Protective Effects of Amburoside A, a Phenol Glucoside from *Amburana cearensis*, against CCl_4-Induced Hepatotoxicity in Rats

Author

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Key words
- Amburoside A
- *Amburana cearensis*
- Fabaceae
- hepatoprotective activity
- carbon tetrachloride toxicity

Abstract

The aim of this study was to investigate the possible beneficial effects of amburoside A, AMB [4-(O-β-D-glycopyranosyl)benzyl protocatechoate], against carbon tetrachloride (CCl_4) toxicity in rats. AMB is a phenol glucoside from the Brazilian medicinal plant *Amburana cearensis*, popularly used for the treatment of respiratory tract affections. Acute AMB (25 and 50 mg/kg, *i.p.* or *p.o.*) treatments of CCl_4-intoxicated rats significantly inhibited the increase in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, as compared to the group treated with CCl_4 only. Histological studies showed less centrolobular necrosis and inflammatory cell infiltrates in the liver of animals treated with AMB plus CCl_4, when compared to the group treated with CCl_4 alone. In hepatic tissues, AMB at both doses inhibited CCl_4-induced thiobarbituric acid-reactive substances (TBARS) formation, indicating a blockade of CCl_4-induced lipid peroxidation. AMB also reversed the decrement in glutathione contents of hepatic tissues in CCl_4-intoxicated rats. Furthermore, it restored catalase activity to normal values, which was significantly increased after CCl_4 treatment. Our results indicate that CCl_4-induced oxidative damage in hepatic tissues is reversed by AMB treatment. The protective effect of AMB is probably due to the phenolic nature of this glucoside.

Introduction

The liver is highly involved in metabolic functions and is frequently a target for a great number of toxicants. Many studies have shown that reactive oxygen species are related to the etiology of degenerative diseases, including some hepatopathies [1], [2]. Carbon tetrachloride (CCl_4) is frequently used as a chemical inducer of experimental liver cirrhosis. This toxic agent elicits liver damage by forming reactive intermediates, such as trichloromethyl free radicals, via cytochrome P450-related functions in the oxidase system [3]. The main causes of CCl_4-induced hepatic damage are related to lipid peroxidation, decreased activities of antioxidant enzymes, and generation of free radicals caused by this agent [2].

The antioxidant activity is an important way of providing protection against hepatic damage, and a number of phenol molecules have been shown to possess hepatoprotective properties by improving the liver's antioxidant status [4]. A recent report [5] showed that the extract of *Polygonum aviculare* L., a species rich in phenols including flavonoids, presents a potent antioxidant effect, probably related to its high content of phenols.

Amburana cearensis belongs to the Fabaceae family and is a medicinal plant used in northeastern Brazil in the treatment of respiratory diseases, including asthma [6]. Phytochemical studies of its trunk bark allowed the isolation of several compounds, such as 3,4-dihydroxybenzoic acid (protocatechuic acid), a mixture of glucosylated β-sitosterol and stigmasterol, coumarin, four flavonoids (isokaempferide, kaempferol, afrormosin and 4′-methoxyfisetin) and the phenol glucosides, amburosides A and B (AMB, **Fig. 1**) [7], [8].

In a previous study [9], we showed that the hydroalcoholic extract, coumarin, and a flavonoid fraction (with isokaempferide as its main constituent) from *A. cearensis* have anti-inflammatory activities and are able to relax the isolated guinea-pig tracheal smooth muscle. Furthermore, we recently [10] demonstrated that the relaxant effect of isokaempferide occurs through several in-

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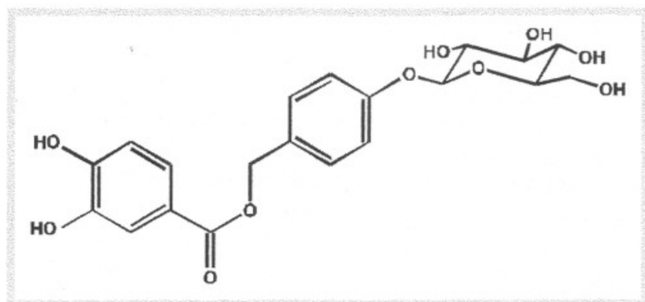

Fig. 1 Chemical structure of amburoside A (AMB) isolated from the trunk bark of *Amburana cearensis* A. C. Smith.

tracellular actions, having a common pathway such as the opening of Ca^{++} and ATP-sensitive K^{+} channels.

In addition, AMB has been shown to possess antimalarial activity against *Plasmodium berghei* and no cytotoxicity against tumor cells lines or sea urchin eggs [8], [11]. We also showed [12] that AMB has a significant neuroprotective effect on rat mesencephalic cell cultures exposed to the neurotoxic agent, 6-hydroxydopamine. Thus, the objective of the present work was to expand our previous findings by evaluating whether AMB possesses hepatoprotective activity in the model of CCl_4-induced hepatotoxicity in rats. For that, we measured liver enzymes (AST and ALT), thiobarbituric acid reactive substances (TBARS), reduced glutathione levels (GSH) and catalase activity. Besides, liver histological analyses were also carried out.

Materials and Methods

Chemicals

Carbon tetrachloride, hydrogen peroxide, sulfhydryl reagent [5,5′-dithio-bis (2-nitrobenzoic acid)] (DTNB), 2-thiobarbituric acid, vitamin E and other reagents were purchased from Sigma Chemical company, USA. Diagnostic kits for assaying alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were from Labtest, Brazil. All other drugs were of analytical grade.

Plant material and isolation of amburoside A

Trunk barks from *A. cearensis* were collected at the Quixeramobim area, Ceará State, in the Brazilian northeastern region. Voucher specimens (nos. 837 and 847) were deposited at the Prisco Bezerra Herbarium of the Federal University of Ceará (UFC), Fortaleza, Brazil and were authenticated by Dr. Afrânio G. Fernandes, Department of Biology, UFC. The powdered trunk bark (3.4 kg) was used for the isolation of amburoside A (AMB, 1.02 g) (**Fig. 1**), according to the method described by Canuto and Silveira [7]. The structure was determined by spectroscopy, including one- and two-dimensional NMR techniques, such as COSY, HMBC and HMQC, physical properties determination, and comparison with data from literature [8].

The purity of AMB (97.3%) was measured by HPLC analysis, based on the relative area of the target peak at five different wavelengths (220, 254, 328, 387 and 540 nm). HPLC analysis was performed on a Waters 1525 chromatograph, rheodyne injector (5 μL loop) and photodiode-array detector (Waters-2996 PDA), utilizing a Waters X-Terra RP-18 column (250 × 4.6 mm, 5 μm). The mobile phase was prepared from an aqueous solution of H_3PO_4/Et_3N (pH 3.0) and HPLC-grade MeOH, at a gradient mode (15 min total run time) varying from 20 to 50% of the latter, and at a flow rate of 1.0 mL/min. AMB was dissolved in an aqueous solution containing 4% Tween 80.

Animals

Male Wistar rats (200 g) from the Animal House of the Federal University of Ceará were maintained at 22 ± 2 °C, 12 h light-12 h dark cycle, and fed with a standard diet (from Purina, Brazil) and tap water ad libitum. Experiments were performed according to the Guide of Care and Use of Laboratory Animals from the European Community. The manuscript protocol used to investigate the pharmacological properties of amburoside A, in rats, was approved by our Institutional Ethics Committee.

CCl_4-induced liver injury *in vivo*

Rats were pretreated with vehicle (aqueous solution of 4% Tween 80), AMB or vitamin E before (30 or 60 min for *i. p.* and *p. o.* administrations, respectively) and 24 h after carbon tetrachloride (50% CCl_4/olive oil, 0.5 mL/kg, *s. c.*) administration. Animals were divided into six groups: (1) negative control (vehicle), (2) CCl_4 plus vehicle, (3) AMB (25 mg/kg, *i. p.*) plus CCl_4 treatment, (4) AMB (50 mg/kg, *i. p.*) plus CCl_4 treatment, (5) AMB (50 mg/kg, *p. o.*) plus CCl_4 treatment and (6) vitamin E (250 mg/kg, *p. o.*) plus CCl_4 treatment. All animals were anesthetized with ether, and the blood was collected from the orbicular plexus in tubes containing heparin 48 h after CCl_4 administration (that is 24 h after the 2nd drug administration). Animals were sacrificed, and livers were dissected and utilized for biochemical analyses.

Determination of serum transaminases activities

AST and ALT activities in the rat serum of all groups were measured according to the manufacturer's instructions.

Measurement of malondialdehyde

The liver was homogenized in 10 vol. of ice-cold 1.15% KCl. The malondialdehyde (MDA) formation in liver homogenates was determined by quantification of thiobarbituric acid reactive substances (TBARS), using the method of Buege and Aust [13] slightly modified. The reaction mixture (0.5 mL tissue homogenate, 0.9 mL of 50 mM phosphate buffer – pH 7.4 and 0.5 mL of the chemical system generating free radicals – 0.01 mM $FeSO_4$ plus 0.1 mM ascorbic acid) was incubated at 37 °C for 30 min. The reaction was terminated by adding 1 mL of 10% (w/v) trichloroacetic acid (TCA) to the mixture that was centrifuged at 8.000 × g for 10 min. Supernatants were incubated with 1 mL of 0.8% (w/v) thiobarbituric acid at 100 °C for 15 min, and after a cooling period TBARS contents were spectrophotometrically determined at 532 nm using MDA as the standard.

Determination of the reduced glutathione content

L-γ-Glutamyl-L-cysteinyl-glycine (GSH) levels were determined by the method of Ellman et al. [14]. Livers were homogenized (400 μL) in 1 : 10 volumes of 0.02 M EDTA followed by the addition of 10% TCA, in order to precipitate the protein contents of homogenates. After centrifugation for 15 min at 4 °C, the DTNB solution (Ellman's reagent) was added to the supernatants, and the absorbance was measured at 412 nm. Different concentrations of a standard GSH solution (1 mg/mL) were used to calculate GSH contents, and results were expressed as μg/mg protein. The protein concentration was measured by the Lowry et al. method [15].

Catalase assay

The method of Aebi [16] was used and consisted in measuring the changes in absorbance of a solution of 10 mM H_2O_2 in phosphate buffer, pH 7.0. The decrease in absorbance per unit time is a measure of catalase activity. The liver homogenate (20 μL) was added to the cuvette containing 980 μL of the substrate mixture (0.30 mL of hydrogen peroxide in 50 mL of 0.05 M phosphate buffer, pH 7.0). After 1 min, the initial absorbance was recorded, and the final absorbance was read after 6 min. The reaction was followed at 230 nm, and the results expressed as mmol/min/mg protein. The protein concentration was measured by the Lowry et al. method [15].

Liver histology

Rats were sacrificed, livers quickly dissected and the pieces immediately fixed in sodium phosphate buffered 10% formalin (pH 7.4). Pieces were then placed in 70% alcohol, followed by 80, 90 and 100% alcohol solutions. Then, pieces were transferred to xylene, embedded in paraffin, and 5 μm sections were prepared for staining with heamatoxylin-eosin [17].

Statistical analysis

Results were expressed as mean ± SEM. Differences among groups were determined by one-Way analysis of variance (ANOVA), followed by Tukey for multiple comparisons, as a *post hoc* test. The significance level was set at $p < 0.05$.

Results

▼

Table 1 shows that, after CCl_4 administration, both serum transaminases were markedly increased by 2.6- and 2.5-fold, respectively (CCl_4, ALT: 89.8 ± 5.5; AST: 137.9 ± 6.1 U/L), as compared to the control (ALT: 33.5 ± 3.5 U/L; AST: 54.5 ± 2.5 U/L). AMB treatment (25 and 50 mg/kg, *i.p.*), in the presence of CCl_4, significantly reduced the activities of ALT by 28% (AMB25 + CCl_4: 53.3 ± 4.9; AMB50 + CCl_4: 36.3 ± 4.8 U/L) and those of AST by 59% (AMB25 + CCl_4: 103.8 ± 7.9; AMB50 + CCl_4: 80.9 ± 6.7 U/L), if compared to the CCl_4 group. The oral administration of AMB (50 mg/kg) or vitamin E (250 mg/kg) caused inhibitions of 40% in both enzymes.

Fig. 2 shows the effect of AMB on TBARS formation (expressed as mmol MDA/mg protein), as a parameter of liver peroxidation. After CCl_4 administration, TBARS contents in the liver homogenate were increased by around 2.4-fold in CCl_4-intoxicated rats (10.2 ± 0.5), as compared to controls (4.2 ± 0.2). The intraperitoneal or oral administration of AMB, at the doses of 25 (6.4 ± 1.0) and 50 mg/kg (7.8 ± 0.3), respectively, reduced significantly the formation of TBARS in liver homogenates in CCl_4-intoxicated rats. In addition, AMB (4.8 ± 0.5) at the dose of 50 mg/kg, i.p. markedly reduced TBARS formation, whose values returned to normal levels. Vitamin E also protected against CCl_4-hepatotoxicity, and values were close to those of controls (5.6 ± 0.7).

Fig. 3 shows changes in reduced glutathione levels (GSH) in liver tissues of CCl_4-intoxicated rats. Hepatic GSH levels (μg/mg protein) significantly decreased from 10.4 ± 0.6 in the control group (without CCl_4 treatment) to 5.7 ± 0.2 in the CCl_4-treated group. The decreased GSH levels observed after CCl_4 treatment were almost completely restored to normal values by intraperitoneal (25 and 50 mg/kg) or oral (50 mg/kg) administrations of AMB, respectively (*i.p.*: AMB25 + CCl_4: 8.2 ± 0.5; AMB50 + CCl_4: 8.7 ± 0.4; *p.o.*: AMB50 + CCl_4: 9.6 ± 0.4). The effect of AMB (50 mg/kg, *p.o.*) was comparable to that of the standard vitamin E (10.6 ± 0.8).

The liver catalase activity (nmol/min/mg protein) was 76.7 ± 8.25 in the control group (**Fig. 4**), and increased to 156.5 ± 14.8 in CCl_4-intoxicated rats (CCl_4 group). AMB (25 and 50 mg/kg, *i.p.*) in the presence of CCl_4 significantly ($p < 0.001$) reduced the enzyme activity (AMB50 + CCl_4: 75.0 ± 10.2), indicating a hepatoprotective effect. No significant effect was seen with AMB, at the lower dose. In addition, both AMB (50 mg/kg, *i.p.*) and vitamin E restored the enzymatic activity to normal levels. The oral administration of AMB, at the dose of 50 mg/kg, also partially restored catalase activity, although the effect was less pronounced than that of AMB at 50 mg/kg, *i.p.*

Liver sections from control, CCl_4- treated, AMB + CCl_4 (25 and 50 mg/kg) and vitamin E (250 mg/kg) groups are presented in **Fig. 5**. The results show that CCl_4 produced considerable necrosis and degeneration of liver cells around the central lobule,

Table 1 Effects of amburoside A (AMB) from *Amburana cearensis* on serum AST and ALT levels in CCl_4-intoxicated rats

Group	AST (U/L)	ALT (U/L)
Control	54.5 ± 2.5	33.5 ± 3.5
CCl_4	137.9 ± 6.1**a**	89.8 ± 5.5**a**
AMB 25 mg/kg, *i.p.* + CCl_4	103.8 ± 7.9**b**	53.3 ± 4.9**b**
AMB 50 mg/kg, *i.p.* + CCl_4	80.9 ± 6.7**b**	36.3 ± 4.8**b**
AMB 50 mg/kg, *p.o.* + CCl_4	77.5 ± 6.0**b**	49.2 ± 7.3**b**
Vitamin E 250 mg/kg, *p.o.* + CCl_4	82.3 ± 7.4**b**	46.0 ± 5.0**b**

CCl_4-intoxicated rats were treated with vehicle or AMB (25 and 50 mg/kg), 30 min before and 24 after CCl_4 administration. The AST and ALT levels were measured in heparinized plasma obtained 48 h after CCl_4 administration. Values are expressed as means ± S.E.M. **a** vs control (without CCl_4 treatment); **b** vs CCl_4 ($p < 0{,}05$, ANOVA and Tukey, as the *post hoc* test).

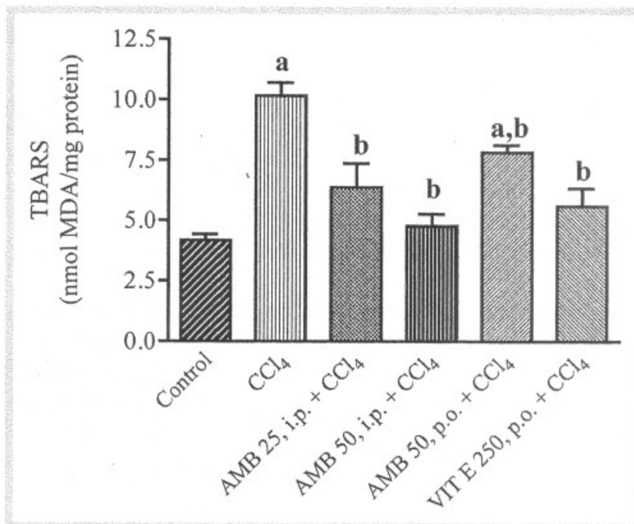

Fig. 2 Effects of AMB on hepatic lipid peroxidation in CCl_4-intoxicated rats. Animals were treated with AMB (25 and 50 mg/kg, *i.p.*; 50 mg/kg, *p.o.*) or vitamin E (250 mg/kg, *p.o.*), before and 24 h after CCl_4 administration. They were sacrificed 48 h after CCl_4 (that is, 24 h after the 2nd drug administration). The content of malondialdehyde (MDA) formation in the liver homogenate was determined by the quantification of thiobarbituric acid reactive substances (TBARS). Values are expressed as means ± S.E.M. **a** vs. control group (without CCl_4 treatment); **b** vs. CCl_4-treated group ($p < 0.05$, ANOVA and Tukey as the *post hoc* test).

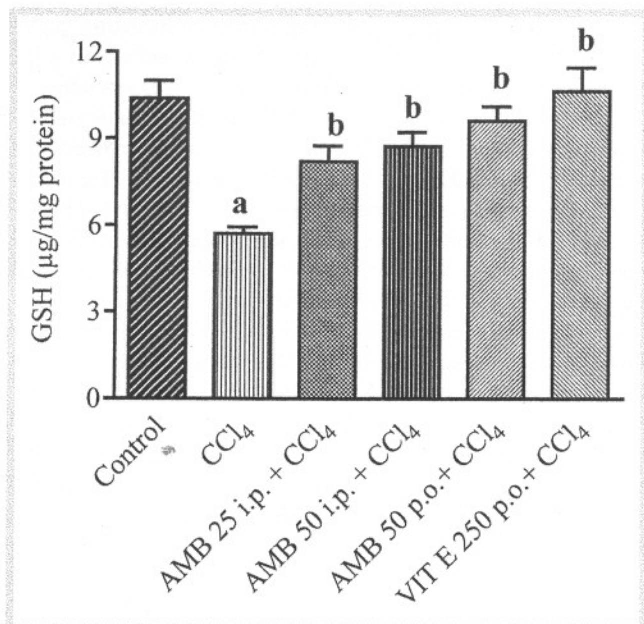

Fig. 3 Effects of AMB on hepatic reduced glutathione (GSH) levels in CCl_4-intoxicated rats. Animals were treated with AMB (25 and 50 mg/kg, *i. p.*; 50 mg/kg, *p. o.*) or vitamin E (250 mg/kg, *p. o.*), before and 24 after CCl_4 administration. They were sacrificed 48 h after CCl_4 (that is, 24 h after the 2nd drug administration). GSH contents were determined by the DTNB conjugation method. Values are expressed as means ± S.E.M. **a** vs. control group (without CCl_4 treatment); **b** vs. CCl_4 group ($p < 0.05$, ANOVA and Tukey as the *post hoc* test).

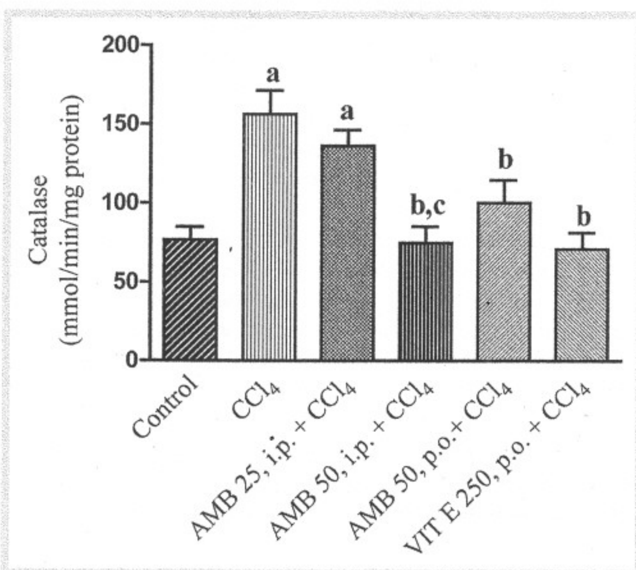

Fig. 4 Effects of AMB on hepatic catalase activity in CCl_4-intoxicated rats. Animals were treated with AMB (25 and 50 mg/kg, *i. p.*; 50 mg/kg, *p. o.*) or vitamin E (250 mg/kg, *p. o.*), before and 24 h after CCl_4 administration. They were sacrificed 48 h after CCl_4 (that is, 24 h after the 2nd drug administration). Catalase activity was determined according to Aebi [16]. Values are expressed as means ± S.E.M. **a** vs. control group (without CCl_4 treatment); **b** vs. CCl_4 group; **c** vs. AMB 25 group ($p < 0.05$, ANOVA and Tukey as the *post hoc* test).

as well as significant increases in serum transaminases activities (◐ **Table 1**), as compared to controls. Infiltration of inflammatory cells was also found in CCl_4-intoxicated rats. On the other hand, the damage was significantly small in rats treated with AMB, prior and after CCl_4 administration. Furthermore, vitamin E also markedly reduced the alterations in acute CCl_4-mediated liver damage.

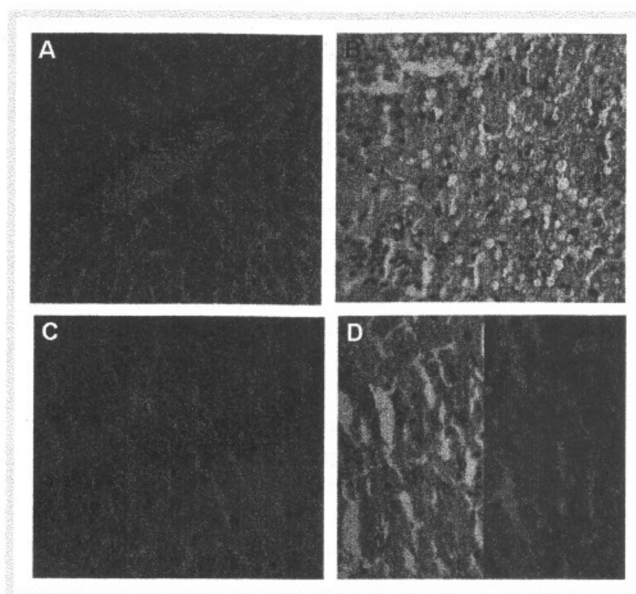

Fig. 5 Effects of amburoside A (AMB) on hepatic morphological analysis, after CCl_4 administration, in rats. Animals were treated with AMB (25 and 50 mg/kg, *i. p.*; 50 mg/kg, *p. o.*) or vitamin E (250 mg/kg, *p. o.*) before and 24 h after CCl_4 administration. They were sacrificed 48 h after CCl_4 (that is, 24 h after the 2nd drug administration). Liver sections were stained with hematoxylin-eosin by standard techniques for photomicrographies (×40). Figure (**A**) is from a normal rat without CCl_4 treatment; Figure (**B**) is from a rat treated with CCl_4 only. Representative Figures (**C**) and (**D**) are from rats treated with vitamin E (250 mg/kg, *p. o.*) and AMB (50 mg/kg, *p.o*), respectively.

Discussion

In the present work, AMB (4-(*O*-β-D-glycopyranosylbenzyl protocatechoate), isolated from *Amburana cearensis*, administered by oral or intraperitoneal routes exhibited hepatoprotective activity against CCl_4-induced hepatotoxicity in rats. AMB is a phenol glucoside and one of the bioactive components present in this Brazilian medicinal plant, popularly used for the treatment of respiratory tract affections.

Polyphenols have been shown to be more effective antioxidants *in vitro* than tocopherols and ascorbate. Antioxidant properties of polyphenols arise from their high reactivity as hydrogen or electron donors, from the ability of polyphenol-derived radicals to stabilize unpaired electrons and to chelate transition metal ions. Another mechanism underlying the antioxidative properties of phenols is the ability of flavonoids to alter peroxidation kinetics by modifying the lipid packing order and decreasing membranes fluidity [18]. Recently, Panda and Kar [19] demonstrated that apigenin (4′,5,7-trihydroxyflavone) has the potential to regulate diabetes mellitus, as well as this disease-induced thyroid dysfunction and lipid peroxidation in mice.

In the liver, the first cells to be damaged by CCl_4 through the CCl_4-derived and highly reactive free radical metabolite trichloromethyl CCl_3 are those in the centrilobular region where enzyme activities are the greatest [20]. Kupffer cells, the major component of the hepatic sinusoid, and macrophages sensitized to

CCl_4 intoxication release pro-inflammatory cytokines, such as the tumor necrosis factor alpha (TNF-alpha). The expression of cytokines occurs through NF-κB activation during hepatocellular injury in the acute CCl_4-mediated liver damage [21].

In the present study, activities of the hepatic transaminases (ALT and AST) were markedly elevated after CCl_4 administration in the rat serum, and AMB significantly reduced these effects. The assessment of hepatic transaminases is the easiest method for estimating liver damage. Necrosis or membrane damage release these enzymes into circulation. AST is present in the liver, heart, muscle, kidney and brain whereas ALT is more specific to the liver. Our results suggest that AMB has a hepatoprotective activity in CCl_4-intoxicated rats, and this AMB action was corroborated by histological data. Thus, CCl_4-treatment caused massive histopathological changes such as necrosis, congested vessels, multifocal areas of fatty changes, nuclear disintegration, sinusoidal dilation, and Kupffer cells hyperplasia. Prevention of these events can be considered as a hepatoprotective activity.

The lipid peroxidative degradation of biomembranes is one of the main causes of CCl_4 toxicity. Lipid peroxidation also leads to a wide range of cytotoxic products (such as malondialdehyde and 4-hydroxynonenal), inhibiting protein synthesis and the activities of certain enzymes [22]. Our results, using the model of CCl_4-induced hepatotoxicity in rats, demonstrated that AMB caused significant decreases in TBARS levels when compared to the group treated with CCl_4 only. These results indicate that AMB protects liver tissues against lipid peroxidation.

Biological systems defend themselves against the damaging effects of reactive oxygen species (ROS) by several means, including free radical scavengers and chain reaction terminators, enzymes such as superoxide dismutase, catalase, and glutathione peroxidase (GPx) systems [23]. The administration of CCl_4 induced a significant increase in the catalase activity when compared to the control group. A significant reversal of this change towards normal values was observed after the administration of AMB or vitamin E used as standard when compared to the CCl_4 treated group alone.

Rohrdanz et al. [24] reported that catalase activity and mRNA expression were induced in hepatocytes after their exposure to hydrogen peroxide. In addition, Szymonik-Lesiuk et al. [25] showed that catalase activity in the liver was significantly increased at 24 and 72 h after CCl_4 intoxication. Thus, increased catalase activity in CCl_4-intoxicated rats, demonstrated in the present study, may be explained by the increase in the enzyme expression caused by the accumulation of ROS in the liver as observed by others [26]. We can then assume that the reduction of catalase activity by AMB may be a consequence of its antioxidant effect.

Glutathione is a ubiquitous, non-essential sulfhydryl amino acid and an antioxidant thiol that plays a crucial role in maintaining redox equilibrium and establishing mechanisms of cellular defense seen during oxidative stress [27]. In our study, CCl_4 administration caused a significant reduction of GSH in the liver that was almost completely reversed by AMB. The depletion of GSH in the CCl_4 group may be explained by the increased utilization of GSH required for the removal of ROS and lipid damaged products. Otherwise, decreases of ROS production in the AMB pretreated groups may reduce the consumption of GSH for ROS scavenger.

Galato et al. [28] investigated the antioxidant capacity of phenols and related compounds and observed that the greater the number of hydroxyls linked to the aromatic ring, the greater is the antioxidant activity of the compounds. Thus, it is possible that hydroxyl groups, present in the AMB molecule aromatic ring, contribute to the drug antioxidant activity.

Our data suggest that the hepatoprotective activity of AMB in CCl_4-induced liver injury is related to its antioxidative property and its ability to scavenge free radicals involved with CCl_4-mediated lipid peroxidation. In a previous study [12], we showed an antioxidant activity of AMB on rat mesencephalic cell cultures after their exposure to 6-OHDA. Inhibition of cytochrome P450 (CYP2E1) was also reported to account for the hepatoprotective actions reported for some compounds [29]. However, determining whether AMB is a cytochrome P450 inhibitor still requires further investigation.

In the present study, we showed that AMB, especially at the higher dose, possesses hepatoprotective properties in the CCl_4-induced liver toxicity model in rats. These effects may be due, at least in part, to a reduction in hepatic peroxidative activities, as well as by a significant restoration to normal levels of the catalase activity and GSH contents observed in CCl_4-intoxicated rats after AMB treatment. These effects are probably related to the presence of hydroxyl groups in the AMB phenol structure, as previously demonstrated to occur with other natural phenols [5]. Finally, the observed hepatoprotective action of AMB from *A. cearensis* may be justified, at least in part, by its antioxidant property [12]. Other plant glucosides are also known to decrease ALT, AST, lipid peroxidation, and nitrite levels in rat serum in the CCl_4-induced hepatotoxicity model [30].

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