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A N   I N T E R N A T I O N A L   J O U R N A L

Bark Extract of *Bathysa cuspidata* in the Treatment of Liver Injury Induced by Carbon Tetrachloride in Rats

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ABSTRACT

The aim of this study was to investigate the effect of bark extract Bathysa cuspidata *(BCE) in the reconstitution of hepatic parenchyma and stroma after dysfunction induced by the CCl₄. Liver lesions were induced by intraperitoneal administration of CCl₄ (1 mL/kg) every 48 h for 12 days. The animals were treated with* B. cuspidata *extract (BCE) administered by gavage for another 12 days. Forty-nine rats were randomized into seven treatment groups with seven animals receiving CCl₄, BCE (200, or 400 mg/kg) and the vehicle DMSO alone, or in different combinations. The extract alone showed no evidence of hepatic toxicity. In general, rats acutely exposed to CCl₄ without the treatment with BCE presented high ALT and AST serum levels and high tissue content of lipid hydroperoxides, malondialdehyde, and lipid droplets. BCE administration, especially at 400 mg/kg attenuated significantly all these parameters. Furthermore, the antioxidant activities of the enzymes superoxide dismutase and catalase were significantly increased in the groups receiving this extract. These results showed that the extract of* B. cuspidata *stem bark stimulated the antioxidant defense system and reduced the morphological and functional liver damage in Wistar rats previously exposed to CCl₄.*

Key words: antioxidant defense, biotechnology, hepatotoxicity, phytotherapy, pathology

INTRODUCTION

The liver is an important organ responsible for various metabolic processes of detoxification of the organism through a complex enzyme system that regulates the antioxidant liver function (Sanmugapriya and Venkataraman 2006; Ranawat et al. 2010). Liver lesions often reduce its antioxidant defense function and the susceptibility of the organism during exposure to the xenobiotics (Lee et al. 2003). Numerous chemical substances used industrially and pharmacologically may result in an increase over the time the risk of liver lesions. These substances are responsible for a broad spectrum of hepatotoxic effects (Roome et al. 2008). A large percentage of liver disorders occur as a result of exposure to chemical compounds capable of inducing the oxidative stress, which leads to an increase in the production of radical compounds. These compounds may cause oxidative damage to bio-molecules such as proteins and lipids present in the cell membranes, culminating in partial or total loss of cell function (Faremi et al. 2008). In

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view of its ability to induce the lesions by increasing the chemically reactive species, primarily through the CCl_3- radical, carbon tetrachloride (CCl_4) has been widely used in experimental models of liver toxicity (Brattin et al. 1985; Lee et al. 2003; Hwang et al. 2009). Moreover, it constitutes a reproducible, inexpensive model in which effects are rapid, thus ensuring its frequent use when investigating the effects of vegetable extracts on liver disorders (Khan and Ahmed 2009; Sreelatha et al. 2009).

The treatment of human diseases using the medicinal plants and their derivatives is an ancient practice that is currently gaining popularity worldwide (Eldin and Dunford 2001). At present, there is considerable interest in natural plant products, particularly those used in traditional medicine. These products may be useful in the development of new drug and applications, including liver diseases induced by the toxic agents (Shen et al. 2009; Ye et al. 2009). This type of investigation has been shown to represent an effective strategy for selecting the medicinal plants for the development of new drugs, since around 80% of the world's population uses the products derived from the plants in their basic healthcare (Farnsworth 1994; Kaur et al. 2005).

Bathysa cuspidata (A. St. Hil.) Hook f. belongs to the Rubiaceae family. The genus *Bathysa* C. Presl includes approximately 15 species of the trees, small trees and scrubs that are found in Panama, French Guyana, Venezuela, Colombia, Peru, Bolivia and Brazil. In Brazil, there are seven species, all in the South and Southeast region of the Atlantic Rain Forest: *B. mendoncaei, B. cuspidata, B. gymnocarpa, B. sylvestrae, B. autralis, B. stipulata and B. nicholsonii* (Germano-Filho 1999). *B. gymnocarpa, B. sylvestrae, B. australis, B. stipulate and B. nicholsonii. B. cuspidata* is a plant popularly known in Brazil as "quina-do-mato" and its bark is used in popular medicine for the treatment of stomach and liver disorders, as well as a healing agent. Studies with an ethanolic extract and the dichloromethane fraction of *B. cuspidata* have shown antinociceptive and antiinflammatory effects in albino mice previously treated enterally (Correa 1984). In toxicity tests, ethanolic extracts of the leaves and bark of this species showed no mutagenic effects at the doses tested (Nunes 2008). Studies have demonstrated the preventive effect of the bark extract of *B. cuspidata* in the injuries to the liver and lung, but its effect in the

treatment of previously established lesions remains poorly understood.

Thus, this study used a stereological approach and biochemical analysis to investigate the morpho-functional adaptations in the liver of Wistar rats exposed to CCl_4 after treatment with the bark extract of *B. cuspidata*. Considering the relationship between the hepatic structure and function, this study investigated the hypothesis that the bark extract of *B. cuspidata* could have the ability to reconstitute the hepatic parenchyma and stroma after induction of lesions by CCl_4.

MATERIALS AND METHODS

Preparation and phytochemical analysis of *B. cuspidata*

The samples of *B. cuspidata* were collected from a biome of Brazilian Atlantic forest in the municipality of Araponga in the state of Minas Gerais, Brazil (20°43`00.0``S e 42°29`10.8``W, 1.200 m altitude) and adequately documented in the herbarium of the Federal University of Viçosa under registration no VIC 21559. Stem bark samples were separated, dried for 48 h at room temperature in a darkened, well-ventilated room, pulverized in a knife mill and stored. The powered air-dried stem bark of *B. cuspidata* (500g) was extracted exhaustively with ethanol (95%) by percolation method. The extract was concentrated under vacuum at 50°C using a rotary evaporator and then lyophilized until complete removal of the solvent, yielding an ethanolic extract (139 g). Phytochemistry of the extract was performed on chromatography plates coated with silica gel GF 254® (Merck, Darmstadt, Germany) using different mobile phases and detection reagents in accordance with the protocol described by Wagner and Bladts (1996).

Estimation the total amount of total phenolics, proanthocyanidin and total flavonoids

For the determination of total phenolics and proanthocyanidin contents, powdered stem bark (1.0 g) was extracted with 200 mL of water at 100°C under reflux for 30 min. After heating, the extract was cooled and transferred to a 250 mL volumetric flask and made up to the volume with water. The extract was left to stand and filtered through the qualitative filter paper. The filtrate was used for the quantification of total polyphenols and of proanthocyanidin contents.

The concentration of total phenolics was determined colorimetrically, using the Folin-Ciocalteau method, as described by Verza et al. (2007).

The estimation of total phenolics was expressed as milligrams of pirogalol equivalents per gram of dry matter (dm). For this purpose, 5.0 mL of filtrate was transferred to a 50 mL volumetric flask and made up to the volume with water. A 1.0 mL aliquot of this extract was added to 6.0 mL of distilled water, followed by the addition of 2.0 mL of Folin-Ciocalteu reagent and 1.0 mL of an aqueous 15% solution of sodium carbonate. After 30 min at ambient temperature, the absorbance was measured at 760 nm using a UV-visible spectrophotometer.

The proanthocyanidin content was determined by the procedure of Price et al. (1980). One milliliter of the filtrate was mixed with 5.0 mL of the 1% vanillin reagent (equal parts of vanillin-methanol solution and 8% hydrochloric acid). The mixture was allowed to stand for 20 min. The absorbance of the sample and standard were measured at 500 nm and the proanthocyanidin was expressed as the milligrams of catechin equivalents per gram of dry matter. The total content of flavonoids was determined by using rutin as a reference compound. This method was based on the formation of flavonoid-aluminium trichloride complexes with a maximum absorption at 420 nm. It was added to 5.0 mL of methanol extract (1.0 g per 200 mL methanol) and then 0.6 mL of acetic acid, 10 mL of methanol, 2.5 mL of aluminum chloride hexahydrate in 8% methanol were added and made up to a total volume of 25 mL with distilled water. After a 15-minute interval, the absorbance was read on a spectrophotometer. The absorption of a standard rutin solution in methanol was measured under the same conditions. All the determinations were performed in triplicate and the results were averaged.

Animals

Male Wistar rats with a mean weight of 235g were kept in an experimental animal house under the controlled light (12 h light/darkness cycles) and temperature ($21 \pm 2^{\circ}C$) conditions, with relative air humidity of 60 to 70%. Water and standard rat chow were supplied *ad libitum*. All the procedures involved in the experimental protocol were approved by the Ethics Committee for the Care and Use of Laboratory Animals of the Federal University of Minas Gerais (UFMG) and were in accordance with the Guiding Principles in the Use of Animals in Toxicology adopted by the American Society of Toxicology in July, 1989 and revised in December 2008.

Chemicals

Carbon tetrachloride (CCl_4), dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA), formaldehyde and glutaraldehyde were acquired from the Sigma Chemical Co. (St. Louis, MO, USA). The diagnostic kits used in the evaluation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT) and alkaline phosphatase (ALP) were acquired from human *in vitro* diagnostics (Itabira, Brazil) and the kits for bilirubin measurement were obtained from Labtest (São Paulo, Brazil). Superoxide dismutase (SOD) activity was measured using a commercial kit (Cayman Chemical Company, Ann Arbor, MI, USA).

Experiment

Liver lesions were induced in the animals in groups 1, 2, 3, 5 and 6 by intraperitoneal administration of CCl_4 (1.0 mL/kg, 60% in olive oil, v/v) every 48 h for 12 days. The ethanolic extract of the stem bark of *B. cuspidata* (BCE) was re-suspended in 700 µL DMSO (w/v), and administered to the animals by gavage. The treatment with this extract at doses of either 200 mg/kg of body weight (BCE 200), or 400 mg/kg of body weight (BCE 400) began immediately after the final application of CCl_4 and was given daily for 12 days. The same protocol used for the groups receiving the extract was followed in the group receiving DMSO vehicle alone. Forty-nine rats were randomized into seven treatment groups with seven animals in each. G1: CCl_4 + 12 days without treatment; G2: CCl_4; G3: CCl_4 + DMSO (700 µL); G4: BCE 400; G5: CCl_4 + BCE 200; G6: CCl_4 +BCE 400; and G7: only DMSO (700 µL). All the animal groups were euthanized 48h following the administration of the final treatment, except G1, which was euthanized 12 days after the termination of CCl_4 administration to investigate the spontaneous recovery of the liver tissue.

Biochemical analysis and histopathology

Forty-eight hours following the end of treatment, the animals were anesthetized with ketamine (10 mg/kg of body weight) and xylazine (2.0 mg/kg of body weight) and euthanized by cardiac puncture.

Aliquots of the blood were collected and centrifuged at 3000 rpm for 15 min and the serum was immediately used for the biochemical measurements of AST, ALT, ALP, GGT and bilirubin levels. Serum samples were processed in accordance with the instructions supplied by the manufacturers of the diagnostic kits. For histopathological analysis and biochemical evaluation of the liver tissue, a median incision was made and the liver was removed intact and weighed using the analytical scales. Then two fragments of the liver of each animal were quickly removed, one of which was frozen in liquid nitrogen (-196°C) and the other immersed in Karnovsky's fixative solution. Aliquots of 500 mg of the frozen liver fragments were homogenized in phosphate buffered saline (PBS) and centrifuged at 5000 rpm at 5°C. The supernatant was then used for the analysis of superoxide dismutase (SOD) and catalase (CAT). Catalase was evaluated according to Aebi's method (1984) by measuring the rate of decomposition of hydrogen peroxide (H_2O_2). Lipid peroxidation in the liver homogenate was assessed by malondialdehyde (MDA) detection as previously described by Gutteridge and Halliwell (1990) and by measuring the lipid hydroperoxides using the methodology of Nourooz-Zadeh et al. (1994). Total protein levels were measured using the Bradford method (Bradford 1976).

For histopathology, the fragments of liver were fixed in Karnovsky's solution for 24 h, then dehydrated in ethanol and embedded in methacrylate (Leica, Germany). Sections (4 µm thick) were cut with a Multicut 2045® rotary microtome (Reichert-Jung, Germany), stained in toluidine blue and hematoxylin-eosin, and mounted using Entellan® mounting medium (Merck, Germany). Seventy photomicrographs taken under 20x magnification were obtained for each group with the use of a BX-60® light microscope (Olympus, Tokyo, Japan). Digitalized images were used to detect the presence of inflammatory infiltrate and areas of necrosis. Histomorphometry was performed to evaluate the lipid droplets within the hepatocytes (% of the histological area) using the Image Pro-plus 4.5® software program (Media Cybernetics, Silver Spring, USA) (Azevedo et al. 2010). The liver somatic index (LSI), which reflected the weight of the liver as a percentage of total body weight, was calculated in accordance with the following ratio: LSI = WL/BW x 100 in which WL was the weight of the liver and BW represented the total body weight.

Statistical analysis

The data were expressed as the measures of central trend, means and standard deviations (S.D.). The normalcy of the distribution of the data was verified using the Kolmogorov-Smirnov test. Based on this test, the biochemical data were submitted to unifactorial analysis of variance, one-way ANOVA, followed by the Tukey's test for multiple comparisons. In addition, the Kruskal-Wallis test was used to analyze the histological area of the lipid droplets, with statistical significance established at $p < 0.05$. All the tests were performed using the GraphPad Prism 5.0® statistical software program (GraphPad Software, Inc, California, USA).

RESULTS

Phytochemical analysis

The total phenol and proanthocyanidin content was 58.7 mg/g of dry matter (expressed as pirogalol) and 37.9 mg/g dry matter (expressed as catechin), respectively. The total flavonoid content was 3.4 mg/g dry matter using the rutin calibration curve. The curative action of the BCE could be associated with the presence of secondary metabolites with antioxidant effects, primarily high levels of phenolic compounds.

Body weight and liver weight

As shown in Table 1, there was no significant variation in the initial weight of the animals in the different groups. The final weight of the animals in group 2 was significantly lower than that of the animals in the other groups ($p < 0.05$). The LSI was also significantly higher in the animals in groups 1, 2 and 3 compared to the groups that received BCE. There were no statistically significant differences in these variables between the animals in the groups treated with the extract.

Biochemical parameters

The animals in the groups 1, 3 and 5 had lower serum ALT levels compared to those in the group 2 ($p < 0.05$), whereas the animals in the groups 4, 6 and 7 had lower serum ALT levels compared to those in the other groups ($p < 0.05$). The animals in the group 2 had higher serum AST levels compared to the animals in the other groups ($p < 0.05$). Groups 1, 3 and 6 did not differ among

themselves but differed from the groups 4 and 5, which showed the lowest values of this enzyme in the blood. Group 7 differed only from the group 2. There were no statistically significant differences in ALP, or GGT levels between the groups (Table 2). Direct bilirubin levels were significantly higher in the group 2, which differed from the groups 1, 3 and 4 ($p < 0.05$) (Table 3).

Lipid peroxidation induced by the CCl_4 in the rat liver (Table 4) led to a significant increase in the quantity of hydroperoxides ($p < 0.05$) in the animals in the groups 1 and 2 that received CCl_4 alone. Values of this marker remained low in the groups 4 and 7 that received BCE and just DMSO. In the group 5, there was a reduction in lipid peroxidation markers following the administration of the extract. Similar results were found in the group 6 ($p < 0.05$). With respect to malondialdehyde, no statistically significant differences were found between the animals in the 1, 2, 3, 4 and 7. On the other hand, there was a reduction in this marker in the animals in the groups 5 and 6 compared to the other groups ($p < 0.05$).

Table 1 - Biometric parameters of rats exposed to CCl_4 (60% v/v, 1 mL/kg) and treated with the bark extract of *B. cuspidata* (200 and 400 mg/kg).

Groups / Treatment	Initial Weight (g)	Final weight (g)	Weight variation (g)*	LSI (%)
G1/ CCl_4+12	251.71 ± 09.69^a	236.85 ± 17.04^a	-14.86 ± 14.91^a	5.17 ± 0.37^a
G2/ CCl_4	271.42 ± 20.55^a	212.85 ± 28.11^b	-58.57 ± 34.96^b	5.54 ± 0.31^a
G3/ CCl_4+DMSO	257.85 ± 11.49^a	237.14 ± 10.59^a	-20.71 ± 17.42^a	5.02 ± 0.54^a
G4/ BCE 400	250.71 ± 10.57^a	252.85 ± 19.54^a	2.14 ± 13.49^a	4.01 ± 0.43^b
G5/CCl_4+BCE 200	249.71 ± 08.86^a	252.46 ± 09.06^a	2.75 ± 9.06^a	4.05 ± 0.50^b
G6/CCl_4+BCE 400	274.28 ± 17.66^a	263.57 ± 22.67^a	-11.00 ± 31.28^a	4.12 ± 0.61^b
G7/ DMSO	261.45 ± 13.49^a	264.33 ± 11.52^a	-2.88 ± 8.73^a	4.27 ± 0.46^b

BCE= *Bathysa cuspidata* extract; LSI= Liver somatic index. (*)Negative sign indicates weight loss. G1= CCl_4 + 12 days; G2= CCl_4; G3= CCl_4 + DMSO; G_4= BCE 400; G5= CCl_4 + BCE 200; G6= CCl_4+ BCE 400; G7= DMSO. Data are expressed as mean $\pm$ S.D. [a,b] Different letters in the columns indicate statistical difference between groups (p 0.05), ANOVA one-way.

Table 2 - Serum levels of transaminases in rats exposed to CCl_4 (60% v/v, 1 mL/kg) and treated with bark extract of *B. cuspidata* (200 and 400 mg/kg).

Groups/ Treatment	ALT (U/l)	AST (U/l)	ALP (U/l)	GGT (U/l)
G1/ CCl_4+12	121.71 ± 31.13^a	166.57 ± 42.34^a	244.00 ± 63.39^a	5.28 ± 1.89^a
G2/ CCl_4	217.42 ± 57.68^b	718.71 ± 318.02^b	234.14 ± 133.77^a	5.14 ± 1.06^a
G3/ CCl_4+DMSO	127.57 ± 50.73^a	166.14 ± 44.99^a	289.57 ± 97.82^a	3.71 ± 0.75^a
G4/ BCE 400	47.14 ± 2.19^c	82.00 ± 2.82^c	198.42 ± 9.16^a	2.00 ± 0.01^a
G5/ CCl_4+BCE 200	74.42 ± 10.78^a	72.28 ± 15.35^c	189.00 ± 38.23^a	2.57 ± 0.97^a
G6/ CCl_4+BCE 400	49.71 ± 5.28^c	113.00 ± 15.73^a	187.71 ± 36.39^a	2.85 ± 1.06^a
G7/ DMSO	52.11 ± 6.09^c	$90.27 \pm 20.18^{a,c}$	225.40 ± 42.37^a	3.80 ± 1.39^a

BCE= *Bathysa cuspidata* extract; ALT= alanine aminotransferase; AST= aspartate aminotransferase; ALP= alkaline phosphatase; GGT= γ-glutamyl transferase. G1= CCl_4 + 12 days; G2= CCl_4; G3= CCl_4 + DMSO; G_4= BCE 400; G5= CCl_4 + BCE 200; G6= CCl_4+ BCE 400; G7= DMSO. Data are expressed as mean $\pm$ SD. [a,b,c] Different letters in the columns indicate statistical difference between groups (p 0.05), ANOVA one-way.

Table 3 - Serum levels of total bilirubin and fractions in rats exposed to CCl_4 (60% v/v, 1 mL/kg) and treated with the bark extract of *B. cuspidata* (200 and 400 mg/kg).

Groups/ Treatment	D-bilirubin (mg/dl)	I-bilirubin (mg/dl)	T-bilirubin (mg/dl)
G1/ CCl_4+12	0.037 ± 0.018^a	0.523 ± 0.484^a	0.560 ± 0.491^a
G2/ CCl_4	0.137 ± 0.116^b	0.489 ± 0.332^a	0.626 ± 0.343^a
G3/ CCl_4+DMSO	0.043 ± 0.014^a	0.331 ± 0.542^a	0.374 ± 0.550^a
G4/ BCE 400	0.013 ± 0.005^a	0.187 ± 0.017^a	0.200 ± 0.016^a
G5/ CCl_4+BCE 200	$0.054 \pm 0.030^{a,b}$	0.123 ± 0.051^a	0.177 ± 0.051^a
G6/ CCl_4+BCE 400	$0.054 \pm 0.034^{a,b}$	0.197 ± 0.067^a	0.250 ± 0.078^a
G7/ DMSO	$0.032 \pm 0.012^{a,b}$	0.122 ± 0.074^a	0.155 ± 0.074^a

BCE= *Bathysa cuspidata* extract, D-bilirubin= direct bilirubin, I-bilirubin= indirect bilirubin, T-bilirubin= total bilirubin. G1= CCl_4 + 12 days; G2= CCl_4; G3= CCl_4 + DMSO; G4= BCE 400; G5= CCl_4 + BCE 200; G6= CCl_4+ BCE 400; G7= DMSO. Data are expressed as mean $\pm$ S.D. [a,b,c] Different letters in the columns indicate statistical difference between groups (p 0.05), ANOVA one-way.

Table 4 - Hydroperoxides and Malondialdehyde levels in the liver tissue from rats exposed to CCl_4 (60% v/v, 1 mL/kg) and treated with the bark extract of *B. cuspidata* (200 and 400 mg/kg).

Groups/Treatment	HPX (µg/mg protein)	MDA (µg/mg protein)
G1/ CCl_4+12	654.52 ± 103.08[a]	0.026 ± 0.018[a]
G2/ CCl_4	755.07 ± 276.91[a]	0.030 ± 0.019[a]
G3/ CCl_4+DMSO	187.04 ± 27.45[b]	0.013 ± 0.012[a]
G4/ BCE 400	82.27 ± 2.92[d]	0.021 ± 0.007[a]
G5/ CCl_4+BCE 200	72.23 ± 15.33[d]	0.001 ± 0.000 [b]
G6/ CCl_4+BCE 400	97.64 ± 3.94[c]	0.00 ± 0.00[b]
G7/ DMSO	76.39 ± 10.25[d]	0.02 ± 0.01[a]

BCE= *Bathysa cuspidata* extract, HPX= hidroperoxides, MDA= Malondialdehyde. G1= CCl_4 + 12 days; G2= CCl_4; G3= CCl_4 + DMSO; G4= BCE 400; G5= CCl_4 + BCE 200; G6= CCl_4+ BCE 400; G7= DMSO. Data are expressed as mean ± S.D. [a,b,c] Different letters in the columns indicate statistical difference between groups (p 0.05), ANOVA one-way.

Antioxidant enzymes

Regarding the levels of SOD, the lowest values were found in the groups 1 and 2; however, in the groups 5 and 6, the SOD levels were significantly higher when compared to the animals in the groups 1, 2 and 3 ($p<0.05$). In the groups 4 and 7, in which the animals received the extract alone, or DMSO respectively without CCl_4, SOD levels were higher compared to the other groups ($p<0.05$) (Fig.1). The CAT activity was significantly lower in the group 2, compared to the other groups ($p<0.05$) but it was significantly lower in the groups 1, 3, 4 and 7 compared to the groups 5 and 6 ($p<0.05$) (Fig. 2).
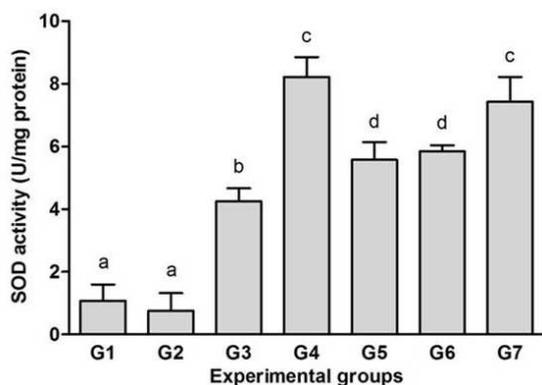

Figure 1 - Activity of Superoxide dismutase (SOD) in the liver tissue from rats exposed to CCl_4 (60% v/v, 1 mL/kg) and treated with the bark extract of *Bathysa cuspidata* (200 and 400 mg/kg). G1= CCl_4 + 12 days; G2= CCl_4; G3= CCl_4+DMSO; G4= BCE 400; G5= CCl_4 + BCE 200; G6= CCl_4 + BCE 400; G7= DMSO. The data are expressed as means ± S.D. [a,b,c,d] Different letters indicate statistical significance between the groups (p 0.05), ANOVA one-way.
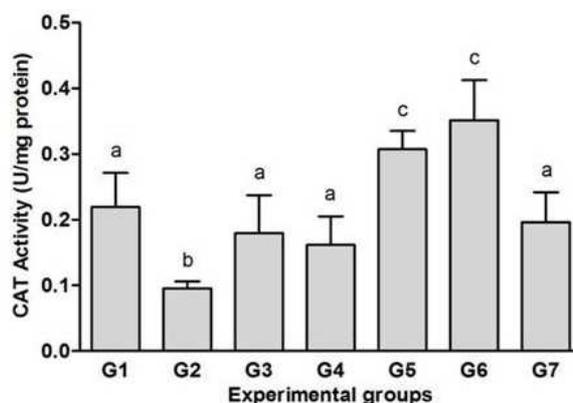

Figure 2 - Activity of Catalase (CAT) in the liver tissue from rats exposed to CCl_4 (60% v/v, 1 mL/kg) and treated with the bark extract of *Bathysa cuspidata* (200 and 400 mg/kg). G1= CCl_4+12 days; G2= CCl_4; G3= CCl_4+DMSO; G_4= BCE 400; G5= CCl_4+ BCE 200; G6= CCl_4+ BCE 400; G7=DMSO. The data are expressed as means ± S.D. [a,b,c] Different letters indicate statistical significance between the groups (p 0.05), ANOVA one-way.

Histopathology

Histopathological alterations such as the accumulation of lipid droplets were found in the three groups in which the animals were not given BCE. Figure 3 showed that the animals in the group 2 had a significantly higher ($p<0.05$) percent area of lipid droplets and inflammatory foci compared to the other groups. Figure 4 showed the main changes in the centrolobular region, which were significantly reduced with BCE treatment. In the animals in the groups 1 and 3, the area of lipid droplet formation was significantly larger compared to the groups 4, 5, 6 and 7 ($p<0.05$).
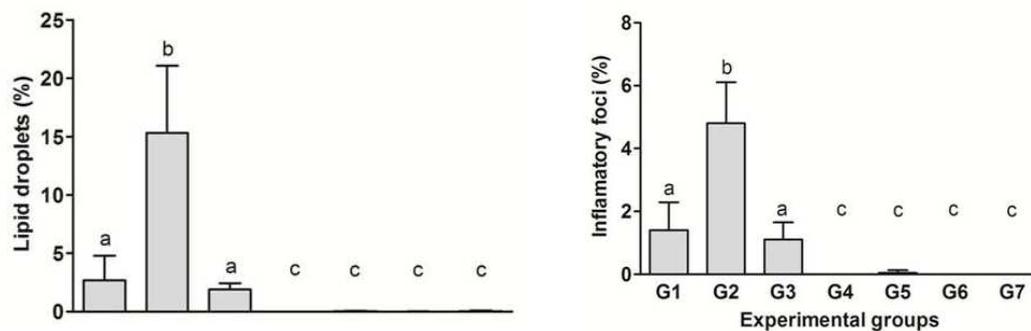
Figure 3 - Distribution of lipid droplets and inflammatory foci in the liver tissue from rats exposed to CCl$_4$ (60% v/v, 1 mL/kg) and treated with the bark extract of *Bathysa cuspidata* (200 and 400 mg/kg). G1= CCl$_4$ + 12 days; G2= CCl$_4$; G3= CCl$_4$ + DMSO; G4= BCE 400; G5= CCl$_4$ + BCE 200; G6= CCl$_4$ + BCE 400; G7= DMSO. The data are expressed as means ± S.D. [a,b,c] Different letters indicate statistical significance between the groups (p 0.05), Kruskal-Wallis test.
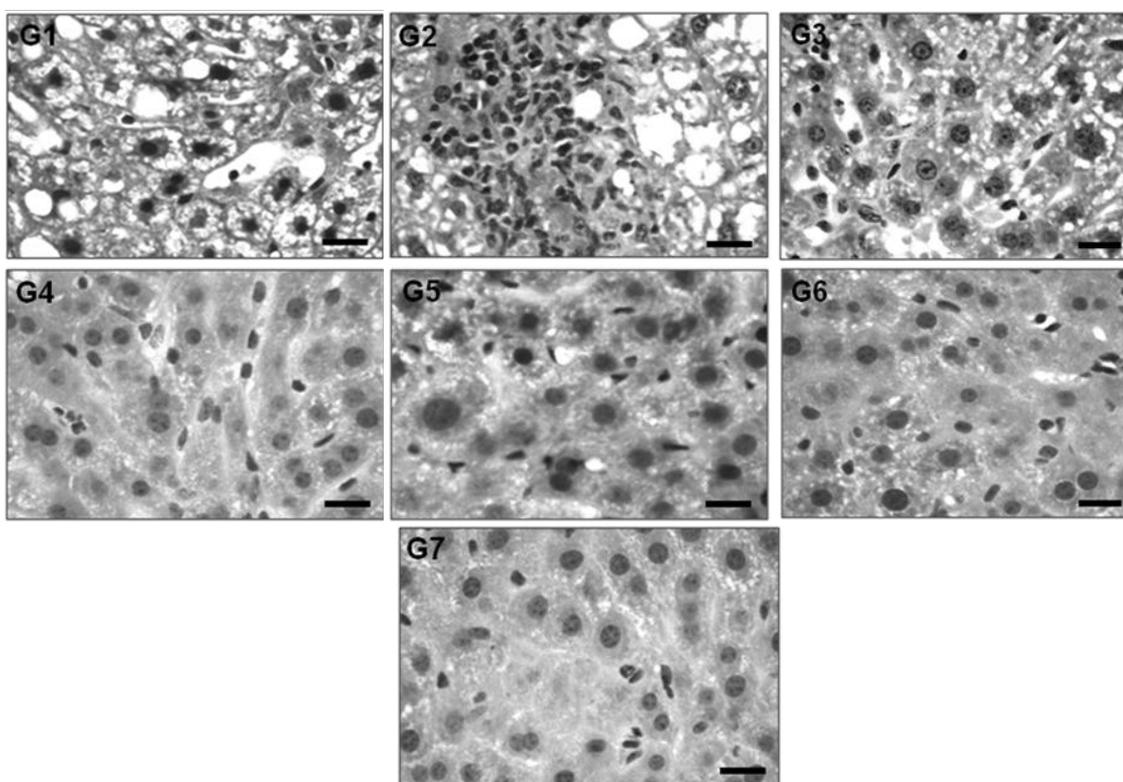

Figure 4 – Representative photomicrography's of the liver tissue from rats exposed to CCl$_4$ (60% v/v, 1 mL/kg) and treated with the bark extract of *Bathysa cuspidata* (200 and 400 mg/kg). Note in G1, G2 and G3 intense damage of the liver tissue with evident lipid droplets deposition. A marked inflammatory infiltrate is observed in G2. G1= CCl$_4$ + 12 days; G2= CCl$_4$; G3= CCl$_4$ + DMSO; G4= BCE 400; G5= CCl$_4$ + BCE 200; G6= CCl$_4$ + BCE 400; G7= DMSO.

DISCUSSION

CCl$_4$ is a known hepatotoxic agent that induces the disorders through the formation of a trichloromethyl radical (CCl$_3$-) and its peroxyl radical derivative, CCl$_3$OO-, formed during the metabolism by the phase 1 enzymes present in the endoplasmic reticulum of hepatocytes. The CCl$_3$-

and CCl$_3$OO- radicals oxidize the unsaturated fatty acids in the plasma membrane of the hepatocytes and cell organelles, leading to the production of lipid peroxyl radicals (LOO-), alkoxy radicals (RO-) and malondialdehyde (MDA), which propagate the oxidative damage in a process, called lipid peroxidation (Wu et al. 2007; Janakat et al. 2008). In addition to their direct toxic effect, lipid peroxides activate an immuno-inflammatory response mediated by the neutrophil chemotaxis, activation of cytokines, chemokines and phospholipases. Both the pathological mechanisms lead to morphological and functional alterations and may result in cell death (Brattin et al. 1985; Geier et al. 2003). In addition to CCl$_4$ exerting an effect on the liver, systemic manifestations such as anorexia, lethargy, malnutrition, and progressive weight loss are common and fibrosis (Kono et al. 2003). In the present study, only the animals in the group 2, euthanized 48 h after the administration of the final dose of CCl$_4$, experienced a significant reduction in body weight. In addition, the highest indexes of liver hypertrophy, as determined by the LSI, were found in the groups that received CCl$_4$. No variations in the above mentioned variables were found in the groups treated with the extract, or in those treated with the vehicle alone (DMSO). This finding suggested that, although the extract did not contribute towards the increase in body weight gain, it might help reduce the weight loss and liver atrophy induced by CCl$_4$.

From the biochemical point of view, due to the alterations in the membrane permeability and cell death triggered by the lipid peroxidation and inflammatory mediators, increases occur in the serum levels of liver-specific enzymes. Increased levels of enzymes such as ALT, AST, ALP and GGT are indicative of loss of functional integrity of the hepatocyte cell membranes and of liver lesions (Ozer et al. 2008; Ranawat et al. 2010). Moreover, bilirubin evaluation constituted a supplementary method of investigating liver lesions triggered by the CCl$_4$, since this liver toxin is capable of increasing the bilirubin levels by inducing the hepatocellular necrosis and cholestasis (Sanmugapriya and Venkataram 2006; Kumar et al. 2009). The present study revealed an increase in ALT, AST and direct bilirubin in the groups that received CCl$_4$ alone, indicating considerable hepatocellular lesioning, which was confirmed by the histopathology. The administration of *B. cuspidata* extract at the doses

used (200 and 400 mg/kg) attenuated the increase in serum levels of these enzymes, resulting in subsequent normalization of these parameters compared to the animals in the groups treated with CCl$_4$ alone, confirming the curative effect of BCE. The experimental model used in this study showed that after the application of CCl$_4$, increases in the levels of these markers occurred, and that 12 days were required to cause significant reductions in the levels of these enzymes (as seen from the values of G1 and G2).

The curative effect of the bark extract of *B. cuspidata* could be observed from the changes in the parameters of lipid peroxidation and the activity of SOD, an important antioxidant enzyme that catalyzed the conversion of superoxide radicals (O$_2$-) to molecular oxygen (O$_2$) and oxygen peroxide (H$_2$O$_2$). In the presence of a liver lesion provoked by the CCl$_4$, the SOD and MDA levels reflected the amount of stress caused by the release of free radicals *in vivo*. As a result of the inflammatory process triggered by the CCl$_4$, the activation of macrophages, neutrophils and hepatic stellate cells occurs, producing a large amount of O$_2$- and, consequently, MDA. This increase in O$_2$- increases the SOD consumption, resulting in a decrease in its tissue levels (Raja et al. 2007; Hwang et al. 2009). In the present study, animals that received BCE (Groups 5 and 6) had lower levels of MDA compared to those in the other groups, which demonstrated the efficacy of this extract in neutralizing the free radicals and protecting the cell membranes from peroxidation. There was a significant reduction in the activity of the SOD enzyme in the animals in the groups 1 and 2, probably associated with the increase in oxidative stress provoked by the CCl$_4$. The decrease in the SOD was reversed in all the treatment groups, particularly those in which the animals received the plant extract in its different concentrations. The highest SOD values were found in the group that received the plant extract alone (group 4), showing that the extract of *B. cuspidata* stem bark stimulated the SOD activity even in the absence of harmful stimuli. The SOD activity was high in the groups G4 and G7; however, these groups had not previously received CCl$_4$. Similar results for the SOD and MDA have been found for the plants such as *Solanum* (false "Jurubeba), *Artemisia capillaries,* and *Decalepis hamiltonii,* among others, after the application of CCl$_4$ (Dani et al. 2009; Wang et al. 2010; Kuo et al. 2010). Catalase, which catalyzes the

degradation of H_2O_2 to molecular oxygen and water, constituted an additional mechanism of antioxidant defense that is supplementary to that of the SOD activity (Hu et al. 2008). In the present study, CAT activity was lowest in the animals in the group 2, but was also lower in the groups 1, 3, 4 and 7 compared to the groups 5 and 6 that received BCE at both doses. This finding indicated the positive modulating effect of the extract on this enzyme, primarily in the presence of harmful liver stimuli.

In addition to modifications in the antioxidant enzymatic system, treatment with the CCl_4 promotes a profound change in the histological architecture of the liver, provoking hemorrhage, leukocyte infiltration, edema, fibrosis, steatosis and necrosis (Kumar et al. 2009; Srivastava and Shivanandappa 2010). In this study, the histological damage to the organ was measured as a function of fat deposition on the liver tissue, which was higher in the groups 1, 2 and 3. It has been well established that CCl_4 interferes with lipoprotein synthesis as a result of lipid peroxidation and oxidative damage to proteins in the smooth endoplasmic reticulum of hepatocytes, the consequence of this being the accumulation of lipids in the cytoplasm of these cells (microvesicular steatosis), central lobular necrosis and fibrosis (Dani et al. 2008). Liver structure was normal in the groups treated with the bark extract of *B. cuspidata* insofar as the arrangement of the cords of hepatocytes, vascularization and disposition of connective tissue were concerned, thereby indicating the efficacy of the BCE treatment, following the exposure of the liver tissue to CCl_4. The architecture of the liver tissue was also normal in the animals of group 4, showing that the administration of the extract alone did not provoke fat deposition in the liver tissue. Together with the analysis of the oxidative stress markers, this finding suggested that the extract helped regulate lipid metabolism by stimulating the enzymatic mechanisms that controlled the oxidative cell damage.

These results showed that the BCE stimulated the antioxidant defense system and reduced the morphological and functional liver damage in the rats exposed to CCl_4. Thus, even after severe injuries induced by the CCl_4, the extract exhibited remarkable activity in restoring the normal morpho-functional characteristics of the liver. Considering these effects, *B. cuspidata* might potentially play a significant role in the discovery

of new drugs for the treatment of liver disease, confirming, in this case, its traditional use in complementary and alternative medicine.

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