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THE PROTECTIVE EFFECTS OF CASSAVA (*MANIHOT ESCULENTA CRANTZ*) LEAF FLAVONOID EXTRACTS ON LIVER DAMAGE OF CARBON TETRACHLORIDE INJURED MICE

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Abstract

Background: Cassava leaf contains many kinds of flavonoids, most of flavonoids function as effective antioxidants *in vivo*. The protective effects of cassava (*Manihot esculenta* Crantz) leaf flavonoid extracts on liver damage were evaluated by carbon tetrachloride (CCl_4)-induced injury in mice.
Materials and methods: The protective effects of cassava leaf flavonoid extracts on liver damage were evaluated using CCl_4-induced injury in mice. The mice were weighted to calculate sample quantity of mice. Bloods were taken to evaluate ALT and AST of serums. Livers were excised and weighted, and fixed for pathological observation. Prepared 10% liver tissue homogenate was used to evaluate MDA, SOD, GSH-PX levels.
Results: Cassava leaf flavonoid extracts significantly decreased ($p < 0.05$) the relative liver weight when compared with the CCl_4-treated group. The contents of ALT and AST in serum of experiment mice declined significantly when compared to those of the CCl_4-treated group, but did not reach normal levels of control group. Pathological observation of livers showed that cassava leaf flavonoid extracts significantly ameliorated the CCl_4-induced pathological changes.
Conclusion These results provided biological evidence that cassava leaf flavonoid extracts indeed expressed potential efficacy of prohibiting liver injury in mice.

Key words: Cassava leaf flavonoid, Carbon tetrachloride, Liver injury, Protective effects.

Introduction

Cassava is an important crop in tropical countries because of the high carbohydrate production in its root. As an all-season crop, cassava when treated as the main food is grown in most region of Africa and cultivated mainly within the south provinces of China (Ravi & Aked., 1996; Christer et al., 2009). In addition to basic nutrients in cassava leaf, recent researches seek to focus on some bioactive compounds (e.g. flavonoid, carotene, anthocyanin) which can help to protect human health (Kubo et al., 2006; Almeida Siqueira et al., 2007; Byamukama et al., 2009). It was reported that cassava leaf contain various kinds of flavonoids (e.g. quercetin, rutin and kaempferol), most of the flavonoids function as effective antioxidants *in vivo* (Kubo et al., 2006). As an important antioxidant bioactive compounds, flavonoids can scavenge oxygen negative ion, hydroxyl free radical and singlet oxygen (Hollman et al., 1999; Montoro et al., 2005).

Oxidation as an important factor induces liver injury, anoxic/re-oxygenation injury, autoimmune hepatitis, viral hepatitis and alcoholic hepatitis (Wasmuth et al., 2005; Gensheng, et al., 1995). As an injury (inducing) agent, CCl_4 is often used extensively to induce reactive oxygen formation. CCl_4 was reported to initiate free radical mediated lipid per-oxidation resulting in the aggregation of lipid-originated oxidation products (Poli et al., 1987; Recknagel et al., 1989), and then hypernymic collagen deposition in the liver induced liver fibrosis. CCl_4 can induce oxidative stress through the inhibition of the junction between antioxidant enzyme and antioxidant substrates (Hung MY et al., 2006). Many researches have indicated that flavonoids prevented CCl_4 toxicity through the repression of lipid per-oxidation in liver (Teselkin et al., 2000), inhibiting ALT and AST enzyme activities (Lin and Huang, 2000), enhancing hepatic antioxidant enzyme activity (Kumaravelu et al., 1995).

Till today, the hepatic protection of cassava leaf flavonoid *in vivo* has not been determined. Therefore, the objective of this study was to investigate the potential protective effects of cassava leaf flavonoid in CCl_4-induced liver damage in mice.

Materials and methods

Plant material

Cassava leaves were harvested from SC5 (cassava variety) developed by Chinese Academy of Tropical Agricultural Sciences (CATAS, Hainan China). They were grown in the experimental field of the Tropical Crops Genetic Resources Institute, CATAS, in 2012. Fresh cassava leaves were washed with distilled water and then left to dry in the oven (60℃) for 48 hr. Powders were obtained by milling (0.8 mm mesh size) the dried leaves.

Chemicals

Macro-porous resins (HPD100) were purchased from Cangzhou Bonchem Co., Ltd. (Hebei, China). Kits of ALT and AST were purchased from BioSino Bio-technology and Science Co., Ltd. (Beijing, China). Food grade olive oil was purchased from supermarket (Beijing, China). Analytical grade ethanol, carbon tetrachloride and other chemicals were purchased from Beijing Chemical Corp (Beijing, China).

Preparation of the cassava leaf flavonoid extracts

Cassava leaves powders (100 g) were extracted with 1000 mL of ethanol-water (1:1, v/v) solution at 60 ℃ for 4 hr (three times). The extracts were purified through membrane filtration and then concentrated in a rotary evaporator (50 ℃) to obtain crude extracts. Supernatants were obtained after centrifuged at 3750 g for 10 min, and further enriched by macro-porous resins (HPD 100). The flavonoids absorbed to the

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column were eluted with 70% ethanol to get flavonoids solutions. Eluants were evaporated at 60 □ to dryness distilled water was added to get suspension liquids. The LD_{50} which represents the concentration of cassava leaf flavonoid extracts causing 50% death of were mice were 30.0 g/kg bw.

Animals and treatment

Male ICR mice (weighting 18-22 g) were obtained from Laboratory Animal Center, Peking University (Beijing, China). They were maintained in a controlled environment at 24 ± 1 ℃ and 55 ± 15% relatively humidity with a 12 hr dark/light cycle, and acclimatized for at least one week prior to use. Before grouping, mice were fasting for 12 hr, mice were randomly divided into five groups (10 mice in each group, n = 10): control group, CCl_4 model group, low dose group (100, mg/kg cassava leaf flavonoid extracts of bw), middle dose group (200 mg/kg cassava leaf flavonoid extracts of bw), high dose group (400 mg/kg cassava leaf flavonoid extracts of bw).

At the 33rd day, all mice were overnight fasting for 12 hr, then mice of CCl_4 group, low dose group, middle dose group and high dose group were treated with CCl_4 [(0.1 mL/kg bw, intra-peritoneal] dissolved in olive oil (0.1%, v/v), control group received the same dose of olive oil. After treated with CCl_4 for 4 hrr, low dose group, middle dose group and high dose group were feed with different concentration of cassava leaf flavonoid extracts. After cassava leaf flavonoid extracts ingestion for 24 hr or 48 hr separately (overnight fasting), animals were anaesthetized with ketamine (100 mg/kg bw). Bloods were taken to measure ALT and AST of serums. All mice were weighted, livers were taken out and weighted, liver were fixed formalin for pathological observation. Prepare 10% liver tissue homogenate to evaluate MDA, SOD, GSH-PX levels. All animal treatments were strictly in accordance with international ethical guidelines. Experiments were carried out with the approval of the Committee of Experimental Animal Administration of the Academy.

Measurement of ALT and AST activities in the serums

ALT and AST activities were measured using spectrophotometric diagnostic kits (BioSino Bio-technology and Science Co., Ltd.) compliance with the manufacturer's instructions. Enzyme activities of ALT and AST in blood serum were evaluated were measured with AUTOLAB Automatic Biochemical Analyzer V1.0 (AUTOLAB Instruments Co., Ltd. Italy).

Pathological observation of livers

After removing livers, liver tissues were rapidly trimmed to a thickness of 3 mm and placed in plastic cassettes, then immersed in neutral buffered formalin for 18 hr. The fixed tissues were processed routinely, and then embedded in paraffin, sectioned, de-paraffinized, rehydrated at last. The CCl_4-induced symptom was evaluated by assessing the morphological changes in liver sections stained with hematoxylin and eosin. Pathological analyses were performed on a DFC300 FX Digital fire-wire color camera system for fluorescence microscopy (Leica Camera Co., Ltd. Germany).

Lipid per-oxidation assays

Take liver tissue and saline solution to prepare 10% liver tissue homogenate (W:V = 1 : 9). The rat liver MDA, SOD, GSH-PX levels were evaluated in order to estimate the extent of lipid per-oxidation in the tissues, the assay was carried out using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The protein content was estimated by the dye binding assay of Bradford (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

All data were represented by means ± standard deviation (SD). Statistical analysis was performed with the SPSS 11.5 for Windows software. Statistical differences between groups under different conditions were analyzed with ANOVA (one-way analysis of variance). p- value < 0.05 was defined as statistically significant.

Results and discussion

The results are shown in Tables 1-4 and Figure 1.

Table 1: Effects of cassava leaf flavonoid extracts on mice weight

Group	0 (day)	7(day)	14(day)	21(day)	28(day)	32(day)
Control group	18.4 ± 1.3	27.0 ± 1.4	29.5 ± 2.3	32.1 ± 2.8	34.4 ± 2.8	34.8 ± 2.2
CCl_4 group	18.6 ± 1.8	27.3 ± 2.0	30.2 ± 2.0	33.9 ± 2.4	36.1 ± 2.5	36.0 ± 2.6
low dose group (100 mg/kg bw)	19.2 ± 1.3	27.6 ± 1.6	30.9 ± 3.1	35.3 ± 2.2*	37.3 ± 2.3	37.4 ± 2.5*
middle dose group (200 mg/kg bw)	18.8 ± 1.5	26.6 ± 2.8	30.2 ± 3.5	32.7 ± 3.0	34.7 ± 3.4	35.3 ± 3.0
high dose group (400 mg/kg bw)	18.9 ± 1.9	26.2 ± 3.1	29.6 ± 2.3	32.0 ± 2.3	33.5 ± 2.2	33.9 ± 2.7

Data are expressed as the mean of ten individual experiments ± SD. * $p < 0.05$ weight of CCl_4 group and different dose cassava leaf flavonoid extracts group compared with the control group.

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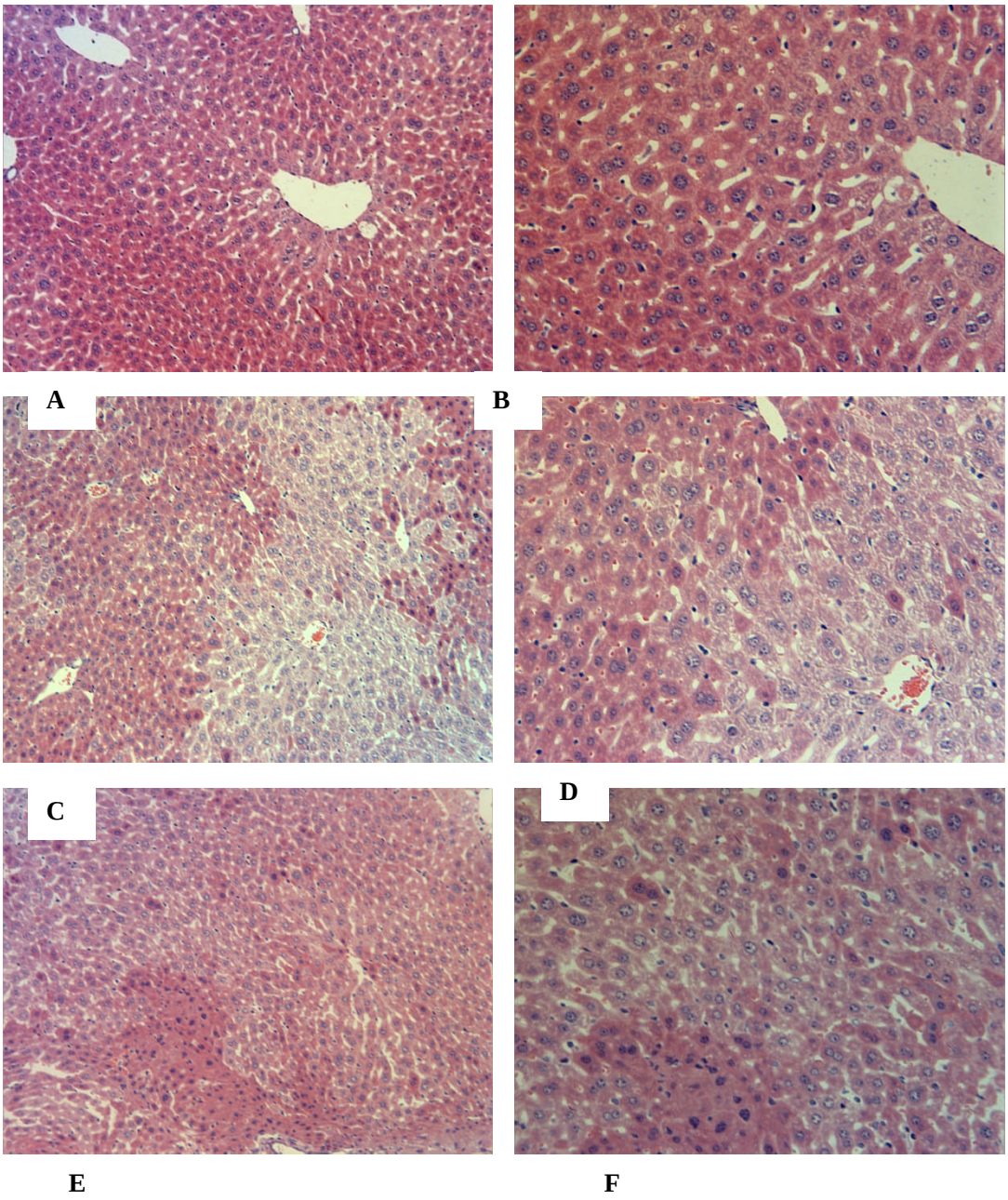

Figure 1: Effects of cassava leaf flavonoid extracts on CCl4-induced liver damage of ICR mice: (A) (B) control group, (C) (D) CCl4 group, (E) (F) Experiment group. Hematoxylin/ eosin staining; magnification: (A)(C)(E) 100×, (B)(D)(F) 200×.

Table 2: Effects of cassava leaf flavonoid extracts on mice relative liver weight

Group	Relative liver weight (%, x ± sd)
Control group	4.18 ± 0.32
CCl_4 group	4.87 ± 0.29*
low dose group (100 mg/kg bw)	4.47 ± 0.25
middle dose group (200 mg/kg bw)	4.33 ± 0.33
high dose group (400 mg/kg bw)	4.31 ± 0.40

Data are expressed as the mean of ten individual experiments ± SD. * $p < 0.05$ relative liver weight of CCl4 group and different dose cassava leaf flavonoid extracts group compared with the control group.

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Table 3: Effects of cassava leaf flavonoid extracts on contents of ALT and AST in blood

Group	24 hr		48 hr	
	ALT (mmol/L, x ± sd)	AST (mmol/L, x ± sd)	ALT (mmol/L, x ± sd)	AST (mmol/L, x ± sd)
Control group	399 ± 21.1	392.0 ± 15.9	44.9 ± 21.1	87.3 ± 25.9
CCl_4 group	1170.9 ± 11.6**	667.5 ± 25.3**	172.5 ± 22.8**	138.5 ± 17.0**
low dose group (100 mg/kg bw)	675.2 ± 23.9*	550.6 ± 16.1*	110.0 ± 43.1*	111.9 ± 21.1*
middle dose group (200 mg/kg bw)	589.5 ± 25.7*	504.7 ± 34.1	87.1 ± 25.9#	102.7 ± 14.3
high dose group (400 mg/kg bw)	482.8 ± 27.9	471.6 ± 27.4	56.4 ± 16.4	97.4 ± 16.7

Data are expressed as the mean of ten individual experiments ± SD. * $p < 0.05$ contents of ALT and AST of CCl_4 group and different dose cassava leaf flavonoid extracts group compared with the control group. ** $p < 0.01$ contents of ALT and AST of CCl_4 group and different dose cassava leaf flavonoid extracts group compared with the control group.

Table 4 Effects of cassava leaf flavonoid extracts on contents of SOD, MDA and GSH-PX in liver homogenate

Group	MDA *(nmol/mL)*	SOD *(U/mgprot)*	GSH—PX *(U/mgprot)*
Control group	0.228 ± 0.073	670.9 ± 13.4	128.7 ± 22.6
CCl_4 group	0.329 ± 0.148**	551.4 ± 15.6**	103.5 ± 25.8**
low dose group (100 mg/kg bw)	0.318 ± 0.173*	567.5 ± 13.5	112.6 ± 20.8
middle dose group (200 mg/kg bw)	0.279 ± 0.060	583.3 ± 11.3	117.1 ± 28.8
high dose group (400 mg/kg bw)	0.255 ± 0.122	587.5 ± 11.6	121.9 ± 26.8

Data are expressed as the mean of ten individual experiments ± SD. * $p < 0.05$ contents of SOD, MDA and GSH-PX of CCl_4 group and different dose cassava leaf flavonoid extracts group compared with the control group. ** $p < 0.01$ contents of SOD, MDA and GSH-PX of CCl_4 group and different dose cassava leaf flavonoid extracts group compared with the control group.

Effect of flavonoid extracts on mice weight

Weight is an initial basic index of medical experiments and preliminary conclusions of extracts on mice were drawn through weight difference. Through measuring mice weight at different stages (Table 1), there were no significant differences on weight between control group，model group, low dose group, middle dose group and high dose group. Weights of cassava leaf flavonoid extracts feeding mice were slightly heavier（$p > 0.05$）than those of control group and CCl_4 group. The experiment showed that flavonoid extracts have no significant pernicious influence on the health of mice.

Effect of flavonoid extracts on relative liver weight

Table 2 showed a significant increase ($p < 0.05$) in the relative liver weights of CCl_4-induced mice compared with the control group. The relative liver weight of experiment group feeding on cassava leaf flavonoid extracts (100, 200, 400 mg/kg of bw) significantly decreased ($p < 0.05$) when compared with the CCl_4 group.

Effect of flavonoid extracts on ALT and AST in the serums

Several hepatic enzymes in the serum were employed as the biochemical markers to show early hepatic injury, such as ALT, AST. Treatment with CCl_4 significantly ($p < 0.05$) elevated the levels of serum ALT and AST compared with the control group (Table 3). Cassava leaf flavonoid extracts (100, 200, 400 mg/kg of bw) during CCl_4 treatment significantly ($p < 0.05$) lowered the serum ALT and AST activities compared with the CCl_4 group. CCl_4 induced model was reported to result in the serum levels of ALT, AST, LDH and ALP increase (Hung et al., 2006; Teocharis et al., 2001). We found that treatment of cassava leaf flavonoid extracts had a significant protective effect against CCl_4-induced hepatotoxicity in mice, as evidenced by decreasing serum ALT and AST activities. Furthermore, the protective effects of cassava leaf flavonoid extracts showed a dose-dependent.

Pathological observation of livers

An experiment involving $CC1_4$ administration was employed to observe liver morphological changes. Representative photographs of the liver morphology were shown in Figure1. Compared to normal rat liver morphology (Figure 1A), CCl_4-induced liver morphological changed evidenced by cell nucleus staining. $CC1_4$-intoxicated mice treated with cassava leaf flavonoid extracts significantly ameliorated the CCl_4-induced pathological changes (such as necrosis, cytoplasmic vacuolization and injury) (Figure 1C–F).

Effect of flavonoid extracts on CCl_4-induced lipid per-oxidation levels

Liver lipid per-oxidation levels are expressed as MDA, SOD and GSH-PX content in Table. 4. Liver MDA contents were significantly increased to 0.329 *nmol/mL* ($p < 0.05$) in mice treated with CCl_4 compared with the control group. Cassava leaf flavonoid extracts (100, 200, 400 mg/kg of bw) during CCl_4 treatment significantly ($p < 0.05$) decreased the MDA contents. Liver SOD and GSH-PX contents were significantly

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decreased ($p < 0.05$) in mice treated with CCl_4 compared with the control group. Cassava leaf flavonoid extracts (100, 200, 400 mg/kg of bw) during CCl_4 treatment significantly ($p < 0.05$) raised the SOD and GSH-PX contents. Flavonoids are antioxidants preventing free radicals oxidation effectively. It was reported that antioxidant capacity is determined by the number and location of hydroxyl groups (Montoro et al., 2005). As a kind of flavonol, rutin content was high in cassava leaf (Kubo et al., 2006). Rutin's chemical structure is characterized by polyphenol hydroxyl groups which are related to scavenge free radicals (Aisling & O'Brien, 2002). And rutin were reported to prevent attacks to liver cells from free radicals, so activities of damaged enzymes in liver cells can resume maintaining the integrity of cell membranes (Janbaza et al., 2002). Therefore, the target in safe-guarding liver cells from a variety of damages may prove viable.

Oxidative stress is a part of the phenomenon in the CCl_4-induced hepatic damage that was evaluated in this study. In the paper, we choose a series of biomarkers to evaluate the liver protection of cassava leaf flavonoid extracts, such as serum biological analysis (including ALT and AST, the major markers of hepatic injury), free radical-mediated lipid per-oxidation capacity (MDA value), and the antioxidant enzymes expression (including GSH-PX and SOD) in the liver. The overall results indicated that cassava leaf flavonoid extracts have potential ability against CCl_4-induced damage. It has been reported that flavonoids can suppress the serum levels of GOT, GPT, LDH and ALP induced by CCl_4-induced (Ohshima et al., 1998; Weber et al., 2003). The data provided in the present study support the above research conclusion.

Conclusion

In conclusion, protective effects of cassava leaf flavonoid extracts were confirmed through a mouse model of CCl_4-induced liver injury. In the present study, the data showed that cassava leaf flavonoid extracts indeed showed potential efficacy *in vivo* (including enhancing the expression of antioxidant enzymes, decreasing lipid per-oxidation, etc.). This study provides biological evidence that cassava leaf flavonoid extracts is beneficial in protecting against liver damage. Although it seems difficult to entirely recuperate the chronic hepatic damage-induced by CCl_4, cassava leaf flavonoid extracts indeed delayed the liver injury by prohibiting the oxidative stress. Therefore, this protective mechanisms of the extracts need to be investigated deeply in the future.

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