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Protective effect of total flavonoids from *Bidens bipinnata* L. against carbon tetrachloride-induced liver injury in mice

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Abstract

Bidens bipinnata L. is well known in China as a traditional Chinese medicine. This study was designed to evaluate the hepatoprotective activity of the total flavonoids of *B. bipinnata* L. (TFB) against carbon tetrachloride (CCl_4)-induced acute liver injury in mice and to determine its mechanism of action. Oral administration of TFB at doses of 50, 100 and 200 mg kg^{-1} for 7 days significantly reduced the elevated relative values of liver weight, serum transaminases (alanine aminotransferase and aspartate aminotransferase) and the hepatic morphologic changes induced by CCl_4 in mice. In addition, TFB markedly inhibited CCl_4-induced lipid peroxidation and enhanced the activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase. Moreover, pretreatment with TFB suppressed nitric oxide production and nuclear factor-κB activation in CCl_4-treated mice. The results suggest that TFB has significant hepatoprotective activity and its mechanism is related, at least in part, to its antioxidant properties. Further research is required to investigate the detailed mechanism of the protective effect of TFB on acute liver injury.

Introduction

There is no doubt that oxidative stress, with reactive oxygen intermediate formation, plays a major role in several liver diseases. Free radicals formed as a result of oxidative stress injure the cell membrane of hepatocytes by lipid peroxidation or other means. They cause extensive damage to DNA, proteins, lipids and carbohydrates, which leads to various acute and chronic liver injuries (Comporti 1985). A large number of studies have focused on the pathogenetic significance of oxidative stress in experimental models of hepatic damage. However, there is no successful treatment in clinical practice for liver injury caused by oxidative stress and subsequent cell death or apoptosis. Interest has therefore focused on the antioxidant and anti-inflammatory properties of many natural products (Flora et al 1996). Natural products have been used effectively for several centuries without obvious toxicity or side-effects. In recent years, the hepatoprotective effects of various natural products have been reported (Park et al 2000; Tang et al 2006). Carbon tetrachloride (CCl_4), the classic hepatotoxin, is widely used to induce liver damage in animal models and to investigate the role of lipid peroxidation as a mediator of hepatic injury (Brattin et al 1985). The toxic effects come from the activation of CCl_4 via the liver microsomal cytochrome P450 system to the trichloromethyl radical ($CCl_3\cdot$), which reacts rapidly with molecular oxygen to produce the trichloromethyl peroxy radical ($CCl_3O_2\cdot$). These radicals initiate lipid peroxidation by withdrawing allylic hydrogens from polyunsaturated fatty acids (Slater 1984; Recknagel et al 1989). When the amount of reactive oxygen species production exceeds the capacity of the endogenous cellular antioxidant system, significant liver injury can occur (Williams & Burk 1990). These effects, in turn, induce the production of inflammatory cytokines by immune cells such as Kupffer cells, and the cytokines then induce injury of the entire liver (Edwards et al 1993). The defence provided by antioxidant systems is therefore crucial in liver injury and disease.

The genus *Bidens* (Asteraceae), widely found in tropical and subtropical areas of the world, has been used in various folk medications. It is used to treat hepatitis, inflammation,

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malaria, hypertension, diabetes, peptic ulcer, snake bite and smallpox (Winkelman 1989; Lin 1992; Alvarez et al 1999; Pereira et al 1999; Dimo et al 2003; Oliveira et al 2004; Nguelefack et al 2005). *Bidens bipinnata* L., commonly known as "po-po-zhen" in China, was first recorded in the "*Supplement to the Herbal*" written by a famous physician in the Tang Dynasty 1000 years ago. It has also been applied in the treatment of jaundice, rheumatism, laryngitis, headache and digestive disorders (Jiangsu New Medical College 1985).

Several ethnopharmacological studies carried out with plants of the genus *Bidens* have demonstrated hepatoprotective activity (Chin et al 1996). In addition, the extract of *Bidens pilosa* L. could inhibit lipopolysaccharide-mediated cytokines, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 expression by blocking nuclear factor-κB (NF-κB) activation in RAW 264.7 macrophages and exhibits significant free radical scavenging activity, comparable with that of α-tocopherol (Chiang et al 2005). Strong effects on the inhibition of lipopolysaccharide-mediated nitric oxide (NO) production in RAW 264.7 cells have also been observed (Chiang et al 2004).

Flavonoids are naturally occurring phenolic compounds, nearly ubiquitous in plants, and have long been recognized to possess antioxidant, hepatoprotective, anti-inflammatory, anti-allergic, antithrombotic, antiviral and anticarcinogenic activities (Middleton et al 2000). In several countries, flavonoids are widely used in the treatment of liver diseases. Moreover, flavonoid dietary intake far exceeds that of vitamin E, a monophenolic antioxidant, and that of β-carotene, on a milligram per day basis (Hertog et al 1993). One of the best known and widely used hepatoprotective plant extracts is silymarin, the most active flavonoid component of which is silybin. It has been reported to prevent liver injuries induced by various chemicals or toxins, and its hepatoprotective effects mainly include antioxidant, anti-inflammatory and antifibrotic activity (Song et al 2006). We extracted the total flavonoids of *B. bipinnata* L. (TFB) and further investigated its hepatoprotective activity in mice treated with CCl_4. In addition, the antioxidant properties of TFB in acute liver injury of mice were studied.

Materials and Methods

Plant material

B. bipinnata L. was purchased from a crude drug market in Bozhou, Anhui Province, China, on October 2005. It was classified by Dr De-qun Wang (Department of Pharmacy, Anhui College of Traditional Chinese Medicine, China) and a voucher specimen (no. AH20051012) was deposited in the herbarium of the College of Pharmacy, Anhui Medical University, China.

Preparation of TFB

The dried aerial parts of *B. bipinnata* L. were cut into small pieces and extracted three times with hot 80% ethanol. The extracts were combined and concentrated in-vacuo to syrup, followed by suspension in water. The suspension was extracted with ligarine to remove the lipophilic constituents. Then, the remaining water fraction was condensed with a rotary evaporator and dried in a vacuum drying oven at 60°C for 5 h, yielding a dark brown mass. The dried crude extract was dissolved again with distilled water (w/v 1:20), undissolved impurities were filtered, and the clarified liquid was collected. The clarified liquid was passed through HPD100 macroporous adsorptive resin columns (Cangzhou Bon Chemical Co., Ltd, Hebei Province, China). An ordered elution was performed using distilled water, 30% ethanol, 50% ethanol and 95% ethanol. Only the 30% ethanol-eluted solution was collected. The 30% ethanol eluate was concentrated in-vacuo and dried by lyophilization to yield a brown powder that reacted intensely with magnesium hydrochloric acid. This powder was referred to as TFB, and the total flavonoid content, measured using a colorimetric assay developed by Zhishen et al (1999), was 66.2%. The powder was suspended in physiological saline and administered orally.

Phytochemistry studies of *B. bipinnata* L. have revealed the presence of several flavonoids, such as hyperoside, rutin, maritimetin, quercetin, okanin, iso-okanin, 7-*O*-(4″,6″-diacetyl)-β-D-glucopyranoside, (*Z*)-6-*O*-(3, 6-di-*O*-acetyl-D-glucopyranosyl)-6, 7,3′,4′-tetrahydroxyaurone and 2′,4′,6′-trimethoxy-4-*O*-D-glucopyranosyl-dihydrochalcone (Li et al 2003, 2005).

Chemicals

CCl_4 was purchased from Shanghai Changjiang Chemistry Plant (Shanghai, China) and dissolved in olive oil to a final concentration of 0.2% before use. Bifendate was purchased from Beijing Union Pharmaceutical Factory (Peking, China) and suspended in physiological saline before use. Commercial kits used for determining alanine aminotransferase (ALT), aspartate aminotransferase (AST), thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), NO activity and total protein were purchased from the Jiancheng Institute of Biotechnology (Nanjing, China). Other chemicals and reagents used in these experiments were of analytical grade and were purchased from commercial sources.

Animals

Five-week-old male Kunming mice, 20 ± 2 g, were obtained from the Animal Department of Anhui Medical University and maintained in a temperature (23 ± 2°C) and humidity (55–60%) controlled room with a 12-h light–dark cycle (lights on from 0600 to 1800 hours). Animals were housed in plastic cages with free access to food and water. All animals received human care in compliance with the Guidelines of the Animal Care and Use of Laboratory Animals as set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Experimental design

Mice were randomly divided into six groups with 10 mice per group: normal control group, CCl_4 group, TFB (50, 100 and 200 mg kg^{-1}) groups and the bifendate (100 mg kg^{-1}) group.

Mice were administered orally by gastric gavage with different doses of TFB or bifendate at a volume of 10 mL kg^{-1} once a day for 7 days; the normal control group and the CCl_4 model group were administered with an equivalent volume of physiological saline. On Day 7, at 2 h after the final administration of TFB or bifendate, the mice were intraperitoneally injected with CCl_4 at the dose of 20 μL kg^{-1} bodyweight, that is 10 mL kg^{-1} as a 0.2% olive oil solution, and the mice in the normal control group were injected with an equivalent volume of olive oil alone (Chen et al 2004). At 16 h after the CCl_4 injection, each mouse was weighed and then killed under ether anaesthesia for blood collection via puncture of the retro-orbital venous plexus. Serum was obtained from the collected blood by centrifugation immediately after death, and livers were well perfused with ice-cold saline and isolated. The isolated livers were washed in ice-cold saline again, blotted on a filter and then weighed to calculate the relative liver weights according to the equation of Lee et al (2006). The left lobes of the livers were sampled for histopathology assessment and NF-κB immunohistochemistry analysis, and the remaining tissue was immediately stored at −80°C until use. Samples for electron microscopy were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (pH = 7.4).

Assessment of liver function

The serum activity of ALT and AST was estimated by spectrophotometry using commercial kits produced by Jiancheng Institute of Biotechnology (Nanjing, China). Activity was expressed as an international unit (units L^{-1}).

Preparation of liver homogenate

Liver tissues were cut into small pieces and homogenized with Tris/HCl (5 mmol L^{-1} containing 2 mmol L^{-1} EDTA, pH 7.4). The homogenates were centrifuged at 3000 *g* for 10 min at 4°C and clear supernatants were used immediately for assessment of liver lipid peroxidation and SOD, GSH-Px and NO activity.

Measurement of lipid peroxidation

The degree of lipid peroxidation in liver tissues was determined by measuring TBARS (Gavino et al 1981). Briefly, a mixture of 1 mL of 10% liver homogenate and 0.67% (w/v) thiobarbituric acid was heated at 100°C for 30 min. The solution was cooled to room temperature using tap water and centrifuged at 3000 *g* for 10 min and then the supernatant was collected. Absorption of the pink supernatant was determined spectrophotometrically at 535 nm. The amount of TBARS was expressed as nmol malondialdehyde (mg protein)$^{-1}$.

Determination of antioxidant enzyme activity

The antioxidant enzyme activity in liver tissues was determined by measuring SOD and GSH-Px using the colorimetric assay according to the manufacturer's instructions. Briefly, the assay for total SOD was based on its ability to inhibit the oxidation of oxymine by the xanthine/xanthine oxidase system. One unit of SOD activity was defined as the amount that reduced the optical density at 550 nm by 50%. GSH-Px was measured by the dithio-bis-nitrobenzoic acid method of Sedlak & Lindsay (1968). One unit of GSH-Px was defined as the amount that reduced the level of GSH by 1 μmol L^{-1} (mg protein)$^{-1}$.

Measurement of NO and protein

The NO level in liver tissues was detected using a commercial kit based on the nitrate reductase method. The protein content in homogenates was assayed by the method of Lowry et al (1951) using bovine plasma albumin as a standard.

Histopathologic analysis

Fresh liver blocks were cut and immediately fixed in 10% phosphate-buffered formalin and then dehydrated in graded alcohol and embedded in paraffin. Paraffin sections of 5-μm thickness were rehydrated and stained with haematoxylin and eosin. Stained sections were observed under light microscopy (Olympus LX70; Olympus Japan) and later subjected to image analysis (BI 2000; TaiMeng Technology, China). The percentage area of necrosis was determined by dividing the area of necrosis by the sum of the reference area of ten low power fields (Tipoe et al 2006).

Immunohistochemistry of NF-κB

Sections of formalin-fixed, paraffin-embedded tissue were cut onto silanized glass slides and stained by means of a SP kit (Zymed, South San Francisco, CA, USA). Rabbit anti-human monoclonal NF-κB (p65) IgG (Santa Cruz, CA, USA) was used as the primary antibody. As immunohistochemistry controls, some sections were incubated in the same way but with normal rabbit serum or with phosphate-buffered saline (0.01 mol L^{-1}, pH 7.4) alone instead of the primary antibody. After being immunostained, the sections were counterstained with haematoxylin.

Electron microscopy

Fresh liver blocks were cut into 1-mm^3 pieces and immediately fixed in 2.5% glutaraldehyde, then postfixed in 2% osmium tetraoxide and embedded in Epon618 resin. Sections of 70-nm thickness were stained with uranyl acetate and lead citrate prior to analysis with electron microscopy (JEM-1230; Jeol, Japan). For quantification of mitochondrial size, five random fields (magnification ×8000) from five random cells in each section were photographed by an electron microscopist blinded to the treatment groups; each photograph contained five to ten mitochondria in a particular cell. Morphometric measurement of the mitochondrial area was performed on the five largest mitochondria per photograph using image analysis software (Kirsch et al 2003).

Statistical analysis

Results were expressed as mean±s.d. Data were analysed by one-way analysis of variance followed by the Students–Newman–Keuls test. $P<0.05$ was considered to be statistically significant.

Results

Effect of TFB on liver weight and liver function

The relative liver weights were significantly augmented after treatment with CCl_4 alone compared with the normal control group. In contrast, pretreatment with TFB (50, 100 and 200 mg kg^{-1}) significantly reduced the relative liver weights compared with mice that received CCl_4 treatment alone (Table 1). Similarly, the CCl_4-treated mice had elevated serum ALT and AST levels, demonstrating marked liver damage. Pretreatment with TFB (50, 100 and 200 mg kg^{-1}) attenuated the CCl_4-induced increase in ALT and AST activity ($P<0.05$) (Table 1).

Effect of TFB on hepatic lipid peroxidation

The level of lipid peroxidation in liver tissues, as measured by the concentration of hepatic TBARS, was significantly increased in mice that received CCl_4 treatment alone compared with the normal control group. Oral administration of TFB (50, 100 and 200 mg kg^{-1}) for 7 days led to a significant decrease in the degree of lipid peroxidation ($P<0.01$) (Table 2).

Effect of TFB on the activity of hepatic antioxidant enzymes

The activity of antioxidant enzymes, including SOD and GSH-Px, were significantly inhibited in the liver tissue of mice treated with CCl_4 alone. Pretreatment with TFB (50, 100 and 200 mg kg^{-1}) markedly increased the levels of SOD and GSH-Px. However, in the positive control group, bifendate (100 mg kg^{-1}) had no effect on GSH-Px activity (Table 2).

Effect of TFB on NO production

Injection of CCl_4 led to an approximately 4-fold increase in the concentration of NO in liver tissues. The mice treated with TFB (50, 100 and 200 mg kg^{-1}) showed a significant decrease in the level of NO (Table 2).

Liver histopathology

No histological abnormalities were observed in normal control mice. The hepatic parenchyma appeared normal and hepatocytes were arranged around the central vein (Figure 1A). The livers of mice treated with CCl_4 alone showed marked centrilobular necrosis in hepatocytes, with marked mononuclear cell infiltration (Figure 1B). The necrotic hepatocytes were characterized by cell enlargement and nuclear dissolution. The percentage area of necrosis was the highest in the CCl_4 group (Figure 1D). In the TFB (50, 100 and 200 mg kg^{-1}) group, the degree of necrosis and inflammation was less extensive than that in livers from mice treated with CCl_4 alone (Figure 1C, D). A significant difference was also observed in the bifendate group (Figure 1D).

Table 1 Effect of total flavonoids of *Bidens bipinnata* L. (TFB) on the relative liver weight and on the activity of serum alanine aminotransferas (ALT) and aspartate aminotransferase (AST) in mice treated with carbon tetrachloride (CCl_4)

Group	Dose (mg kg^{-1})	Liver weight (%)	ALT (units L^{-1})	AST (units L^{-1})
Normal	–	5.01 ± 0.50	17.63 ± 6.41	48.49 ± 10.70
CCl_4	–	6.25 ± 0.72**	177.78 ± 8.50**	187.50 ± 27.75**
TFB	50	5.66 ± 0.63$^{\#}$	106.92 ± 7.39$^{\#\#}$	165.41 ± 20.24$^{\#}$
	100	5.51 ± 0.76$^{\#}$	100.42 ± 12.83$^{\#\#}$	158.10 ± 21.34$^{\#\#}$
	200	5.48 ± 0.65$^{\#}$	82.64 ± 10.07$^{\#\#}$	154.43 ± 10.59$^{\#\#}$
Bifendate	100	5.55 ± 0.54$^{\#}$	75.83 ± 24.15$^{\#\#}$	170.87 ± 24.59

Data are expressed as mean ± s.d., n = 10 per group. **$P<0.01$ compared with the normal control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the CCl_4 group.

Table 2 Effects of total flavonoids of *Bidens bipinnata* L. (TFB) administration on thiobarbituric acid reactive substances (TBARS) formation, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity, and nitric oxide (NO) production in the liver of mice treated with carbon tetrachloride (CCl_4)

Group	Dose (mg kg^{-1})	TBARS (nmol (mg protein)$^{-1}$)	SOD (units (mg protein)$^{-1}$)	GSH-Px (units (mg protein)$^{-1}$)	NO (nmol (mg protein)$^{-1}$)
Normal	–	5.40 ± 2.52	143.59 ± 29.83	97.93 ± 5.60	1.14 ± 0.25
CCl_4	–	8.71 ± 1.38**	80.62 ± 18.21**	50.41 ± 8.48**	4.36 ± 1.02**
TFB	50	6.08 ± 2.45$^{\#\#}$	102.63 ± 18.79$^{\#}$	60.15 ± 8.15$^{\#}$	3.39 ± 0.56$^{\#}$
	100	5.12 ± 2.51$^{\#\#}$	104.41 ± 17.45$^{\#\#}$	79.69 ± 8.81$^{\#\#}$	2.65 ± 0.46$^{\#\#}$
	200	5.57 ± 1.64$^{\#\#}$	122.88 ± 25.24$^{\#\#}$	86.82 ± 9.35$^{\#\#}$	1.75 ± 0.49$^{\#\#}$
Bifendate	100	6.16 ± 1.60$^{\#\#}$	98.67 ± 19.29$^{\#}$	56.56 ± 6.92	3.33 ± 0.73$^{\#}$

Hepatic TBARS, SOD, GSH-Px and NO were measured in liver homogenates collected 16 h after treatment with CCl_4. Data are expressed as mean ± s.d., n = 10 per group. **$P<0.01$ compared with the normal control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the CCl_4 group.

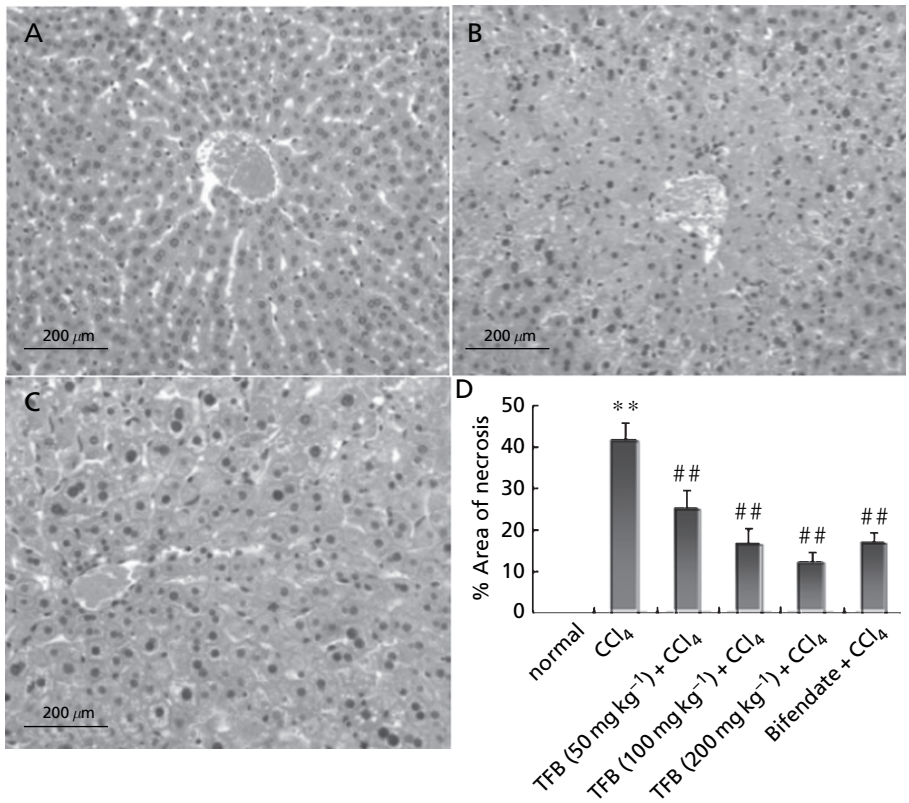

Figure 1 Histopathological examinations performed under a light microscope (magnification ×200) on liver specimens obtained 16 h after carbon tetrachloride (CCl_4) treatment. A. Liver section from mice treated with saline and olive oil (normal control group) showing normal liver architecture. B. Liver section from CCl_4-treated mice showing severe hepatocellular necrosis. C. Liver section from mice pretreated with 200 mg kg^{-1} total flavonoids of *Bidens bipinnata* L. (TFB) + CCl_4 showing a marked reduction in the severity of hepatocellular necrosis. D. The percentage area of necrosis was further characterized by using image analysis. Data are expressed as mean ± s.d., n = 10 per group. $^{**}P < 0.01$ compared with the normal control group; $^{\#\#}P < 0.01$ compared with the CCl_4 group.

Effect of TFB on NF-κB expression in liver tissue

The effect of TFB pretreatment on CCl_4-induced expression of NF-κB was assessed by immunohistochemistry. In the normal mice, occasional hepatocytes were positive for NF-κB protein (Figure 2A). After treatment with CCl_4, NF-κB expression was significantly increased, predominantly in the centrilobular regions of the liver (Figure 2B). Mice pretreated with TFB showed a marked reduction in the number of NF-κB-positive hepatocytes (Figure 2C). The image analysis of the number of cells showing positive staining for NF-κB confirmed the effect of TFB in reducing NF-κB protein expression (Figure 2D).

Ultrastructural morphology

Mice treated with CCl_4 alone showed evidence of ultrastructural injury that was not observed in normal control mice. Such changes included profound mitochondrial swelling, even vacuolization (some were 3–4-fold the normal size), and disruption of mitochondrial inner membranes, loss of the normal relationship between the rough endoplasmic reticulum and mitochondria, blebbing of the nuclear membrane and increased lipid droplets in the cytoplasm (Figure 3A, B). Morphometric analysis confirmed differences in mitochondrial size in treated mice and normal control mice (average mitochondrial surface area in the normal group and CCl_4-treated group was $0.09 \pm 0.03\ \mu m^2$ and $0.38 \pm 0.09\ \mu m^2$, respectively; $P < 0.001$) (Figure 3D). Mice pretreated with TFB (50, 100 and 200 mg kg^{-1}) had a marked improvement in the degree of damage to hepatocyte organelles (Figure 3C, D).

Discussion

Acute liver injury is a common pathology with very high morbidity and mortality. It is mostly induced by viral hepatitis, alcoholism, iron overload or drug toxicity. Among these types of liver injuries, there is consistent evidence of enhanced production of free radicals and/or a significant decrease in antioxidant defence mechanisms (Hoek & Pastorino 2002). Many drugs have been used in the treatment of liver damage. However, it is still difficult to achieve satisfactory therapeutic effects. In recent years, the therapeutic benefits of traditional Chinese medicine have been recognized and

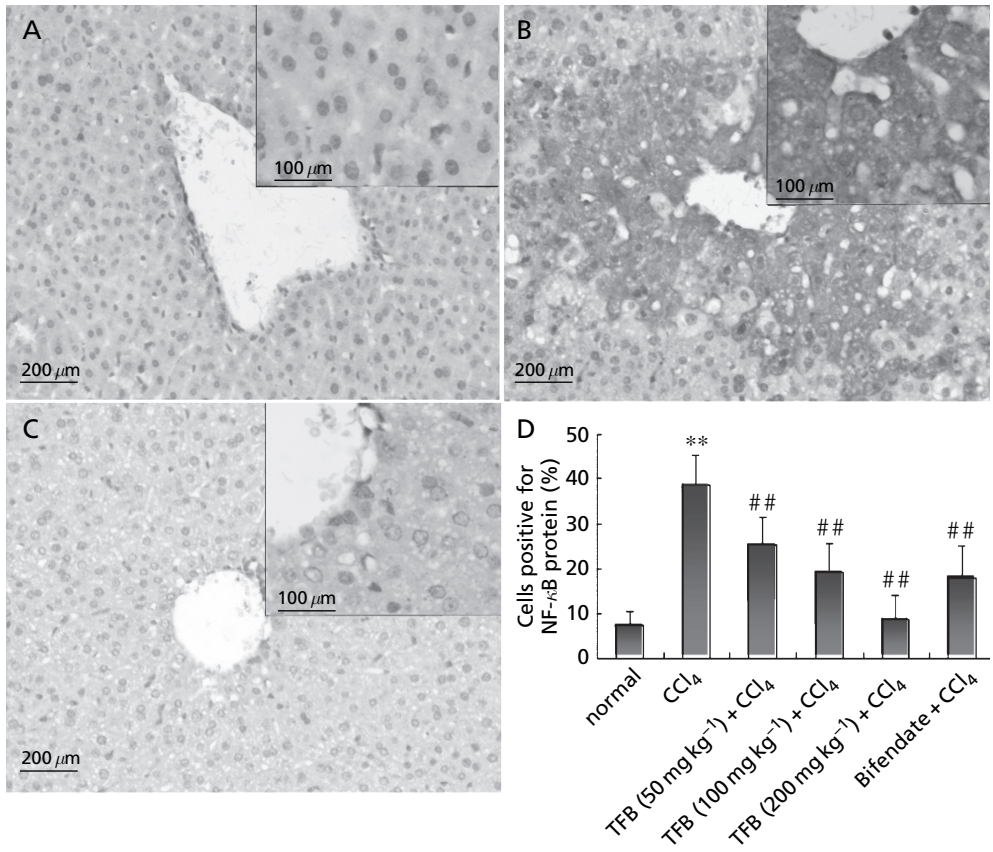

Figure 2 Immunohistochemistry of nuclear factor κB (NF-κB) p65 in the liver (magnification ×200, ×400). Liver specimens were obtained from mice treated with saline and olive oil (normal control group; A), mice treated with carbon tetrachloride (CCl_4) alone (B), and mice pretreated with 200 mg kg^{-1} total flavonoids of *Bidens bipinnata* L. (TFB) + CCl_4 (C). The presence of NF-κB was further characterized by image analysis (D). Data are expressed as mean ± s.d., n = 10 per group. $^{**}P < 0.01$ compared with the normal control group; $^{\#\#}P < 0.01$ compared with the CCl_4 group.

widely accepted. *B. bipinnata* L. is commonly used on the basis of clinical experience for the treatment of hepatitis, liver fibrosis and other inflammations in Asia. We show here for the first time the hepatoprotective effects of TFB in the CCl_4-induced liver injury model in mice.

Our results demonstrated that the oral administration of TFB effectively protected mice against CCl_4-induced acute liver injury. CCl_4 is known to cause hepatic damage, with a marked elevation in the serum levels of the aminotransferases enzymes AST and ALT, because these enzymes are cytoplasmatic and are released into the blood after cellular damage (Recknagel et al 1989). Our results also showed a significant increase in the activity of ALT and AST in CCl_4-treated mice. The increase in the activity of these enzymes in serum was found to decrease after pretreatment with TFB. In addition, TFB ameliorated the severe hepatic pathological abnormalities induced by CCl_4. These results indicate that TFB can exert a hepatoprotective effect on CCl_4-induced acute liver injury in mice.

In the liver, CCl_4 is converted to trichloromethyl free radicals by its dehalogenation by the cytochrome P450 system, which forms trichloromethyl peroxy radicals (Slater 1984). These reactive oxygen species generate lipid peroxides that induce hepatocellular injury and activate Kupffer cells by releasing increased amounts of active oxygen species and other bioactive agents. It has been shown that TBARS, an index of lipid peroxidation and oxidative stress, damages cells and tissues. SOD is responsible for neutralizing the most common free radical, known as superoxide. It also aids the body's utilization of the minerals copper, zinc and manganese. GSH-Px, along with SOD, is one of the body's endogenous antioxidants, and is well known to protect liver cells against oxidative damage through chemical or enzymatic reactions. In this study, we found an increase in TBARS content and a decrease in SOD and GSH-Px activity in the liver of mice treated with CCl_4 alone. Pretreatment with TFB (50, 100 and 200 mg kg^{-1}) was found to prevent both the increase in liver TBARS content and the decrease in liver SOD and GSH-Px activity. These results suggest that administration of TFB may decrease lipid peroxidation, improve antioxidant status and thereby prevent damage to the liver and leakage of the enzymes ALT and AST. We also found changes in the ultrastructural morphology of liver tissues in CCl_4-treated mice, including mitochondrial injury, loss of the rough endoplasmic reticulum and an increase in lipid droplets. The degeneration may be caused by oxidative stress and consequent lipid

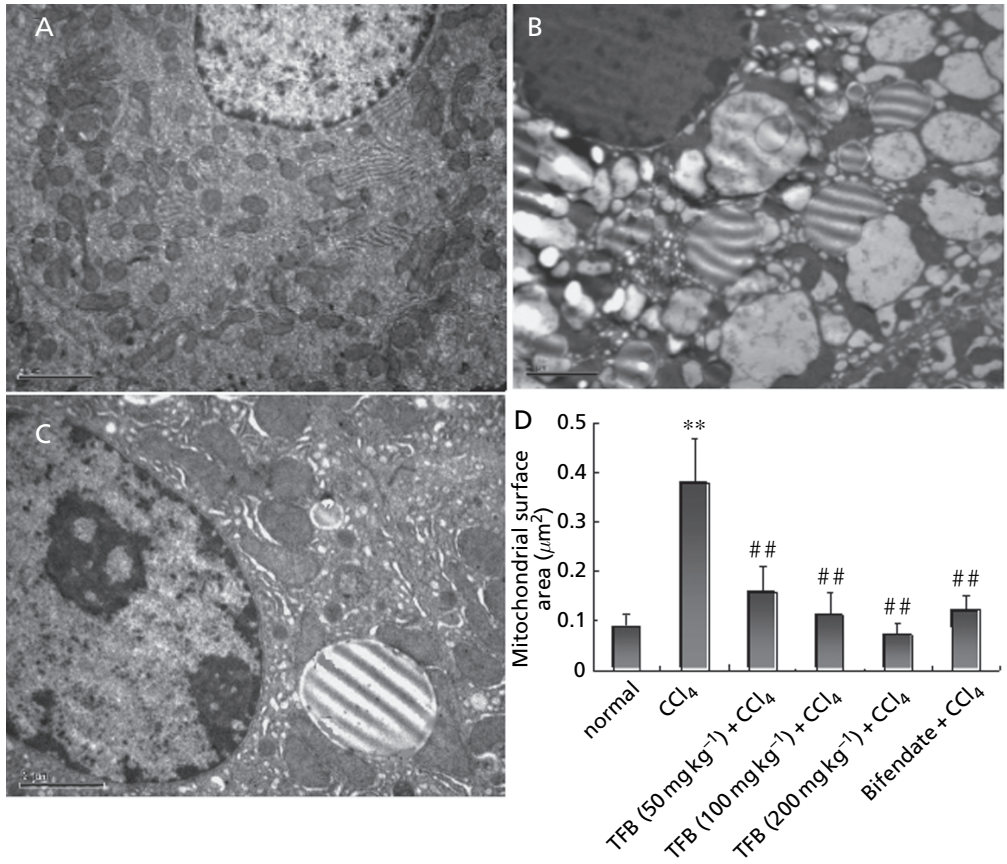

Figure 3 Ultrastructural morphology examination performed on liver specimens under a transmission electron microscope (magnification ×8000). A. Representative hepatocyte from a normal control animal. B. Hepatocyte from a mouse treated with carbon tetrachloride (CCl_4) alone. C. Hepatocyte from a mouse pretreated with 200 mg kg^{-1} total flavonoids of *Bidens bipinnata* L. for 7 days before CCl_4 injection. D. Morphometric analysis showing mitochondrial size in the different groups. Data are expressed as mean ± s.d., n = 10 per group. $^{**}P < 0.01$ compared with the normal control group; $^{\#\#}P < 0.01$ compared with the CCl_4 group.

peroxidation, which decomposed the membrane phospholipids of the cell organelles due to free radical attack (Kirsch et al 2003). Mice pretreated with TFB (50, 100 and 200 mg kg^{-1}) showed a significant improvement in the degree of damage to hepatocyte organelles. Therefore, the results clearly show that the protective effect of TFB on CCl_4-induced acute liver injury is attributed to its antioxidant properties.

It has been reported that NO is involved in acute liver injury based on several observations that toxin-induced hepatic damage is associated with increased NO production by the liver (Li & Billiar 1999). High concentrations of NO, as a free radical molecule, can directly cause lipid peroxidation and deplete cellular energy via disruption of mitochondrial enzymes and nucleic acids. NO also reacts with the superoxide anion to form peroxynitrite, a highly toxic reactant, capable of producing extensive cellular and tissue damage (Hon et al 2002). However, whether the augmented production of NO serves a protective or deleterious role in the liver remains a controversial issue (Muriel 1998; Zhu & Fung 2000; Sass et al 2001). In recent years, there has been growing evidence that excessive NO production by iNOS plays an important role in the induction of toxic-induced liver injury (Chen et al 2004; Tipoe et al 2006). Our results confirm that CCl_4 treatment induced severe hepatocellular damage, with a significant increase in the level of NO in liver. TFB blocked augmentation of the liver NO level in CCl_4-treated mice. Thus, the down-regulation of NO production by TFB may be a useful therapeutic strategy to protect against CCl_4-induced liver injury by decreasing the formation of free radicals.

Oxidative stress enhances NF-κB activity, which has been shown to enhance the expression of cytotoxic cytokines (Liu et al 1995). In unstimulated cells, including liver cells, NF-κB proteins are localized in the cytoplasm, bound to an inhibitory protein, IκB. A variety of stimuli, including oxidative stress, result in the degradation of IκB and the nuclear translocation of NF-κB, then NF-κB stimulates the expression of a variety of genes, including those involved in toxin-induced liver injury (Xie et al 1994; Pahl 1999). Thus, reduction of NF-κB activity may decrease cell necrosis. Moreover, several reports suggest that some of the biological activities of flavonoids may be mediated by their inhibition of the NF-κB pathway (Yamamoto & Gaynor 2001). We speculated that TFB might eliminate increased NF-κB expression. As shown in Figure 2D,

the expression of NF-κB increased after treatment with CCl_4 alone compared with the normal control group. However, NF-κB expression in the CCl_4-treated mice gradually decreased after pretreatment with TFB compared with mice treated with CCl_4 alone, indicating that TFB down-regulates the expression of NF-κB. In addition, NF-κB can stimulate the expression of enzymes including iNOS, which generates NO. They contribute to the pathogenesis of the inflammatory process (Yamamoto & Gaynor 2001). From the above results, we deduced that CCl_4 induces oxidative stress, which activates the redox-sensitive NF-κB, and then the activated NF-κB can stimulate the expression of the iNOS gene as an inflammatory response in hepatocytes and Kupffer cells. Hepatic damage occurs following the excessive release of NO. Although we did not study the relationship between NF-κB and iNOS genes, TFB clearly blocked NF-κB expression and NO production, and then inhibited CCl_4-induced hepatic damage.

In conclusion, our study indicates that TFB has a remarkable protective effect against CCl_4-induced liver injury in mice and its mechanism is related, at least in part, to its antioxidant properties. Furthermore, TFB can inhibit expression of NF-κB and NO production, which may enhance the activity of cytotoxic cytokines in CCl_4-induced mouse liver injury. More research is needed to clarify the mechanisms of the hepatoprotective effect of TFB at the molecular level.

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