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Antioxidant and hepatoprotective action of cassava leaf flour extract against injury induced by CCl_4 in rats

Mírian Aparecida Isidro Santos*, Angelita Duarte Corrêa, Anderson Assaid Simão, Ana Paula de Carvalho Alves, Raimundo Vicente de Sousa and Adelir Aparecida Saczk

Chemistry Department, Universidade Federal de Lavras – UFLA, PO Box 3037, zip code 37200-000, Lavras, MG, Brazil.

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In the present study, we assessed the effect of the cassava leaf flour (CLF) extract on the antioxidant activity and liver injury in rats. For the extract preparation, the CLF was kept under maceration in 50% ethanol at a 1:40 ratio (w/v) for 30 min. and then centrifuged at 2,500 x g for 15 min. The supernatant was collected and the precipitate was again subjected to the extraction process with 70% acetone, as described above; the supernatants were collected, rotoevaporated for removal of solvents and lyophilized to give the extract. The animals received intraperitoneal doses of carbon tetrachloride (CCl_4) and daily doses of the extract by gavage. After the treatment, we tested the activity of the enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT), and lipid peroxidation, determining the concentration of albumin and serum protein, total lipids and liver histopathology. Treatment with the CLF extract was capable of inhibiting damage induced by CCl_4, presenting a lower degree of steatosis. Protection was demonstrated by the lower activities of serum enzymes in liver damage, such as AST, ALT and GGT, low levels of lipid peroxidation and histopathological observation. These actions were attributed to phenolic compounds, such as gallic acid, gallocatechin, catechin and chlorogenic acid found in the CLF extract.

Key words: *Manihot esculenta,* leaves, phenolic compounds, carbon tetrachloride, enzyme, lipid peroxidation.

INTRODUCTION

Carbon tetrachloride (CCl_4) is a hepatotoxin that has been used to induce liver fibrosis in animals. One of the major consequences of liver injury caused by CCl_4 is lipid peroxidation, which is mediated by the production of free radicals derived from CCl_4 and, when repeatedly administered in a low dose, induces liver fibrosis and then cirrhosis (Muriel and Escobar, 2003). The hepatotoxic effects of CCl_4 are largely due to its active metabolite, trichloromethyl radical. These activated radicals can bind covalently to macromolecules and induce peroxidative degradation of lipids in the endoplasmic reticulum membrane, rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides. The toxicity of this compound can be evidenced by an elevation of serum marker enzymes, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) (Palanivel et al., 2008).

Although no therapeutic approach has been successful in the pathogenetic mechanism of liver disease,

*Corresponding authors. E-mail: miriansantos.a@gmail.com, Tel: +55-35-3829-1272; Fax: +55-35-3829-1271.

antioxidant therapies have proven effective to achieve some positive effects. Natural products have been prominent in alternative treatments for some diseases. It is known that cassava leaves exhibit several compounds that have diverse activities, such as phenolic compounds, which present antioxidant activity (Corrêa et al., 2004; Melo et al., 2007). Among the various classes of naturally occurring antioxidants, phenolic compounds have received much attention in recent years, especially by inhibiting *in vitro* lipid peroxidation (Naczk and Shahidi, 2004; Soares, 2002).

Melo et al. (2007) reported that diets containing 10 and 15% cassava leaf flour (CLF) were associated with a reduction in plasmatic levels of Thiobarbituric acid reactive substances in rats, confirming the *in vivo* antioxidant activity of CLF. Therefore, the present study was performed to investigate the antioxidant and hepatoprotective activity of the CLF extract against liver injury induced by CCl_4 in Fischer rats.

MATERIALS AND METHODS

Mature cassava leaves (*Manihot esculenta Crantz*, Pão da China cultivar) free from pests and diseases were collected at 12 months of age, transported to the laboratory, washed in tap and distilled water, and then placed in air-circulating ovens for drying during 48 h at temperatures between 30 and 35°C. After drying, the leaves had their petioles removed and were milled to obtain the flour.

For the extract preparation, the CLF was kept under maceration in 50% ethanol at a 1:40 ratio (w/v) for 30 min, and then centrifuged at 2,500 x g for 15 min. The supernatant was collected and the precipitate was again subjected to the extraction process with 70% acetone, as described above, and the supernatants were collected, rotoevaporaded for removal of solvents and lyophilized to give the extract.

The biological assay was developed in accordance with the ethical principles in animal experimentation, and the project was approved by the Ethics Committee on Animal Use of Universidade Federal de Lavras (UFLA - Protocol 075/11). Thirty Fischer albino rats (*Rattus norvegicus*) were used, in growth phase, with initial body weight of approximately 115 g.

Throughout the experiment the animals were kept in polyethylene boxes (49 x 34 x 16 cm) with wood shavings bedding, with at most six animals per box, in a room with a temperature of 25°C ± 3°C, light/dark cycle of 12 h and with access to food and water *ad libitum*. Daily cleaning, feeding and gavage were performed by the same person. For the induction of hepatic injury, amounts of 1.5 mL kg^{-1} carbon tetrachloride (CCl_4), solubilized in olive oil in a 1:1 ratio, were administered to the animals intraperitoneally on the third, fifth and seventh days of the last week of the 21-day treatment.

The animals were divided into 5 groups of 6 animals each: group 1- Negative control (water), group 2- Positive control (water and CCl_4), group 3- 50 mg CLF extract kg^{-1} and CCl_4, group 4- 150 mg CLF extract kg^{-1} and CCl_4 and group 5- 450 mg CLF extract kg^{-1} and CCl_4. The CLF extract was administered to animals at the concentrations mentioned above, by gavage, once daily, for 21 days. The negative control received water, and the positive control received water and CCl_4 by the same route. On the 22nd day, the animals were anaesthetized with thiopental sodium, intraperitoneally, at a dose of 40 mg kg^{-1}, for the removal of blood by cardiac puncture and of the liver by median laparotomy. The blood was centrifuged at 2370 x g for 15 min to separate the serum, which was used to determine concentrations of albumin and total proteins, activity of AST, ALT and GGT. The liver was washed with saline solution and stored at -20°C for ether extract, antioxidant activity and histopathological analyses.

Albumin was determined by a colorimetric method using a commercial kit (Labtest), and the concentration of total serum protein was determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as a standard. The activities of AST, ALT and GGT were measured by the kinetic colorimetric method using commercial kits (Labtest). The lipid peroxidation was determined by the formation of thiobarbituric acid reactive substances (TBARS), according to Winterbourn et al. (1985). The amount of ether extract (total lipids) was determined using the methodologies proposed by AOAC (2005). Livers were dried at 65°C to constant weight, finely ground and defatted with ethyl ether on cellulose cartridges in a Soxhlet-type extractor for 6 h.

For histopathological analyses, a liver fragment from each animal was fixed in 10% formalin for the first 24 h for conservation until the inclusion procedure. Subsequently, the fragments were processed for inclusion in paraffin blocks, then underwent microtomy, yielding approximately 4 µm sections, which were stained with hematoxylin-eosin (HE method) and mounted on glass slides. The samples were examined under a light microscope and identified for the presence of hepatic steatosis, considering mild (+), moderate (+ +) or severe (+ + +).

The experimental design was completely randomized with five treatments and six replications, each animal representing an experimental unit. Data were subjected to analysis of variance and, when significant, the Scott-Knott test at 5% probability was applied for comparison of means using Sisvar (Ferreira, 2003), a program for statistical analysis and design of experiments.

Chromatographic analyses were performed using an Agilent HPLC equipment model 1100, equipped with a binary pump, an auto injector and a detector with diode array at a wavelength of 280 nm. The CLF extract and the standards were separated on an Ascentis C18 column (25 cm x 4.6 mm, 5 µm), attached to an Ascentis C18 pre-column (2 cm x 4.0 mm, 5 µm). The mobile phase was composed of the following solutions: 2% acetic acid (A) and methanol : water : acetic acid (70:28:2 v/v/v) (B). Analyses were performed in a total time of 50 min at 15°C, in a gradient-type system: 100% solvent A from 0.01 to 5 min, 70% solvent A from 5 to 25 min, 60% solvent A from 25 to 43 min, 55% solvent A from 43 to 50 min. Solvent A was increased to 100%, seeking to maintain a balanced column. The flux used in all tests was 1 mL min^{-1} and the injection volume was 20 µL.

The phenolic standards used were: gallic acid, gallocatechin, 3,4-dihydroxybenzene, catechin, chlorogenic acid, epigallocatechin, vanillic acid, epicatechin, syringic acid, p-coumaric acid, ferulic acid, m-coumaric acid, o-coumaric acid, resveratrol, ellagic acid, salicylic acid, all Sigma-Aldrich (St. Louis, MO, USA). The stock standard solutions were prepared in dimethyl sulfoxide and/or methanol (Merck) in a concentration range of 0.1 a 177.15 mg L^{-1}. Acetic acid and methanol (Merck, Darmstadt, Germany) were used to prepare the mobile phase and ultrapure water was obtained by the Milli-Q system (Millipore, Billerica, MA, USA).

The CLF extract and the standards were filtered on a 0.45 µm nylon membrane (Millipore®) and directly injected into the chromatographic system. Each solution was injected three times on the HPLC system, with the purpose of obtaining concentration means and retention times. The phenolic compounds in the CLF extract were identified by comparison with retention times of standards. Quantitation was performed by the construction of analytical curves, in which each point represents the mean of three replicates.

RESULTS AND DISCUSSION

It was possible to notice that all tested doses of the CLF

Table 1. Content of thiobarbituric acid reactive substances (nmol MDA mg^{-1} protein) and activity of enzymes ALT, AST and GGT ($U\ L^{-1}$) in control groups and in the ones treated with cassava leaf flour (CLF) extract in different doses.

Treat*	TBARS	ALT	AST	GGT
1	0.157 ± 0.03[c]	41.71 ± 2.73[c]	115.35 ± 17.21[c]	2.76 ± 0.27[c]
2	0.359 ± 0.10[a]	235.93 ± 3.97[a]	357.22 ± 19.77[a]	13.70 ± 0.73[a]
3	0.253 ± 0.06[b]	127.37 ± 2.83[b]	238.91 ± 20.98[b]	7.64 ± 0.51[b]
4	0.210 ± 0.07[b]	132.15 ± 2.75[b]	241.35 ± 21.13[b]	6.05 ± 0.65[b]
5	0.231 ± 0.09[b]	135.59 ± 2.98[b]	233.77 ± 19.85[b]	7.22 ± 0.49[b]

*Treatments: 1- Negative control (water), 2- Positive control (water and CCl_4), 3- 50 mg CLF extract kg^{-1} and CCl_4, 4- 150 mg CLF extract kg^{-1} and CCl_4 and 5- 450 mg CLF extract kg^{-1} and CCl_4. Values represent the average of 6 replicates ± standard deviation. Means followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability.

extract inhibited the formation of malonic dialdehyde (MDA), and no antioxidant action was observed for the compounds present in the extract, although there was no statistical difference between the three doses (Table 1).

These results are in agreement with those reported by Melo et al. (2007), who treated rats with diets plus 5, 10 and 15% CLF for 7 weeks; in all cases, isocaloric diets contained 1% cholesterol, and the authors observed that the diets containing 10 and 15% CLM were associated with the decrease in plasma levels of thiobarbituric acid reactive substances, corroborating the results of the present study.

The group treated with water (negative control) showed a significantly smaller activity of AST, ALT and GGT than the group treated with CCl_4 (positive control), indicating that the administration of this compound caused a significant raise in the levels of these enzymes (Table 1). In all groups treated with the CLF extract, there was a decrease in serum levels of AST, ALT and GGT compared to the group treated with CCl_4. However, the recorded contents did not equal the negative control.

The results for AST and ALT differed from those reported by Melo et al. (2008), who observed an increase in the ALT activity, and AST showed no significant difference when compared to the control; in this study, the authors treated rats for 7 weeks with diets plus 5, 10 and 15% CLF; in all cases, isocaloric diets contained 1% cholesterol. However, Huo et al. (2011) studied the antioxidant and hepatoprotective effect of licorice aqueous extract against the oxidative damage induced by CCl_4 (3 mL kg^{-1}) in rats and the results showed that all tested concentrations (100, 150 and 300 mg kg^{-1}) effectively protected the liver of animals, and the protective effect of the extract was evidenced by a decrease in the levels of AST and ALT.

Frazini et al. (2008) and Teixeira et al. (2009) reported that ALT is found primarily in the liver, and considered a more sensitive indicator than AST, since it exists in all body tissues, especially the heart, liver, skeletal muscle, kidneys, brain, pancreas, leukocytes, erythrocytes. GGT is a glycoprotein enzyme regularly attached to the cell membrane and participates in the transport of amino acids and peptides to cells and tissue glutathione levels. It is found predominantly in the liver, kidneys and plasma. Thus, it is clearly important to simultaneously employ tests with different marker enzymes of liver function, because the results obtained are complementary.

Albumin is the most abundant protein in blood plasma, constituting about 50 to 65%. It is synthesized in the liver and the most important factor for its blood concentration is the liver ability to synthesize it (2000). Albumin synthesis is affected in a number of disorders, especially those of the liver. The plasma of patients with liver diseases often presents a decrease in the albumin: globulin ratio. Hypoalbuminemia is promoted by a decrease or defect in the synthesis, due to hepatocellular damage, among others (Ribeiro et al., 2006).

The negative control showed a higher albumin level than the positive control, indicating that CCl_4 decreased albumin levels in the serum of animals (Table 2). The decrease in albumin production appears to be related to hepatic fat accumulation because, according to Nicoluzzi et al. (2000), this build-up causes a reduction in the synthesis ability of the liver and the consequent reduction in albumin concentration. Groups that received treatment with different CLF extract doses had significantly higher levels than the positive control group and lower than the negative group, but no significant difference between the doses was observed. These results demonstrate that the CLF extract conferred protection to the liver of animals in all tested doses. The total protein test assesses plasma concentration of protein (albumin + globulin). In a model of experimental cirrhosis developed with CCl_4, Díaz-Gil et al. (2009) observed a decrease in serum albumin and total protein levels, corroborating data from this study.

The significant difference between the positive and negative control groups showed that CCl_4 administration led to a decrease in serum levels of total protein (Table 2). Treatment with CLF extract, in all doses, caused an increase in serum protein concentration, but they did not reach the baseline levels recorded for the negative control. Total proteins are reduced in the case of liver injury, due to the increase in capillary permeability and decrease in the liver ability to synthesize mainly albumin

Table 2. Serum albumin and total protein levels (g dL^{-1}) and total lipids in the liver (g 100 g^{-1} dry matter) in control groups and in the ones treated with cassava leaf flour (CLF) extract in different doses.

Treatment*	Albumin	Total proteins	Total lipids
1	6.31 ± 0.87[a]	7.73 ± 1.09[a]	30.67 ± 1.47[c]
2	4.30 ± 0.98[c]	4.87 ± 0.73[c]	47.46 ± 1.21[a]
3	5.33 ±1.05[b]	6.45 ± 1.13[b]	44.29 ± 1.13[b]
4	5.46 ± 1.11[b]	6.39 ± 1.19[b]	43.96 ± 1.50[b]
5	5.37 ±1.02[b]	6.43 ± 1.17[b]	39.48 ± 1.39[b]

*Treatments: 1- Negative control (water), 2- Positive control (water and CCl_4), 3- 50 mg CLF extract kg^{-1} and CCl_4, 4- 150 mg CLF extract kg^{-1} and CCl_4 and 5- 450 mg CLF extract kg^{-1} and CCl_4. Values represent the average of 6 replicates ± standard deviation. Means followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability.

(Adhal and Manning, 2008).

Hepatic steatosis is caused by fat accumulation in the liver, and this condition may be caused by the presence of toxic substances, such as CCl_4. The total lipid content of the group treated with water (negative control) was significantly lower than the group treated with CCl_4, indicating that this xenobiotic increases the rate of hepatic lipids (Table 2).

When compared with the positive control, groups pre-treated with CLF extract, in combination with CCl_4, showed lower lipid levels. Among the different CLF extract doses, there was no significant difference. Fat accumulation in the liver was prevented by the CLF extract independently of dose, but the recorded contents did not equal the negative control. The liver can be protected from fat accumulation by agents capable of combating oxidative stress, therefore, the reduction in hepatic fat may have been caused by the phenolic compounds present in CLF extract.

In the chromatographic profile of CLF extract, the following phenolic compounds were identified: gallic acid (33.00 mg 100 g^{-1} ± 0.42), gallocatechin (9.10 mg 100 g^{-1} ± 2.37), catechin (155.96 mg 100 g^{-1} ± 14.07) and chlorogenic acid (104.80 mg 100 g^{-1} ± 3.32); catechin presented the highest content, followed by chlorogenic acid. There are no published data on the characterization of phenolic compounds in CLF.

However, there are numerous reports associating the presence of phenolic compounds with antioxidant activity. Sotelo-Félix et al. (2002) studied the effect of rosemary extract orally administered in rats in a concentration of 200 mg kg^{-1} for five days, and observed a hepatoprotective effect against severe injury induced by CCl_4. Rosemary played a role as an antioxidant, eliminating trichloromethylperoxyl radicals formed by hepatic metabolization of the chemical aggressor. According to Silva et al. (2011), the antioxidant activity of rosemary extracts depends on their phenolic composition.

Balasubashini et al. (2004) observed that the administration of a ferulic acid supplementation to diabetic rats for 45 days resulted in a decrease in thiobarbituric acid reactive substances; results were more pronounced when ferulic acid alone was employed. Hypercholesterolemic rats fed on diets containing 0.3% of strawberry phenolic compounds showed a decrease in lipid peroxidation (Mateos et al., 2005). A decrease in thiobarbituric acid reactive substances and in transaminases (AST and ALT) was also reported in diabetic rats. In this study it was demonstrated that olive phenolic compounds are effective in inhibiting oxidative stress (Tung et al., 2009); besides, the hepatoprotective effect of *Acacia confusa* bark and gallic acid, its active constituent, was tested against CCl_4 induced hepatotoxicity and it was found that the treatment was effective against damage induced by CCl_4, which was evidenced by the significant decrease in AST, ALT and inhibition of lipid peroxidation. These data corroborate the results found in this study, and allow to infer that the phenolic compounds present in the CLF extract are responsible for their action.

Fat accumulation is more frequently observed in the liver, since this is the main organ involved in lipid metabolism. Lipid content in hepatocytes is regulated by the activities of cellular enzymes that catalyze lipid uptake, synthesis, oxidation and externalization from the cell. When the fat amount that enters into hepatocytes exceeds the capacity for their oxidation or externalization, hepatic steatosis settles (Koteish and Diehl, 2001).

The histopathological study revealed hepatic steatosis with hepatocyte vacuolization or degeneration (Figure 1). With the exception of the negative control group, all groups had this injury; however, the intensity was considered severe in the positive control, and mild or moderate in the other groups.

Conclusion

The administration of ethanol extract/CLF acetone in concentrations of 50, 150 and 450 mg kg^{-1} decreases the activity of marker enzymes of liver damage, such as AST, ALT and GGT and lipid peroxidation, and increases the levels of albumin and total proteins, demonstrating the hepatoprotective action of this extract, which contains the

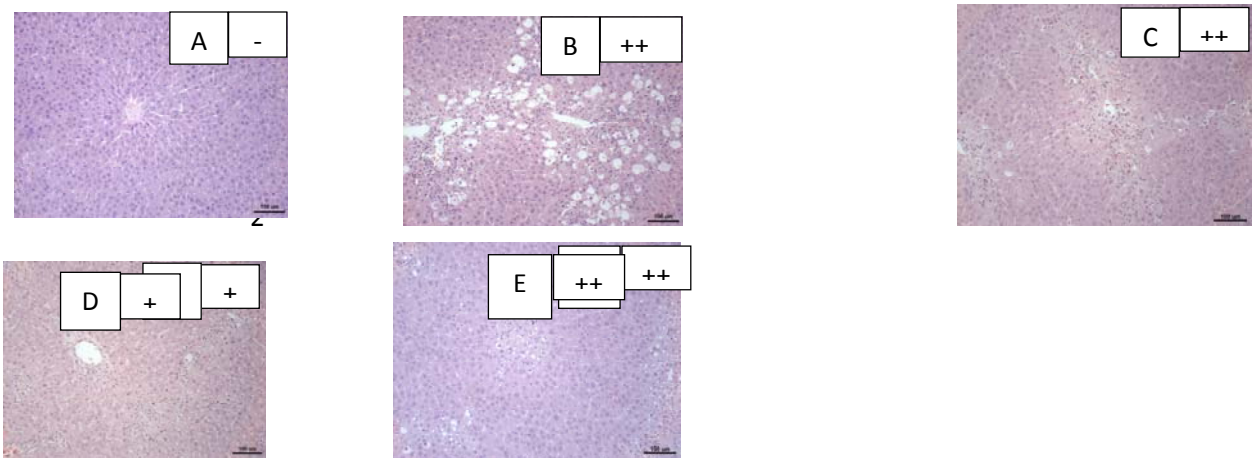

Figure 1. Cytoplasmic vacuolization of animal hepatocytes for five treatments (-) absent, (+) mild, (++) moderate and (+++) severe. A = rats received water; B = rats received water and CCl_4; C = 50 mg cassava leaf flour (CLF) extract kg^{-1} and CCl_4; D = 150 mg CLF extract kg^{-1} and CCl_4 and E = 450 mg CLF extract kg^{-1} and CCl_4.

following phenolic compounds: gallic acid, gallocatechin, catechin and chlorogenic acid.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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ABBREVIATIONS

ALT, alanine aminotransferase; **AST,** aspartate aminotransferase; **BSA,** bovine serum albumin;**CCL_4,** carbon tetrachloride; **CLF,** cassava leaf flour; **GGT,** gamma glutamyl transferase; **HPLC,** High performance liquid chromatography; **MDA,** malondialdehyde; **TBARS,** thiobarbituric acid reactive substances.

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