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Protective effect of *Panax ginseng* against serum biochemical changes and apoptosis in liver of rats treated with carbon tetrachloride (CCl_4)

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ABSTRACT

The purpose of this study was to investigate possible beneficial effects of *Panax ginseng* (PG) on carbon tetrachloride (CCl_4)-induced acute hepatotoxicity in rats. CCl_4 challenge elevated serum enzyme activities of liver and some biochemical parameters, but these effects were prevented by the pretreatment of rats with PG. Histologically, a great amount of mononuclear cells infiltration, necrotic cells and few fibroblasts were observed in liver of CCl_4 group. Also, $CD68^+$ and caspase-3 staining cells were diffused in both lobular and portal areas. However, PG pretreatment had a little influence on the number of caspase-3 immunpositive staining cells in the liver, but $CD68^+$ staining areas were significantly decreased in the PG + CCl_4 when compared to CCl_4 group. We conclude that PG treatment may play a protective role by enhancing liver enzyme activities and recovering biochemical parameters, and improving the changes in histological structure against CCl_4-induced liver damages in rats.

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1. Introduction

The liver is a vital organ that plays a key role in many toxication cases. The hepatotoxicants including carbon tetrachloride (CCl_4), nitrosamines, and polycyclic aromatic hydrocarbons are transformed into the intermediate reactive oxygen species (ROS), including oxygen free radicals then, they show their hepatotoxic effects in experimental animals and humans [1,2].

Carbon tetrachloride is a common industrial solvent used as hepatotoxin in the experimental studies for liver diseases. It is metabolized by cytochrome P450 in liver cells to yield the reactive metabolic hepatotoxic metabolites that trichloromethyl free radicals ($CCl_3^{\bullet}$) and/or trichloromethyl peroxyl radicals ($CCl_3O_2($). The toxicity of CCl_4 probably depends on formation of the trichloromethyl radical ($CCl_3($), which in the presence of oxygen interacts with it to form the more toxic trichloromethyl peroxyl radical ($CCl_3O_2($) [3]. $CCl_3O_2($ is capable of abstracting hydrogen from polyunsaturated fatty acids to initiate lipid peroxidation. Therefore, both $CCl_3($ and $CCl_3O_2($ causes damage in cell membrane, change enzyme activity and finally induce hepatic injury or necrosis [4]. The number of infiltrated neutrophils, macrophages, Kupffer cells, lymphocytes and natural killer cells are significantly increase after liver injury induced by hepatotoxins such as CCl_4, alcohols, D-galactosamine, *etc.* most of which causes activation of liver resident macrophages and/or chemoattraction of extrahepatic cells (*e.g.* neutrophils and lymphocytes) [5]. The activated macrophages are release to cell death ligand (CD95L and TNF alfa, *etc.*) that mediated apoptosis might be contributed to liver fibrosis, inflammation and injury [6,7]. Following damages and inflammation in the liver tissue, repairing hepatocytes by anti-inflammatory agents take places where death necrotic and apoptotic cells [8].

Herbal compounds obtained from plant extracts that reduce chemical activating enzymes could be considered as good candidates for protection against chemically induced toxicities such as CCl_4 and cisplatin. *Panax ginseng*, a traditional multipurpose herb in Asia, has become the World's most popular herbal supplements in recent years. Ginseng has a variety of beneficial biological processes that include anti-carcinogenic, anti-diabetic and anti-inflammatory effects, as well as cardiovascular- and neuro-protection [9–11]. Most of the pharmacological actions of ginseng are attributed to a variety of ginsenosides, which are phenolic acids, flavonoids and triterpenoid saponins [12,13]. These properties of the ginseng are thought to provide many beneficial effects against organ damages. Thus, we investigated effects on

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serum lipid profile and liver enzymes to determine the protective effect of *P. ginseng* against CCl_4-induced damage in male rats.

2. Material and methods

2.1. Chemicals

P. ginseng was purchased from trade cooperation SGM (Ankara, Turkey). CCl_4 and all other chemicals of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO) and IBL Chemical Co. (Ankara, Turkey).

2.2. Animals and hausing

Forty adult male Wistar albino rats ($n = 10 \times 4$) weighing about 250 g were used. The animals were given standard rat pellets and tap water *ad libitum*. The rats were housed in individual cages (360 mm × 200 mm × 190 mm), each containing 2 or 3 animals from 15 days before the start of the experiment. All animals were housed in stainless steel cages under standard laboratory conditions (light period 07.00 a.m. to 8.00 p.m. h, 21 ± 2 °C, relative humidity 55%), and received humane care according to the criteria outlined in *the Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institute of Health.

2.3. Experimental design

Rats were divided into four groups, each containing 10 animals. Control group (C) was injected intraperitoneally (i.p.) with 1 ml physiological saline for 7 consecutive days. CCl_4 toxication group (CCl_4) was given a single i.p. dose of CCl_4 10 ml/kg. *P. ginseng* + CCl_4 toxication group (PG + CCl_4) was injected i.p. with ginseng (300 mg/kg) for 7 consecutive days prior to CCl_4 injection. *P. ginseng* group (PG) was injected i.p. with ginseng (300 mg/kg) for 7 consecutive days.

2.4. Blood sampling and analysis

All treated animals were anesthetized by ether inhalation for blood sample collection 24 h after administration of CCl_4. Blood samples were collected from hearts of rats using a syringe with 24-gauge needle under ether anesthesia. The samples were centrifuged at 3200 g for 10 min within 1 h after collection. The sera were stored in the −20 °C freezer before they were analysed. Serum enzyme activities [aspartate aminotransferase (AST), alanine aminotransferase (ALT), gama-glutamil transferase (GGT)], biochemical parameters [urea, blood urea nitrogen (BUN), glucose, creatinine, total protein, calcium and phosphorus] and serum total triglyceride and cholesterol levels were analysed using diagnostic kits (IBL Chemical Co., Ankara, Turkey).

2.5. Histochemical and immunhistochemical examination

The liver tissue samples were fixed in 10% buffered neutral formalin, and embedded in paraffin. The paraffin blocks were cut 5–7 μm thick and stained with Mallory's triple stain modified by Crossman. CD68 (universal marker for monocyte/macrophage lineage cells) and capase-3 (apoptotic marker) positive cells were determined with streptavidin-biotin-peroxidase staining method. For immunohistochemistry examinations were used monoclonal mouse anti-CD68 (Clone KP1, Invitrogen, 08-0125) and monoclonal caspase-3 (Biovision-3015-100, dilution: 1/25) primary antibodies and biotinylated secondary antibody (DAKO-Universal LSAB Kit-K0690). The binding sites of antibody were visualized with DAB (Sigma), and evaluated by high-power light microscopic (Nikon i50). For each specimen, $CD68^+$ and caspase-3 immunoreactivity were determined in 10 randomly selected areas of approximately X20 objective.

2.6. Statistical analysis

The results were expressed as mean ± SEM of ten animals in each group. The data were subjected to one-way ANOVA followed by Tukey's multiple comparison tests. Student's *t*-test was used in the two-group comparison. Statistical significance was accepted for all tests at $p < 0.05$.

3. Results

3.1. Biochemical results

The activities of AST, ALT and GGT were estimated in serum samples as the liver function markers. These results are given in Table 1. The CCl_4 treatment markedly affected the liver specific enzymes. It was found that a significant increase in serum AST, ALT and GGT activities of rats given alone CCl_4 ($p < 0.05$). This result suggests that liver function markers are elevated in the serum due to release of the enzymes from damaged liver. However a significant decrease was observed in above serum activities of rats given PG + CCl_4 compared with the alone CCl_4 treated groups ($p < 0.05$).

The levels of serum biochemical parameters of rats in all groups are presented in Table 2. Cholesterol, triglyceride and glucose levels were increased in the CCl_4 groups compared with the control group ($p < 0.05$). There were decreases in cholesterol, triglyceride and glucose levels in the PG + CCl_4 group compared with the CCl_4 treated groups ($p < 0.05$).

A significant change was not determined in calcium and phosphorus levels of control and all treated groups ($p > 0.05$). However, a noticeable increase in protein levels of rats given alone CCl_4 was observed compared with control groups ($p < 0.05$). But, this increase was reduced in pretreated with PG compared with alone CCl_4 treated group ($p < 0.05$).

The level of kidney markers of rats in control and all treated groups are showed in Table 3. A significant increases change in creatinine, urea and BUN levels of rats given CCl_4 were determined ($p < 0.05$). On the contrary, a marked reduction was observed in the amount of creatinine, urea and BUN of rats given PG + CCl_4 group compared with the alone CCl_4 treated groups ($p < 0.05$).

3.2. Histochemical and Immunohistochemical findings

The livers of the control and the PG-only groups were seen normal histological structure. However, a great amount of mononuclear cells infiltration, necrotic cells, steatozis and few fibroblasts were significantly determined in both lobular and portal areas in the CCl_4 group. On the other hand, these changes were slightly decreased in the PG + CCl_4 treated animals.

Table 1
Effects of CCl_4 and *Panax ginseng* on serum enzyme activities of liver in rat.

Groups	Parameters		
	AST U/L	ALT U/L	GGT U/L
C	126.19 ± 10.30	67.63 ± 6.57	130.84 ± 12.35
CCl_4	395.43 ± 20.35[a]	258.46 ± 8.52[a]	542.24 ± 20.25[a]
PG + CCl_4	133.69 ± 14.59[b]	77.67 ± 7.15[b]	118.72 ± 14.26[b]
PG	247.09 ± 20.24	66.46 ± 9.25	246.18 ± 15.20

Each value represents the mean ± SEM of 6 animals.
[a] Significantly different from control group ($p < 0.05$).
[b] Significantly different from CCl_4 group ($p < 0.05$).
C: Control, PG: *Panax ginseng*, CCl_4: Carbon tetrachloride.

Table 2
Effects of CCl_4 and *Panax ginseng* on serum biochemical parameters in rat.

Groups	Parameters					
	Cholesterol mg/dl	Triglyceride mg/dl	Glucose mg/dl	Total protein g/dl	Calcium mg/dl	Phosphorus g/dl
C	145.56 ± 10.25	169.62 ± 15.25	218.73 ± 10.98	62.10 ± 5.65	13.56 ± 3.56	8.14 ± 1.25
CCl_4	264.98 ± 15.24[a]	320.62 ± 12.35[a]	357.6 ± 12.58[a]	44.26 ± 6.24[a]	11.85 ± 4.58	7.94 ± 2.03
PG + CCl_4	113.17 ± 15.55[b]	145.76 ± 14.65[b]	223.36 ± 16.5[b]	61.54 ± 7.21	14.12 ± 5.32	8.4 ± 1.64
PG	200.77 ± 16.48	216.44 ± 12.05	294.43 ± 14.56	65.97 ± 6.58	13.65 ± 5.78	7.91 ± 2.58

Each value represents the mean ± SEM of 6 animals.
[a] Significantly different from control group ($p < 0.05$).
[b] Significantly different from CCl_4 group ($p < 0.05$).
C: Control, PG: *Panax ginseng*, CCl_4: Carbon tetrachloride.

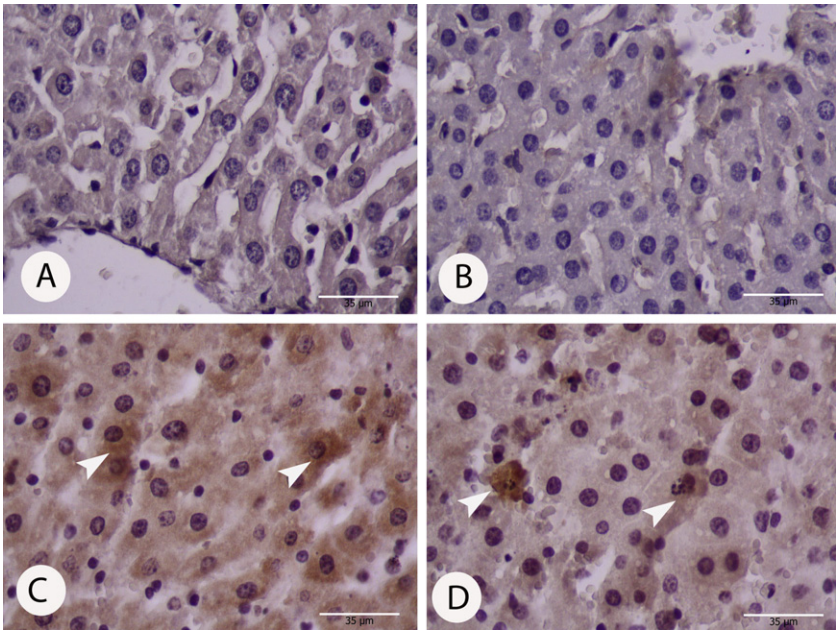

Fig. 1. Caspase-3 positive reactions in rat livers. (A) Control, (B) PG, (C) CCl_4, (D) PG + CCl_4 Arrow heads: Caspase-3 positive apoptotic cells (streptavidin-biotin peroxidase staining), Bar 35 μm.

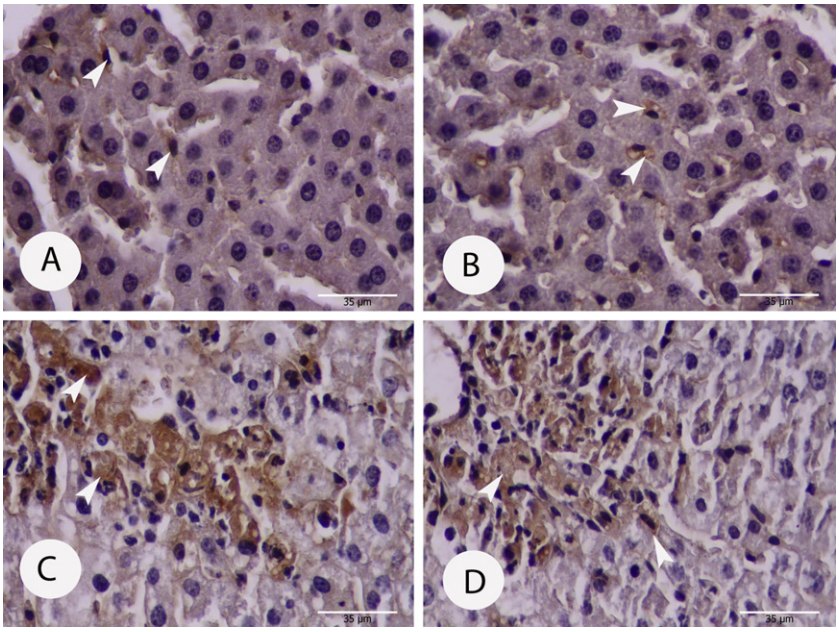

Fig. 2. $CD68^+$ positive reactions in rat livers. (A) Control, (B) PG, (C) CCl_4, (D) PG + CCl_4 Arrow heads: $CD68^+$ positive phagocytic cells (streptavidin-biotin peroxidase staining), Bar 35 μm.

Table 3
Effects of CCl_4 and *Panax ginseng* on the kidney markers in rat.

Groups	Parameters		
	Creatinine mg/dl	Urea mg/dl	BUN mg/dl
C	0.44 ± 0.12	45.78 ± 5.62	16.81 ± 5.25
CCl_4	1.80 ± 0.15[a]	89.20 ± 7.25[a]	79.69 ± 4.87[a]
PG + CCl_4	0.53 ± 0.08[b]	44.63 ± 6.32[b]	14.02 ± 6.59[b]
PG	0.68 ± 0.10	59.54 ± 5.58	44.42 ± 8.25

Each value represents the mean ± SEM of 6 animals.
[a] Significantly different from control group ($p < 0.05$).
[b] Significantly different from CCl_4group ($p < 0.05$).
C: Control, PG: *Panax ginseng*, CCl_4: Carbon tetrachloride.

Table 4
Semiquantitative analysis of caspase-3 and $CD68^+$ reactivity in liver.

Groups	Apoptotic and CD68 immune staining density	
	Caspase-3 Liver	CD68 Liver
C	−/+	+
PG	+	+
CCl_4	+++	++++
PG + CCl_4	++	+++

Caspase-3 and $CD68^+$ reaction density was estimated as follows: none = −, weak = +, moderate = ++, strong = +++, very strong = ++++. C: Control, PG: *Panax ginseng*, CCl_4: Carbon tetrachloride.

In this study, apoptotic and monocyte/macrophage lineage cells in the liver tissue were investigated with caspase-3 and $CD68^+$ antibodies, respectively. The caspase-3 and $CD68^+$ activities are showed in Figs. 1 and 2, respectively. Caspase-3 immune-reactive cells were high levels observed around of the central vein and lobuler areas in the CCl_4 group (Fig. 1). We determined that the numbers of apoptotic cell were increased in the CCl_4 group when compared with other all groups, but it was slightly decreased in the PG + CCl_4 group (Table 4). In control and PG treated animals, slightly immune-reactive $CD68^+$ cells were seen diffusely throughout the hepatic tissue. In liver of rats treated with CCl_4 observed a significant increased numbers of $CD68^+$ cells when compared to PG + CCl_4 group (Table 4). $CD68^+$ cells were mainly localized within the damaged lobuler and portal area (Fig. 2).

4. Discussion

Previous studies have reported that herbal medicines have a significant contribution to the treatment of liver fibrosis which seems to be related to their antioxidant potentials [14,15]. *P. ginseng*, a traditional Asia and Chinese herb, has been used to important roles in maintaining oxidative status, by possessing either direct or indirect antioxidant functions, and has been a component of effective formulations for treatment of liver disease [16].

Carbon tetrachloride is one of the most commonly used hepatotoxins in the experimental liver studies. CCl_4 induced liver injuries are the best characterized system of xenobiotic-induced hepatotoxicity and commonly used model for the screening of hepatoprotective activities of drugs [17–19]. Liver fibrosis induced by CCl_4 is associated with the severity of lipid peroxidation and the depletion of antioxidant status which causing by damage in the cell membrane and the organelles of the hepatocyte [20,21].

In the present study, showed that serum AST, ALT and GGT activities which hepatic markers were greatly increased in rats with the CCl_4 treatment alone in comparison with control group. The increased serum levels of hepatic markers have been attributed to the liver injury, because these enzymes are place in cytoplasmic area of the cell and are released into circulation in case of cellular damage [18,22]. Zimmerman et al. [23] stated that the CCl_4 induced the increase of serum ALT and AST levels which source from cell membrane and mitochondrial damages in liver cells. There are many authors' reports that these enzymes activities were significantly elevated after CCl_4 treatment [24–27]. The first reports about of hepatotoxic effects by CCl_4, are lipid peroxidation origin, and are largely due to its active metabolite CCl_3(. This metabolite can abstract hydrogen from fatty acids, initiating the lipid peroxidation, lead to cell injury, and finally liver damage [28,29]. On the other hand, pretreatment with ginseng was found to significant suppressed an increase in serum AST, ALT and GGT activities induced by CCl_4 in rats. This finding implies that ginseng challenge to protect liver tissue from CCl_4 injury. Current studies have provided a considerable support for evidencing the protective effects of ginseng on liver damage [30–34]. Also, these studies declared that the antioxidant properties of ginsenosides those phenolic acids, flavonoids and saponins contribute to protection against CCl_4 induced hepatotoxicity in rats. These compounds may be responsible for its hepatoprotective action by scavenge and destroy lipid peroxyl radicals and reactive oxygen species such as like the superoxide anion (O_2^-), the hydrogen peroxide (H_2O_2) and the hydroxyl radical ((OH) [35].

In addition to the increased level of hepatic marker, the results of the present study have also established that the CCl_4 treatment could have affected the lipid metabolism of liver (triglyceride and cholesterol levels), renal markers (creatinine, urea, BUN, levels) and glucose level in rats. This is evidenced from our observations that, CCl_4 was caused a significant increase in the levels of lipid parameters and renal markers, but it decreased to the total protein levels. Muller et al. [34] stated that CCl_4 intoxication is similar the hepatitis in case of the triglycerides catabolism. This situation could be also attributed to the reduction of lipase activity, which could lead to decrease in triglyceride hydrolysis [35]. On the other hand, it can be assumed that hypercholesterimea in CCl_4 intoxicated rats has resulted in damage of hepatic parenchymal cells that lead to disturbance of lipid metabolism in liver [36]. However, in group of pretreatment with ginseng showed a significant decline in above parameters compared with CCl_4-intoxicated group. The mechanisms of lipid lowering effects of ginseng are mainly unknown, but recently ginseng sapogenins which produced from ginseng saponins, were shown to exert a strong inhibitory activity on microsomal acyl coenzyme A: cholesterol acyltransferease *in vitro* [37]. This enzyme is responsible for acylation of cholesterol to cholesterol esters in liver [38]. However, there are some authors reported that ginseng may have a supportive effect as a antiatherosclerotic agent by reducing elevated serum total cholesterol level and enhancing antioxidant capacity [39,40]. Also, Inoue et al. [41] stated that oral administration of ginseng saponins are decreased the elevated serum triglycerides and cholesterol levels in cyclophosphamide treated rabbit. This result indicates that ginseng or sapogenins might be affecting the pathway of cholesterol biosynthesis.

The histological changes in the liver injury induced by CCl_4 are know as apoptosis, necrosis, steatosis and mononuclear cell infiltration in both lobuler area and portal septa [6,42,43]. As similar with above reports, our findings were revealed high level inflammation, steatosis, necrosis, CD68 and caspase-3 positive cells within the lobuler areas in the CCl_4 group. Caspases enzymes have remarkably a role on apoptotic pathway of endoplasmic reticulum induced by CCl4, various injuries and stress that these protein expressions are accelerated to cells death [7,44,45]. In agreement with above report, we observed an expansion of the caspase-3 positive cell population in CCl_4 treated rats when compared with PG + CCl_4 group. These findings indicated that the PG pretreated may contribute to inhibition of apoptosis

There are many studies about of monoclonal antibody CD68 that showed as immunhistochemically in the mononuclear phago-

cyte lineage cells (monocytes and most macrophages) [7,45,46]. Numerously researchers had shown that growth of the Kupffer cells and other mononuclear phagocytes population had higher in the administration of CCl_4 compare with control animals [46–49]. Released mediators from cytotoxic cells cause an increase in the numbers of activated phagocytic cell in hepatotoxic CCl_4 treatment [47]. Edwards et al. [45] have shown that gadolinium chloride were protected from CCl_4-induced liver damage in rats that it extensively decreased infiltration of neutrophils and destroys mononuclear phagocytes resulting from exposure to CCl_4. In our experiments, the numbers of CD68 and caspase-3 positive cells were slightly decreased within the lobuler areas and portal tracts in the PG + CCl_4 group when compared to CCl_4 group, which showing anti-inflammatory effects and inhibition of hepatocyte apoptosis through PG.

As shown in our results, CCl_4 treated rats have much higher blood glucose levels than control rats. By ginseng treatment to CCl_4 rats, however, blood glucose levels were declined to a certain degree. The main mechanism of hypoglycemic activity for *P. ginseng* is not clearly, but three possibilities mechanisms can be suggested that modulation of glucose transport [50], glucose disposal [51] and insulin secretion [52]. On the other hand, Ragunathan and Sulochana [53] stated that some components such as phenolics and flavonoids are known to be responsible for hypoglycemic activity.

The main signs of kidney damage induced by CCl_4 treatment are the high levels of creatinine, urea and BUN in serum. In this study we showed that CCl_4 treatment caused a noticeable injury in kidney functions. Serum creatinine, urea and BUN concentrations were significantly higher in the CCl_4 treated rats compared with control group. Khan et al. [54] stated that the CCl_3(radical initiates the of lipid peroxidation which is supposed to the most important mechanism in the pathogenesis of kidney injury induced by CCl_4. Also, there are many authors reported that the nephrotoxic effects of CCl_4 are connected with oxidative damage of the lipids and proteins in rat kidney tissue as well as humans [55–58]. However *P. ginseng* significantly decreased the elevated levels of creatinine, urea and BUN in our study. Ginsenosides are present in the *P. ginseng*, which may have improved the kidney functions through different activity properties such as scavenging of reactive oxygen species and inhibition of the free radicals generation. Similar results were also documented that different plant extracts significantly improved the kidney injuries induced by CCl_4 treatment [59–61].

In conclusion, we found that *P. ginseng* caused a protective effect against CCl_4-induced liver damage and improved the biochemical parameters. Also, we showed that *P. ginseng* has a hepatoprotective effect against apoptosis, and increased $CD68^+$ cell activation in the liver of CCl_4-treated rats. We suggest that *P. ginseng* may be used to protect against toxic effects of CCl_4 and other chemical agents in liver.

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