

Mechanisms of Apigenin-7-glucoside As a Hepatoprotective Agent¹

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Objective *Ixeris chinensis* (Thunb.) Ankaï has been used as a Chinese folk medicine, but only scanty information is available on the physiological and biochemical functions of the compounds extracted from *I. chinensis*. In the present study the effects of apigenin -7-glucoside (APIG) isolated from *I. chinensis* against liver injury caused by carbon tetrachloride (CCl₄) were investigated. **Methods** The contents of malondialdehyde (MDA), glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), and reduced glutathione (GSH) were evaluated by spectrophotography. The content of 8-Hydroxydeoxyguanosine (8-OHdG) was measured with high-performance liquid chromatography (HPLC) equipped with electrochemical and UV detection methods. The antioxidant activity of APIG was evaluated using chemiluminescence single photon counting technology. **Results** CCl₄ significantly increased the enzyme activities of GPT and GOT in blood serum, as well as the level of MDA and 8-OHdG in liver tissue, and decreased the levels of GSH. Pretreatment with APIG was able not only to suppress the elevation of GPT, GOT, MDA and 8-OHdG, and inhibit the reduction of GSH in a dose-dependent manner *in vivo*, but also to reduce the damage of hepatocytes *in vitro*. On the other hand, we also found that APIG had strong antioxidant activity against reactive oxygen species (ROS) *in vitro* in a concentration-dependent manner. **Conclusion** The hepatoprotective activity of APIG is possibly due to its antioxidant properties, acting as scavengers of ROS. These results obtained *in vivo* and *in vitro* suggest that APIG has protective effects against hepatic oxidative injury induced by chemicals. Further studies on the pharmaceutical functions and immunological responses of APIG may help its clinical application.

Key words: Apigenin-7-glucoside; Malondialdehyde; Glutathione; 8-Hydroxydeoxyguanosine; Hepatotoxicity

INTRODUCTION

Ixeris chinensis (Thunb.) Ankaï, a Chinese folk medicine, has been used as an anti-inflammation, anti-atherosclerosis and anticancer agent which can invigorate blood circulation, normalize menstruation and eliminating blood stasis in relieving pain. The isolation and identification of various compounds from this plant and the same genus have been reported by several studies^[1-3]. However, the information on the physiological and biochemical functions of these compounds remains scanty. We isolated apigenin-7-glucoside (APIG) (Fig. 1) from *I. chinensis* as previously reported^[4-6] and investigated pharmaceutical effects on protection against liver injury caused by CCl₄.

CCl₄ is a hepatotoxin, from which trichloromethyl radicals are generated *in vivo*. These radicals stimulate a sequence of reactions that culminate in the initiation of the peroxidation of membrane

lipids^[7] and aggravate the liver damage. Trichloromethyl radicals are believed to be the immediate product of the reductive dechlorination of CCl₄, catalysed by certain cytochrome P450 isoenzymes^[8] particularly the ethanol inducible isoform of cytochrome^[7]. CCl₄-induced lipid peroxidation process provides a model with which APIG can be assessed for its antioxidant property and protective effects against liver injury.

In this study, we examined the protective effects of APIG on liver injury induced by CCl₄, and its inhibitory effects on ROS generation in hepatocytes.

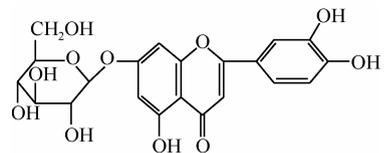


FIG.1. Chemical structure of apigenin-7-O-β-D-glucoside isolated from *Ixeris chinensis* (Thunb.) Ankaï.

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MATERIALS AND METHODS

Chemicals

5, 5'-dithio-bis (2-nitrobenzoic) (DTNB), proteinase K, thiobarbituric acid, sodium dodecyl sulfate, were obtained from Sigma (St. Louis, MO). Apigenin-7-glucoside isolated from *Ixeris chinensis* (Thunb.) Ankaï, as described previously^[4-6], had a high purity (97.3%). All other reagents purchased from Shanghai Biochemical Co. (Shanghai, China) were of analytical grade, unless otherwise noted.

Animals

Male Wistar rats (150-180 g) obtained from Experimental Animal Center of Lanzhou Institute of Biological Products were provided with tap water and rodent chows *ad libitum*, and housed in a controlled-environment with a 12 h day and light cycle.

Experimental Protocol

The experimental animals were divided into three groups: the control group (10 rats) which received the vehicle and normal saline (10 mL/kg) orally, the second group (10 rats) which received CCl₄ to induce chemical hepatitis followed 6 h later by oral saline administration, the third group which was treated similarly to group 2 except that APIG was administered instead of saline to evaluate its curative effects. Three dosages of APIG (10, 20, and 30 mg/kg) dissolved in dimethyl sulphoxide (DMSO) (final concentration 0.1%) were used. Ten animals received one dose of APIG. The CCl₄ was dissolved in corn oil and administered orally to the stomach of rats through an intragastric tube. All treated animals were sacrificed 24 h after receiving APIG or hepatotoxin. All the experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in for example the European Community Guidelines (EEC Directive of 1986; 86/609/EEC).

Assays for activities of GPT and GOT
Animals were anaesthetized with ether, and blood (5 mL) was withdrawn with sterile disposable syringes equipped with hypodermic needles from posterior vena cava. Plasma was separated by centrifugation at 1 100×g for 15 min, and then diluted to 10-fold with 0.9% (v/v) saline. The plasma enzyme levels of GPT and GOT were estimated according to the method of Reitman and Frankel^[9].

Analyses of 8-hydroxy-2'-deoxyguanosine (8-OHdG)
To minimize the artificial 8-OHdG generation during sample preparation, DNA isolation

from livers was achieved according to the pronase/ethanol method described by Kendall *et al.*^[10]. Briefly, DNA was extracted from livers by homogenization in 1 mL of buffer containing 1% sodium dodecyl sulfate, 10 mmol/L Tris, 1 mmol/L EDTA (pH 7.4) and overnight incubated in 0.5 mg/mL proteinase K at 55°C. Homogenates were incubated with RNase (0.1 mg/mL) at 50°C for 10 min and extracted twice with chloroform/isoamyl alcohol (24:1, v/v). The extracts were mixed (1:15 v/v) with 3 mol/L sodium acetate (pH 7.0) and 2 vols of 100% cold ethanol to precipitate DNA at -20°C for 1 h. The samples were centrifuged at 17 000 g for 10 min. The resultant DNA pellets were washed twice with 70% ethanol, air-dried for 3 min and dissolved in 100 mL of 10 mmol/L Tris/1 mmol/L EDTA (pH 7.4)^[11].

DNA digestion was performed as described by Kasai *et al.*^[12]. The oxidative DNA adduct, 8-OHdG, was measured with high-performance liquid chromatography (HPLC) equipped with electrochemical and UV detection^[13] using a CoulArray system (Model 5600). Analytes were detected on two colorimetric array cell modules, each containing four electrochemical sensors attached in series. UV detection was set at 260 nm. HPLC was controlled and the data were acquired and analyzed using CoulArray software. The mobile phase was composed of 50 mmol/L sodium acetate/5% methanol at pH 5.2. Electrochemical detector potentials for 8-OHdG were 120/230/280/420/600/750/840/900 mV (vs Pd) and the flow rate was 1.0 mL/min (31°C). The amount of 8-OHdG was expressed as the number of 8-OHdG for each 10⁵ dG in DNA^[13].

Measurement of MDA

MDA, the marker of oxidative lipid damage, was measured to estimate the extent of lipid peroxidation. Liver tissue homogenates (prepared in 0.5 mL of PBS with 1% SDS) were mixed with 40% TCA to precipitate protein, and the tubes were covered with foil, incubated at 95°C for 60 min with 0.75 mL of 1% thiobarbituric acid (TBA) in 50 mmol/L NaOH, and then placed in an ice bath for 10 min before extraction with *n*-butanol. Then 375 µL *n*-butanol was added to each sample, the tubes were vortexed vigorously for 10 s, and centrifuged at 5 000×g for 10 min. The upper *n*-butanol layer was transferred to a glass tube, the amount of TBA-reactive substances (TBARS) in each sample was calculated from the fluorescence intensity at an excitation wavelength of 532 nm and an emission wavelength of 553 nm, using malondialdehyde (MDA) derived from tetraethoxypropane (TEP) as a standard^[14]. Thus, TBARS were calculated as MDA equivalents and expressed as

nmol/mg protein. TBARS formation was used as an index of lipid peroxidation^[14].

Measurement of reduced glutathione (GSH)
GSH was assessed with DTNB based on the method as described by Jocelyn^[15]. The liver tissue was homogenated in 300 μ L 5% of 5-sulfosalicylic acid and centrifuged for 10 min at 1 000 \times g. The resultant acid thiol extract was assayed for nonprotein sulfhydryls by quantifying the reduction of DTNB through its conversion to 5-thiol-2-nitrobenzoic acid (TNB) at 412 nm by spectrophotometer. Sample values were then calculated from a standard curve generated with known amounts of GSH.

Evaluation for in vitro hepatoprotective activity
Hepatocytes were prepared from livers of intact rats using the two-stage collagenase perfusion method described by Hung *et al.*^[16]. The hepatocytes were suspended in Krebs-Henseleit buffer (pH 7.4), treated with 5% CO₂ at 37°C and incubated in siliconised round-bottom flasks at a cell density of 1 \times 10⁶ cells/mL. Viability of hepatocytes was assessed by trypan blue exclusion method. APIG was dissolved in dimethyl sulphoxide (DMSO) (final concentration 0.1%). To evaluate the protective activity of APIG, cells were incubated with various concentrations of APIG for 30 min before administration of CCl₄ (10 mmol/L). The control group contained 0.1% of DMSO.

Measurement of ROS formation in hepatocytes
ROS formation in hepatocytes was determined at 37°C using chemiluminescence single photon counting technology^[16]. Placing hepatocytes suspension close to the cathode of the thermally insulated photomultiplier maximized the hemiluminescence collection efficiency. Cooling the detector to -20°C reduced its photoemission to less than 100 cpm in the dark. The photon counting was integrated at intervals of one min. The instrument was calibrated to read values of the emitted light by using a radioactive standard solution consisting of tritiated hexadecane in toluene with diphenyloxazole and p-phenyl-bis (5-phenyloxazole) as scintillators.

The activity of APIG on inhibiting ROS formation in hepatocytes was examined by determining chemiluminescence. Hepatocytes were pretreated with various concentrations of APIG for 30 min, and then incubated at 37°C with 10 mmol/L of CCl₄.

Statistical Analysis

Statistical analysis of data was carried out as previously described^[17]. Quantitative difference in values between groups was statistically analyzed using ANOVA (analysis of variance) with a multiple comparison post-test by the Bonferroni method. $P < 0.05$ was considered statistically significant. All

values were expressed as $x \pm s$, and each sample was analyzed three times.

RESULTS

Effects of APIG on CCl₄- induced Hepatotoxicity

The enzyme assays of the serum transaminases showed that the levels of GPT and GOT significantly raised (Table 1) in the group receiving a toxic dose of CCl₄ (1.25 mL/kg) compared with the control (saline).

The values of GPT and GOT serum enzymes in the APIG-treated group were lower ($P < 0.01$) than those in the CCl₄ group. When the rats were administrated with the highest dose (30 mg/kg) APIG alone, no effects of hepatotoxicity were observed (Table 1).

TABLE 1

Effects of APIG (at dosages of 10, 20, and 30 mg/kg) on CCl₄-induced Elevation of GPT and GOT Activities (Each Value Represents the $\bar{x} \pm s$ 10 Treated Rats)

Group	GPT(IU/l)	GOT (IU/l)
Control	22.7 \pm 1.9	53.4 \pm 3.1
APIG (alone, 30 mg/kg)	24.6 \pm 2.8	55.1 \pm 2.9
CCl ₄ (alone, 1.25 mL/kg)	1946 \pm 133 ^{a, b}	2351 \pm 158 ^{a, b}
CCl ₄ + APIG 10 mg/kg	1501 \pm 77.1 ^c	1611 \pm 151.2 ^c
CCl ₄ + APIG 20 mg/kg	1215 \pm 62.2 ^c	1402 \pm 73.3 ^c
CCl ₄ + APIG 30 mg/kg	924.7 \pm 58.1 ^c	1086 \pm 62.3 ^c

Note. ^aCCl₄ group vs control, $P < 0.01$; ^bCCl₄ group vs APIG group, $P < 0.01$; ^cCCl₄ group vs CCl₄ + APIG group, $P < 0.01$.

Protection of APIG on DNA Damage Induced by CCl₄

After the pretreatment with APIG, the 8-OHdG level in DNA (223/10⁵ dG) in the animals administered CCl₄ was 2.7 times higher than in the control (82/10⁵ dG). Protective activity of APIG evaluated at various doses showed that DNA damage could be almost fully protected by APIG at the highest dose of 30 mg/kg (Fig. 2).

Protection of APIG on Lipid Peroxidation Caused by CCl₄

Pretreatment with APIG reduced MDA concentration in the liver tissue in a dose-dependent manner compared to the CCl₄ groups (Fig. 3).

APIG Inhibiting the Reduction of GSH Concentration Induced by CCl₄

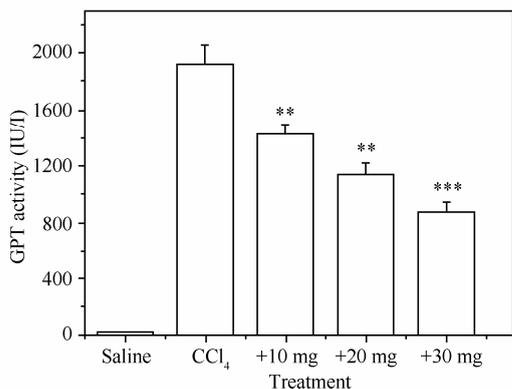


FIG. 2. Effects of LUTG (at dosages of 10, 20, and 30 mg/kg) on CCl₄-induced elevation of MDA content. Each value represents the $\bar{x} \pm s$ of 10 treated rats. Values statistically significantly different from that of CCl₄ control group were indicated by ** $P < 0.01$, *** $P < 0.001$.

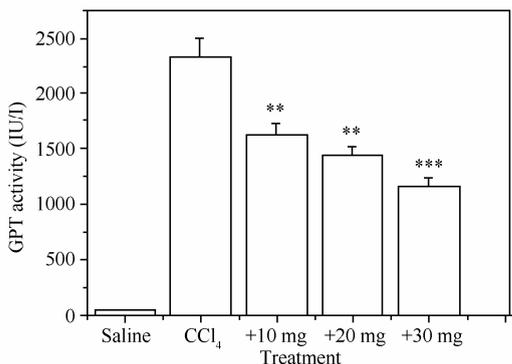


FIG. 3. Effects of APIG (at dosages of 10, 20, and 30 mg/kg) on CCl₄-induced elevation of MDA content. Each value represents the $\bar{x} \pm s$ of 10 treated rats. Values statistically significantly different from that of CCl₄ control group were indicated by ** $P < 0.01$, *** $P < 0.001$.

The GSH concentration in the CCl₄ groups significantly reduced in the liver tissue when compared with the control, while GSH concentration increased in a dose-dependent manner in the APIG groups compared with the CCl₄ groups (Fig. 4).

APIG Reducing the Damage of Hepatocytes *in vitro*

Hepatocytes incubated with 10 mmol/L CCl₄ demonstrated a loss of cell viability. The decrease of hepatocyte viability (%) induced by CCl₄ was significantly elevated by adding APIG to the incubation media in a concentration-dependent manner (Fig. 5).

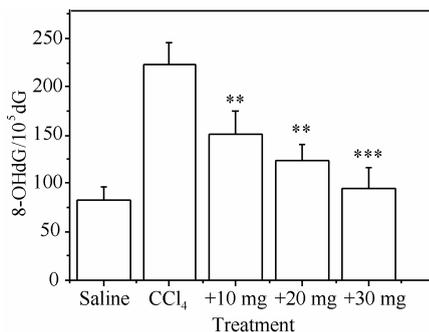


FIG. 4. The effects of various doses of APIG combined with CCl₄ given to rats on accumulation of 8-OHdG in livers. ** $P < 0.01$; *** $P < 0.001$ vs CCl₄ group, $n=10$.

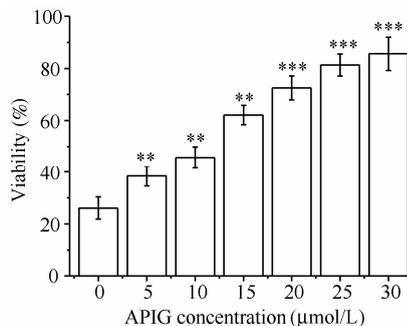


FIG. 5. The effects of APIG on viability of hepatocytes. Hepatocytes were preincubated with various concentrations of APIG 30 min before CCl₄ administration. The initial viability of hepatocytes was 90% (control). ** $P < 0.01$; *** $P < 0.001$ vs control. Results are expressed as $\bar{x} \pm s$ of 3 experiments.

Effects of APIG on Formation of Reactive Oxygen Species (ROS)

Spontaneous intensity of chemiluminescence was significantly inhibited by APIG in a concentration dependent manner (Fig. 6).

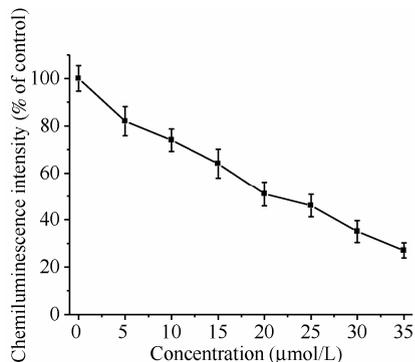


FIG. 6. The effect of APIG on chemiluminescence of hepatocytes in the presence of CCl₄.

DISCUSSION

The present study revealed that APIG had protective effects against hepatic injury induced by CCl₄. The protective effects were evidenced by a blockage of the CCl₄-induced increase in serum GPT and GOT activities, MDA and 8-OHdG formation, and GSH depletion in the liver tissues of rats. LUFT alone did not affect the hepatic functional parameters. This fact indicated that APIG derived from *I. chinensis* had low toxicity. CCl₄ caused oxidative DNA damage in the target organ liver, which could be blocked by APIG. It is well known that oxidative DNA damage is involved in chemical induced carcinogenesis, and APIG may have a protective effect against such carcinogenesis.

The possibility of protecting DNA against oxidative damage is of great importance because of the growing recognition that such damage can both initiate and promote carcinogenesis^[18-20]. The reduction of hepatic GSH could significantly influence the susceptibility of the liver to the effects of hepatotoxic agents^[21] and lead to cell death. Hepatocyte damage is a key process in the pathogenesis of various liver diseases. One of the possible mechanisms for the hepatoprotection by drugs is believed to be stabilization of hepatocyte membranes through their antioxidant action when used for pretreatment^[22].

The model of hepatotoxicity induced by CCl₄ is commonly used to evaluate the curative effects of drugs against hepatotoxicity^[23,24]. CCl₄ could produce hepatotoxicity when taken at a suitable dose (1.25 mL/kg) (Slater, 1966). Enzyme activities of glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) in blood serum served as parameters to illustrate the extent of hepatotoxicity in rats. These enzymes, especially GPT, are highly localized in hepatocyte cytosols. The increases in GPT and GOT serum levels have been attributed to damage to the structural integrity of the liver^[25]. The mechanism of CCl₄-induced hepatotoxicity is considered to result from the activation of CCl₄ by respective specific isozymes of the cytochrome P-450 system in hepatocytic endoplasmic reticulum to the reactive metabolite, CCl₃, which can form covalent products with protein and lipid and interact with O₂ to generate CCl₃O₂, which in turn initiates lipid peroxidation of the endoplasmic reticulum^[23,24] and oxidative DNA damage. APIG probably acts to preserve the structural integrity of the plasma cellular membranes of hepatocytes to protect them from breakage by the reactive metabolites produced.

CONCLUSIONS

Our study demonstrates that the hepatoprotective activity of APIG against CCl₄ is due to its antioxidant properties, acting as scavengers of ROS. The preventive action of APIG on hepatic injury induced by CCl₄ is due to protection against oxidative damages in liver tissue. The possible mechanisms of protection are rather speculative at this stage and more studies are needed.

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