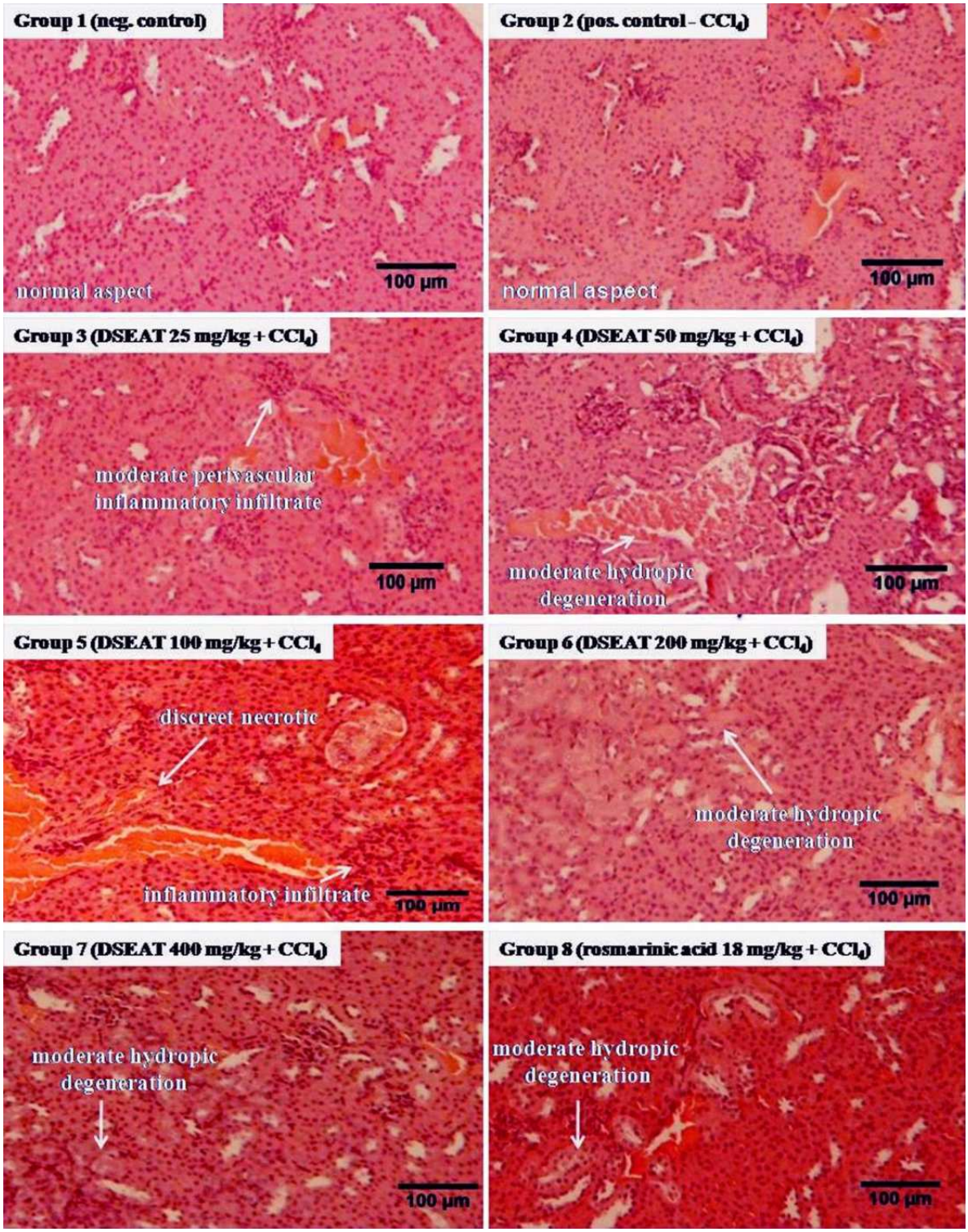


Figure 3. Kidney from mice treated with dry standardized extract of *Apeiba tibourbou* leaves (DSEAT) and CCl_4, for 7 days. Arrows indicates the histological alterations (n = 6 mice per experimental group). Hematoxylin and eosin; scale bar = 100 µm.

The animals pre-treated with DSEAT that underwent CCl_4-induced hepatotoxicity exhibited marked decrease in plasma level of MDA compared to Group 2 (Fig. 4B). This may have happened due to the presence of rosmarinic acid, a phenolic compound (derived from caffeic acid) with natural antioxidant activity which scavenges radical species found in plant tissues and in preventing CCL_4 induced hepatotoxicity by scavenging harmful free radicals and activating physiological defense mechanisms (16, 24). In the positive control (Group 2), MDA level in the liver increased by 222.30% compared to the negative control (Group 1) ($p < 0.05$) and the animals treated with DSEAT (groups 3, 5, 6, and 7) presented MDA levels in the liver higher or similar to Group 2, reaching an increase by 40.62% in Group 3 (Fig. 4A). Compared to the negative control (Group 1), MDA level in the liver increased by 353.23% in Group 3, presenting statistical significance ($p < 0.0001$).

In contrast, lower doses of DSEAT prevented and/or reduced complications in the gallbladder due to normalization of GGT levels (Group 3, 25 mg/kg), and reduced MDA levels in the liver by 27.45% (Group 4, 50 mg/kg) compared to Group 2 ($p < 0.05$) (Fig. 1D and Fig. 3, respectively). MDA reduction in plasma with simultaneous increase in the liver, in the groups treated with DSEAT, possibly occurred because this metabolite is produced in the presence of lesions on the cell membrane and binds to tissue organic compounds, therefore reaching highest level in the tissue than in the plasma.

The dose of 50 mg/kg proved to be the most favorable to reduce the harmful effects of CCl_4 in relation to AST and ALT, presenting significant antioxidant activity in liver samples (Fig. 1A and 1B). Similarly, rosmarinic acid (Group 8, 18 mg/kg) reduced oxidative aggressions in liver and plasma (Fig. 4A and 4B). Reacting with ROS, *in vitro,* rosmarinic acid protects tissues against lesions caused by CCl_4 (7, 23). Nevertheless, at higher doses, rosmarinic acid does not present the same protection, possibly due to the marked presence of other metabolites that may harm the action of this compound. Therefore, in the present study, the combination of DSEAT (at higher doses) with CCl_4 induced significant increase in ALT, AST, ALP, and GGT in plasma, as well as in MDA in liver tissue, with histological alterations in the liver and kidneys. However, at lower doses of DSEAT, it was possible to observe a significant tendency of its hepatoprotective effect.

The extract of *A. tibourbou* standardized with 6.5% rosmarinic acid was capable of protecting the liver against CCl_4-induced hepatotoxicity at low doses. However, further dose-response studies are necessary to verify whether high doses of this extract also induce hepatotoxicity, since in the histopathological exams we observed liver alterations ranging from discreet to severe. More detailed studies should be carried out in order to define the dose of this extract that produces the best result with no adverse reactions.

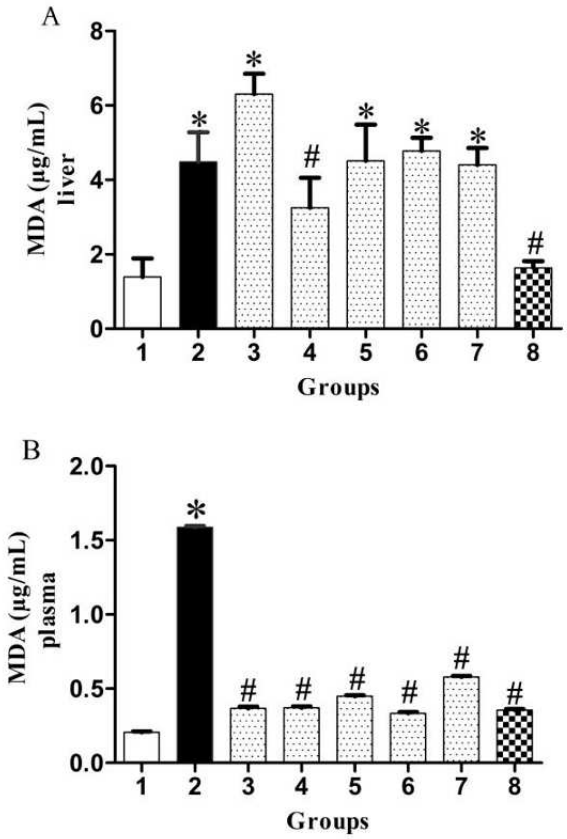


Figure 4. Effects of DSEAT on the levels of MDA in livers (µg/mL) (A) and MDA in plasma (µg/mL) (B). Group 1 = negative control; Group 2 = positive control (CCl_4); Group 3 (DSEAT 25 mg/kg + CCl_4); Group 4 (DSEAT 50 mg/kg + CCl_4); Group 5 (DSEAT 100 mg/kg + CCl_4); Group 6 (DSEAT 200 mg/kg + CCl_4); Group 7 (DSEAT 400 mg/kg + CCl_4); Group 8 (rosmarinic acid 18 mg/kg + CCl_4). MDA = malondialdehyde; CCl_4 = carbon tetrachloride; DSEAT = dry standardized extract of *Apeiba tibourbou* leaves. Values are expressed as the mean ± standard error of the mean (mean ± SEM) (n = 6 mice per experimental group). $^{*}p < 0.05$ compared to Group 1; $^{\#}p < 0.05$ compared to Group 2.

Conclusion

According to the method herein used, it is possible to conclude that both DSEAT standardized with 6.5% rosmarinic acid, at low doses, and pure rosmarinic acid exhibited, *in vivo* (mice) antioxidant activity. Compared to treated groups with DSEAT, the rosmarinic acid showed best response in the biochemistry assays evaluated in this study. Therefore, further studies are suggested to evaluate the activity of this substance isolated in other doses to affirm its hepatoprotective activity *in vivo*.

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