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Full Length Research Paper

Anti-hepatotoxic and anti-oxidant effects of extracts from *Piper nigrum* L. root

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The aim of this study was to investigate the effect of *Piper nigrum* L. root extracts on carbon tetrachloride (CCl₄)-induced rat liver injury. Among the three different extracts (water, ethanol and chloroform extract), ethanol extract exhibits the highest hepatoprotective activity ($p < 0.05$). When using the ethanol extract at a dose of 120 mg/kg to treat the CCl₄-intoxicated rat, the activities of alanine transaminase (ALT) and aspartate transaminase (AST) in rat serum decreased to 65.7 and 84.5%, respectively. At the same time, the lipid peroxidation (MDA) decreased to 52.3% and glutathione (GSH) increased to 55.8% in the rats liver homogenate, as compared with those of the CCl₄ positive control rats. The hepatoprotective effect of ethanol extract was also supported by the histopathological observations. Moreover, the ethanol extract was studied for its *in vitro* antioxidant activity using the methods of ferric thiocyanate (FTC) and thiobarbituric acid (TBA). The findings indicate that the ethanol extract of *P. nigrum* L. root is an efficient hepatoprotective and antioxidant agent against CCl₄-induced liver injury.

Key words: *Piper nigrum* L. root, ethanol extract, carbon tetrachloride (CCl₄), hepatoprotective, antioxidant.

INTRODUCTION

The liver is the most important organ of human being, which plays a vital role on regulating various physiological processes in the body. It has great capacity to detoxicate toxic substances and synthesize useful ones. Therefore, the damage which is caused by hepatotoxic agents is of grave consequence to the body as it deprives the liver of its principal functions (Subramoniam and Pushpangadan, 1999). Most of the liver damages are induced by lipid peroxidation and other oxidative damages which are caused by the hepatotoxic chemicals (Dianzani et al., 1991; Muhtaseb et al., 2008; Appiah et al., 2009). Carbon

tetrachloride (CCl₄) is a well know hepatotoxin that is widely used to study the induction of toxic liver injury in a range of laboratory animals. Antioxidative action plays an important role in protecting the liver against CCl₄-induced liver injury (Ardanaz and Pagano, 2006).

In recent years, Chinese herbal medicine has attracted many attentions from the researchers in various areas. A number of medicinal preparations using Chinese herb has been recommended for the treatment of liver disorders (Chatterjee, 2000). *Piper nigrum* L. is widely distributed in the Asian continent, especially in Hainan and Yunnan, China. The plant is used in many Asian countries as a stimulant, for the treatment of colic, rheumatism, headache, diarrhoea, dysentery, cholera, menstrual pain, removal of excessive gas in the system and increase in the flow of urine (Wee and Hsuan, 1990). It is also used in folk medicine for stomach disorders and digestion problems, neuralgia, scabies, etc (Chatterjee et al., 2007). Piperine from *P. nigrum* exerts a significant protection effect against tertiary-butyl hydroperoxide and CCl₄ induced hepato- toxicity by reducing both *in vitro* and *in vivo* lipid peroxidation, enzymatic leakage of alanine

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Abbreviations: **FTC**, Ferric thiocyanate; **TBA**, thiobarbituric acid; **GSH**, reduced glutathione; **MDA**, malondialdehyde; **ALT**, alanine aminotransferase; **AST**, aminotransferase; **CCl₄**, carbon tetrachloride; **Vit C**, vitamin C; **vit E**, vitamin E; **WE**, water extract; **EE6**, 60% ethanol extract; **CE**, chloroform extract of dried and powdered root of *P. nigrum* L.

amino- transaminase and alkaline phosphatase (AP) and by preventing the depletion of reduced glutathione (GSH) and total thiols in the intoxicated mice. Piperine showed lower hepatoprotective potency than silymarin, a known hepato-protective drug (Koul and Kapil, 1993). Rats treated with piperine and fed with a high fat diet and antithyroid drug had normalized erythrocyte osmotic fragility, reduced lipid peroxidation and show an improvement in the enzymatic and non-enzymatic antioxidant status compared to control rats (Vijayakumar et al., 2006). The fruit of *P. nigrum L.* has been acclaimed for its native and medicinal values. Its root has been used as traditional drugs in China for a long time. It has also been reported that it is being used as folk remedy for adult diseases, such as potent anti-hepatotoxic, anti-pyorrhea and anti-gingival inflammation (Indu and Aruana, 1993; Singh and Rao, 1993; Nalini et al., 1998; Firoza et al., 2000). However, few studies have been reported on the effect of the *P. nigrum L.* root extracts on liver damage caused by hepatotoxicants and anti- hepatotoxic chemical constituents. Investigations have not been, previously, undertaken in a systematic way. This study focused on evaluating the hepatoprotective and antioxidant potentials of the root against CCl₄-induced liver injury in rats.

MATERIALS AND METHODS

Design

The rats were divided into different groups and treated with different extracts, from which serum parameters and hepatic parameters were assayed using the standard method, the liver histopathology was also observed. Moreover, *In vitro* antioxidant, activity of extract was investigated using the methods of ferric thiocyanate (FTC) and thiobarbituric acid (TBA).

Drugs and chemicals

All biochemicals and chemicals used for the experiments were of analytical grade.

Animals

Wistar albino rats (140 ± 20 g) of either sex, procured from Hainan Medical University (Hainan China) were used for the study. The animals were housed in large polypropylene cages and allowed free access to Purina Rodent Chow and tap water. They were kept in a controlled environment at 20 ± 2°C and 50 ± 5% relative humidity with a 12-h dark/light cycle and were acclimatized for at least one week before use.

Collection of plant material

The roots of *P. nigrum L.* root were collected from the plants grown in the campus of School of Medicine, Hainan University (Hainan Province, China) in November, 2008 and identified by Prof. Liu Zhengdao of the Department of Botany, Hainan University. A

voucher specimen was deposited in The Key Laboratory of Food Science and Safety, Hainan University, Hainan China, Vide accession No. 2008036. Root materials were separated, washed, cut into small parts, air-dried (moisture 10% in weight), ground by a miller (A11 basic, ZKA®-WERKE, Germany), and screened by sieve. The particles of (0.5 - 1.5) × 10⁻² mm diameter were selected.

Preparation of plant extracts

Each 1 kg dried and powdered root of *P. nigrum L.* was extracted twice each time with 8 L water, 60% ethanol or chloroform for 3 days, then the extracts were filtered through muslin. The filtrate was concentrated under the reduced pressure environment of (45°C, 0.1 MPa, 3 h) and freeze-dried (24 h) to produce the water extract (WE), 60% ethanol extract (EE6) and chloroform extract (CE). The yields on the dry root corresponded to 180, 200 and 80 mg/ g, respectively. The extracts were dissolved in normal saline prior to oral administration.

CCl₄-induced hepatotoxicity

The animals were divided into six groups, each group with six animals. Group I served as normal control and received saline water (1 mL/ kg, p.o.) daily for 5 days. They also received olive oil (1 mL/ kg, s.c.) on days 2 and 3 (Rasheeduz and Mujahid, 1998). Group II served as CCl₄ control and received saline water (1 mL/ kg, p.o.) daily for 5 days. They also received CCl₄: olive oil (1: 1, 2 mL/ kg, s.c.) on days 2 and 3. Group III was treated with the reference drug silymarin (60 mg/ kg, p.o.) daily for 5 days and they also received CCl₄: olive oil (1: 1, 2 mL/ kg, s.c.) on days 2 and 3, respectively, 30 min after administration of reference drug. Groups IV - VI were treated with the WE, EE6 and CE, at doses of 60 mg/ kg (p.o.), respectively, for 5 days, they also received CCl₄: olive oil (1: 1, 2 mL/ kg, s.c.) on days 2 and 3, 30 min after administration of extracts. The activities of the different dose of EE6 were also investigated. The animals were treated as mentioned above except that Groups IV - VI were treated with EE6 at doses of 30, 60 and 120 mg/ kg (p.o.), respectively.

Biochemical estimations

The rats were sacrificed on the sixth day by cervical decapitation and their blood was collected in plain tubes. The serum was obtained by centrifugation. After bleeding, their livers were weighed and a thin slice preserved in a buffered formalin solution for obtaining histological sections. The remaining livers were frozen quickly in dry ice/methanol and stored at -70°C for analysis. The activities of serum aspartate transaminase (AST) and alanine aminotransferase (ALT) were assayed by the standard method using commercially available kits (Nanjing Biomedical.Co., Ltd., China) on an auto-biochemical analyzer (BTS-370 plus, Spain). The hepatic GSH and lipid peroxidation (malondialdehyde, MDA) levels were assayed by the standard method using commercially available kits (Nanjing Biomedical.Co., Ltd., China).

Assessment of antioxidant activity

In vitro antioxidant activity of the EE6 was determined based on the ferric thiocyanate (FTC) method of Kikuzaki et al. (1993) and the thiobarbituric acid (TBA) method of Ottolenghi (1959), compared with that of vitamin E and vitamin C.

The inhibition of lipid peroxidation in percentage was calculated

Table 1. Effect of the differently extracts against CCl₄-induced hepatotoxicity in rats.

Groups	Dose (mg/kg)	Serum ALT (U/L)	Serum AST (U/L)
Control		85 ± 4.7	94 ± 3.8
CCl ₄ /olive oil	2	282 ± 26.0 ^a	762 ± 46.4 ^a
Silymarin+ CCl ₄	60	89 ± 11.4 ^b	99 ± 10.7 ^b
WE + CCl ₄	60	196 ± 18.1 ^{a b c}	597 ± 23.8 ^{a b c}
EE6 + CCl ₄	60	124 ± 12.7 ^{a,b}	329 ± 35.4 ^{a,b}
CE + CCl ₄	60	228 ± 16.4 ^{a b c}	686 ± 41.3 ^{a b c}

Values are expressed as mean ± standard error of mean of six animals in each group; symbols represent statistical significance: ^a p < 0.01, significantly different from the control; ^b p < 0.01, significantly different from the CCl₄/olive oil and ^c p < 0.05, significantly different from the EE6+CCl₄.

Table 2. Effects of the EE6 against CCl₄-induced hepatotoxicity in rats.

Groups	Dose (mg/kg)	Serum ALT (U/L)	Serum AST (U/L)	Lipid peroxidation (MDA: nmol/g liver)	Glutathione (mg/g liver)
Control		82 ± 6.5	95 ± 4.0	83 ± 9.1	1.91 ± 0.22
CCl ₄ /olive oil	2	279 ± 25.6 ^a	752 ± 65 ^a	186 ± 15.5 ^a	1.20 ± 0.12 ^a
Silymarin+ CCl ₄	60	97 ± 16.3 ^b	105 ± 30.4 ^b	94 ± 10.6 ^b	1.89 ± 0.19 ^b
EE6 + CCl ₄	30	242 ± 24.1 ^{a b}	607 ± 41.7 ^{a b}	167 ± 16.9 ^{a b}	1.39 ± 0.14 ^{a b}
EE6 + CCl ₄	60	126 ± 12.2 ^{a,b}	317 ± 43.7 ^{a,b}	122 ± 14.5 ^{a,b}	1.78 ± 0.16 ^{b c}
EE6 + CCl ₄	120	96 ± 16.5 ^{b c}	116 ± 10.3 ^{b c}	89 ± 11.6 ^{b c}	1.87 ± 0.17 ^{b c}

Values are expressed as mean ± standard error of mean of six animals in each group; symbols represent statistical significance: ^a p < 0.01, significantly different from the control; ^b p < 0.01, significantly different from the CCl₄/olive oil and ^c p < 0.05, not significantly different from the silymarin+ CCl₄.

using the following equation:

$$\text{Percent inhibition} = [(A_0 - A_1) / A_0] \times 100$$

Where A₀ is the absorbance of control and A₁ is the absorbance of sample at 500 nm (Duh et al., 1999).

Statistical analysis

The data were expressed as mean ± standard error of mean (n = 6). Results were analyzed statistically using one-way ANOVA followed by Tukey's multiple comparison using SPSS software student's version. The difference was considered significant since p < 0.05.

RESULTS AND DISCUSSION

Effects of the different extracts on CCl₄-induced hepatotoxicity (*in vivo*)

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in normal, CCl₄ control and treated groups were represented in Table 1. CCl₄ treatment significantly (p < 0.01) increases to 232 and 712% of ALT and AST activities in the rat serum, respectively, as compared with those of the normal control group.

This result indicates that a single dose of CCl₄: olive oil (1:1, 2 ml / kg, s.c.) caused hepatotoxicity in the rats. The activities of ALT, AST were significantly (p < 0.01) increased in CCl₄ control compared to normal and control groups (Table 1). Also, the different extracts have differently hepatoprotective effect, ethanol extract have higher hepatoprotective effect than water extract and chloroform extract at the dose of 60 mg/ kg (p < 0.05) (Table 1). Of the three different extracts, ethanol extracts have been proven to be more efficient to the reduction of ALT and AST. With treatment of ethanol extracts, the ALT and AST activities in rat serum from 282 ± 26.0 U/ L and 762 ± 46.4 U/ L decrease to 124 ± 12.7 U/ L, and 329 ± 35.4 U/ L, decreased to 56.1 and 56.9%, as compared with those of the CCl₄ positive control group, respectively.

Effects of the dosage of ethanol extract on CCl₄-induced hepatotoxicity (*in vivo*)

The effects of different doses of ethanol extract on CCl₄-induced hepatotoxicity in rats were investigated; the levels of serum ALT, AST and hepatic reduced glutathione (GSH), malondialdehyde (MDA) of the EE6 treats CCl₄-induced rats are shown in Table 2. The levels of ALT,

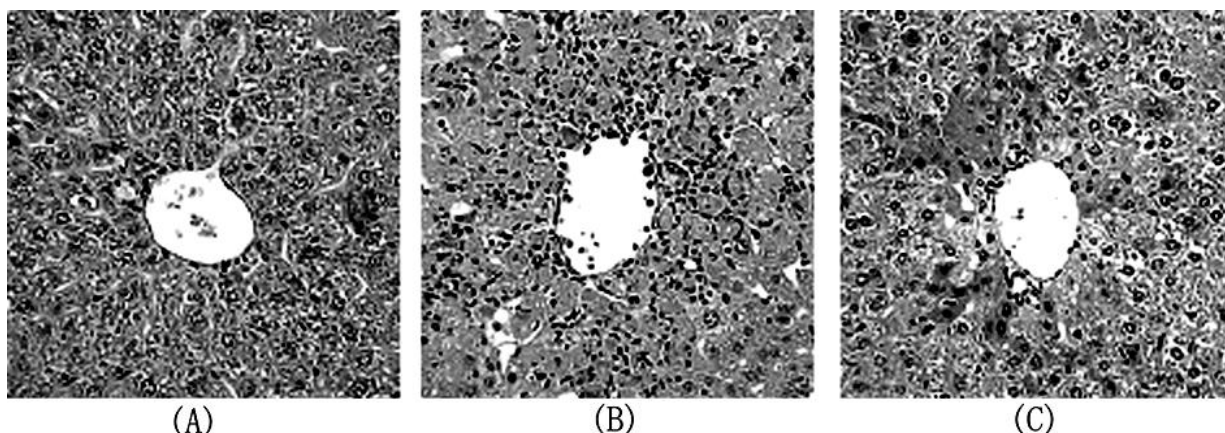


Figure 1. Livers histological slices of different groups of rats. (A) Liver from control group rat; (B) liver from CCl₄/olive oil control group rat (1:1, 2 mL/kg); and (C) liver from CCl₄ + EE6 (at a dose of 120 mg/kg) group rat.

AST and MDA decreased with the increase of the dosage of the EE6. The levels of ALT, AST and MDA decreased by 65.7, 84.5 and 52.3%, respectively, as compared with those of the CCl₄ positive control group. However, the levels of ALT, AST and MDA only increased by 17.2, 22.1 and 7.3% respectively, as compared with those of normal (CCl₄ negative) control group, when CCl₄-intoxicated rats were treated with 120 mg/kg EE6. Table 2 also indicates that the EE6 can prevent the depletion of GSH. Liver GSH decreasing rate reaches 37.2% when normal rats were treated with CCl₄, however, its decreasing rate only reaches 2.1% when 120 mg/kg of EE6 was used. Moreover, the EE6 has similar hepatoprotective effect as that of silymarin at the dose of 60 mg/kg.

On the other hand, the protective effects of ethanol extract were confirmed using the rat liver histological slices observation (Figure 1). It revealed that CCl₄:olive oil induced the rat liver ballooning degeneration, centrilobular necrosis, thereby bridging neurosis and apoptosis of the hepatocytes (Figure 1 B), as compared with those of normal rat (Figure 1 A). Significant protective effect was observed when 120 mg/kg EE6 was administered to the CCl₄-intoxicated rats (Figure 1 C), as compared with those of CCl₄: olive oil treated rats (Figure 1 B). The necrotic hepatocytes were of different sizes and much larger than normal hepatocytes. They occasionally appeared at con-fluent areas (Figure 1).

Antioxidant effect of ethanol extract on lipid peroxidation (*in vitro*)

The antioxidant effect of ethanol extract *in vitro* was determined using ferric thiocyanate (FTC) and thiobarbituric acid (TBA) method and illustrated in Figures 2 (FTC) and 3 (TBA). FTC method was used to determine the amount of peroxide that was generated at the initial stage of lipid

peroxidation. During the linoleic acid oxidation, peroxides are produced and these compounds oxidize Fe²⁺ to Fe³⁺. The Fe³⁺ ions react with thiocyanide (SCN⁻) to form a complex, which has a maximum absorbance at 500 nm. In this method, the concentration of peroxide decreases with the increase of the antioxidant activity, while the absorbance values are much smaller with higher antioxidant activities of the samples. Ethanol extract has demonstrated a significant antioxidant activity on lipid peroxidation compared with Vitamins E and C. The highest absorbance value (0.892) was observed for the control, but the absorbance values are 0.403, 0.537 and 0.583 for the EE6, vitamins E and C, respectively (Figure 2). Accordingly, the highest percent inhibition, 54.8 ± 3.3% is achieved for EE6. However, the percent inhibitions of vitamins E and C are 39.8 ± 1.9 and 34.6 ± 1.2%, respectively. The result shows that the EE6 demonstrates a significant antioxidant activity on lipid peroxidation ($p < 0.01$), as compared with those of vitamins E and C.

In the process of the thiobarbituric acid (TBA) method, the formation of malonaldehyde is the basis for evaluating the extent of lipid peroxidation. The conditions of low pH and high temperature (100°C), malonaldehyde could bind TBA to form a red complex which could be determined at 532 nm. The increase in amount of red pigment formed correlates with the oxidative rancidity of the lipid. Figure 3 shows antioxidant effect of the EE6 on lipid peroxidation by TBA method. The results are similar to those detected by ferric thiocyanate (FTC) method. The highest absorbance value (0.375) was achieved for the control group and it was 0.208, 0.162 and 0.212 for the EE6, vitamins E and C, respectively. Based on the results, the highest inhibition percentage 56.8 ± 3.2% was calculated for EE6. However, the inhibition percentage of vitamins E and C are 44.5 ± 1.6 and 43.5 ± 1.4%, respectively. Hence, EE6 shows higher antioxidant activity than that of vitamins E and C ($p < 0.01$).

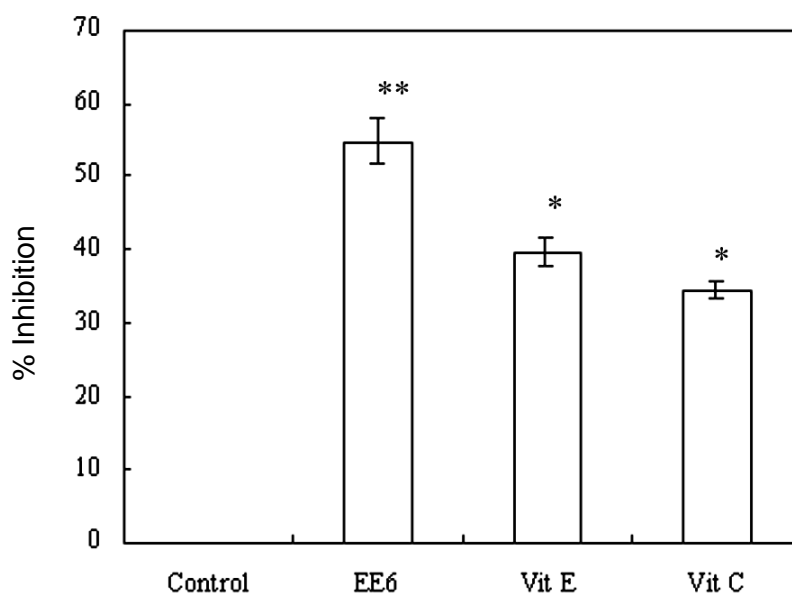


Figure 2. *In vitro* antioxidant activity of EE6 by ferric thiocyanate method. ** $p < 0.001$ compared to control group and * $p < 0.01$ compared to EE6 group (one-way ANOVA followed by Tukey's multiple comparison test). Data represents mean \pm standard error of mean of six samples.

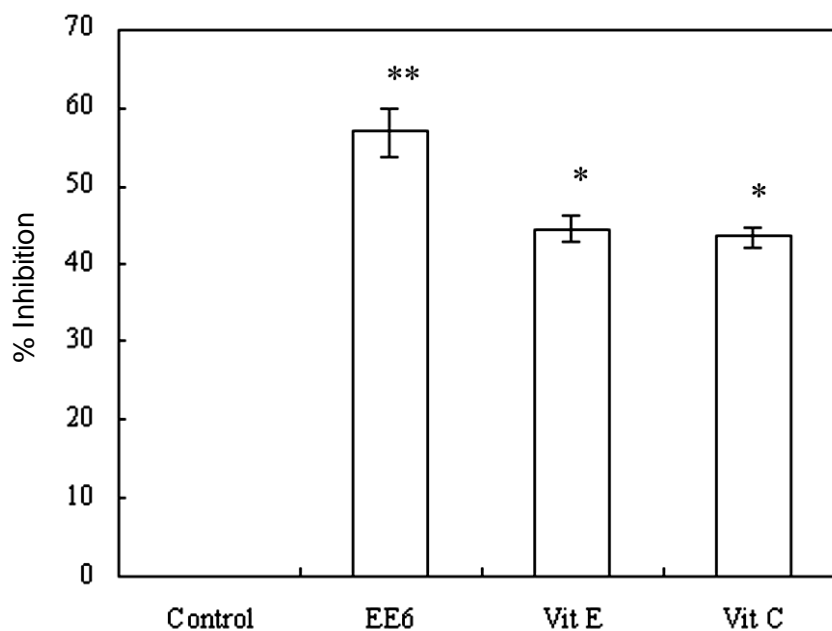


Figure 3. *In vitro* antioxidant activity of EE6 by thiobarbituric acid method. ** $p < 0.001$ compared to control group and * $p < 0.01$ compared to EE6 group (one-way ANOVA followed by Tukey's multiple comparison test). Data represents mean \pm standard error of mean of six samples.

Conclusion

This study has proved that the extracts of *P. nigrum L* root

have hepatoprotective activities against CCl_4 -induced liver damage. Of the three different extracts (water, ethanol and chloroform extract), the hepatoprotective

activities of ethanol extract are higher than those of the other extracts ($p < 0.05$). Ethanol extract exhibits significantly hepato-protective effect against CCl_4 -induced liver damage in a dose-dependent manner. Similar hepatoprotective effect was observed when 120 mg/kg ethanol extract was used, as compared with that of the reference drug silymarin. Furthermore, antioxidant activity was also observed in ethanol extract.

The above results indicate that the EE6 has higher hepatoprotective activities than WE and CW ($p < 0.05$) and are in a dose-dependent manner. Similar hepatoprotective effect was observed when EE6 was used at 120 mg/kg, as compared with that of the reference drug. Furthermore, the *in vitro* antioxidant activities of EE6 was higher than those of vitamins E and C ($p < 0.01$).

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