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Protective effects of luteolin-7-glucoside against liver injury caused by carbon tetrachloride in rats

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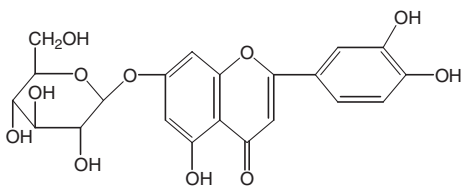
Ixeris chinensis (Thunb.) Nakai has been used as a Chinese folk medicine; the information on the physiological and biochemical functions of the compounds extracted from *I. chinensis* is still scanty. We investigated the effects of luteolin -7-glucoside (LUTG) isolated from *I. chinensis* against liver injury caused by carbon tetrachloride (CCl_4). CCl_4 significantly increased the enzyme activities of glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) in blood serum, as well as the level of malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-OHdG) in liver tissue, and decreased the levels of reduced glutathione (GSH). Pretreatment with LUTG was not only able 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 reduce the damage of hepatocytes *in vitro*. On the other hand, we also found LUTG has strong antioxidant activity against reactive oxygen species (ROS) *in vitro* in a concentration-dependent manner. The hepatoprotective activity of LUTG was possibly due to its antioxidant properties, acting as scavengers of ROS. These results obtained *in vivo* and *in vitro* suggest that LUTG had protective effects against hepatic oxidative injury induced by chemicals. Further studies on the pharmaceutical functions and immunological responses of LUTG may help in the development of a clinical application.

1. Introduction

Ixeris chinensis (Thunb.) Nakai has been used as a Chinese folk medicine in anti-inflammation, anti-atherosclerosis and anticancer, invigorating blood circulation, normalizings menstruation and eliminating blood stasis to relieve pain. The isolation and identification of various compounds from this plant and the same genus have been reported by several researchers (Asada et al. 1984a; 1984b; Chung et al. 2002; Lu et al. 2002). However, the information on the physiological and biochemical functions of theses compounds is still scanty. We isolated luteolin-7-glucoside (LUTG) from *I. chinensis* as previously reported (Meng et al. 1998; Ma et al. 1998; Saito et al. 1994). The pharmaceutical effect of this compound on protection against liver injury caused by CCl_4 has not been studied.

CCl_4 is a hepatotoxin, trichloromethyl radicals are generated from it *in vivo*. The radicals stimulate a sequence of reactions that culminate in the initiation of the peroxidation of membrane lipids (Reinke et al. 1988) and enhance liver damage. The trichloromethyl radical is believed to be the immediate product of the reductive dechlorination of CCl_4, catalysed by certain cytochrome P450 isoenzymes (Sipes et al. 1977) particularly the ethanol inducible iso-



LUTG

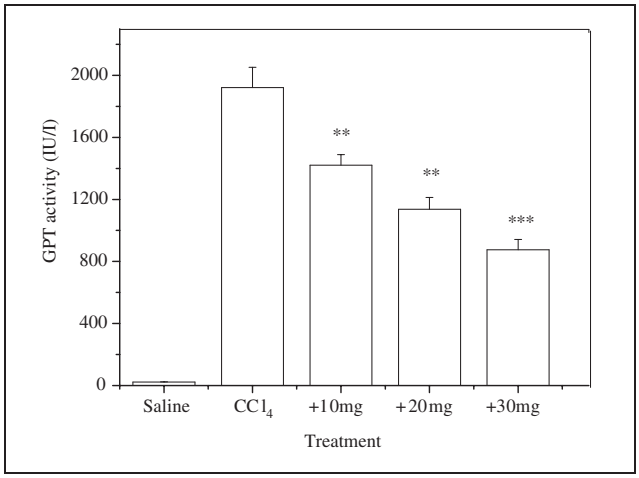


Fig. 1: Effects of LUTG (at dosages of 10, 20 and 30 mg/kg) on CCl_4-induced elevation of GPT activity. Each value represents the mean ± S.E.M. of 10 treated rats. Values statistically significantly different from that of CCl_4 control group indicated by $^{**}P < 0.01$ and $^{***}P < 0.001$. n = 10

form of the cytochrome (Reinke et al. 1988). CCl_4-induced lipid peroxidation process provides a model with which LUTG can be assessed for antioxidant property and protective effects against liver injury.
In this study, we examined the protective effects of LUTG on liver injury induced by CCl_4, and the inhibitory effects of LUTG on ROS generation in hepatocytes.

2. Investigations, results and discussion

2.1. Effects of LUTG on CCl_4-induced hepatotoxicity

CCl_4 produces hepatotoxicity when taken in 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 the rats. These enzymes, especially GPT, are highly localized in hepatocyte cytosols. The enzyme assays of the serum transaminases showed that a toxic dose of CCl_4 (1.25 ml/kg) significantly raised the levels of GPT (Fig. 1) and GOT (Fig. 2) to 1921 ± 131 and 2347 ± 164 IU/l ($n = 10$), respectively, compared with the control (saline) which had corresponding values of 22 ± 1.7 and 51 ± 3.2 IU/l, respectively.

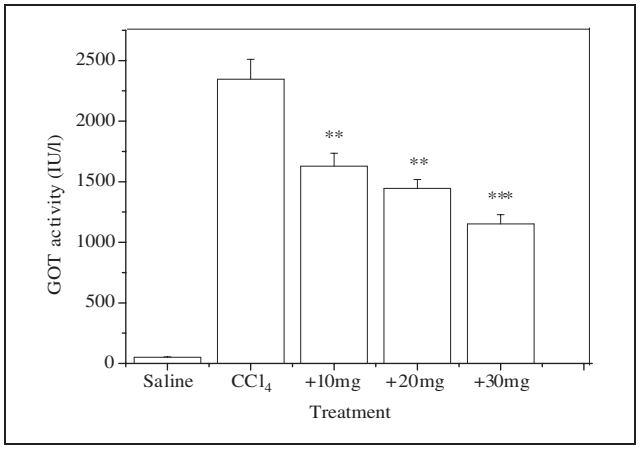


Fig. 2: Effects of LUTG (at dosages of 10, 20 and 30 mg/kg) on CCl_4-induced elevation of GOT activity. Each value represents the mean ± S.E.M. of 10 treated rats. Values statistically significantly different from that of CCl_4 control group indicated by $^{**}P < 0.01$ and $^{***}P < 0.001$. n = 10

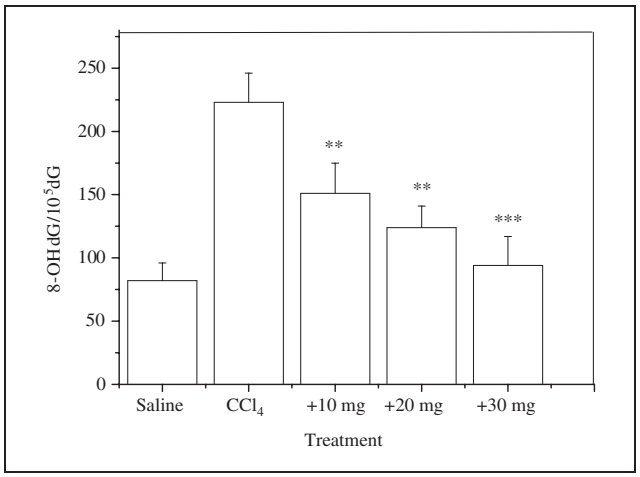


Fig. 3: Effects of various doses of LUTG combined with CCl_4 given to rats on accumulation of 8-OHdG in livers. $^{**}P < 0.01$; $^{***}P < 0.001$ vs CCl_4 group, n = 10

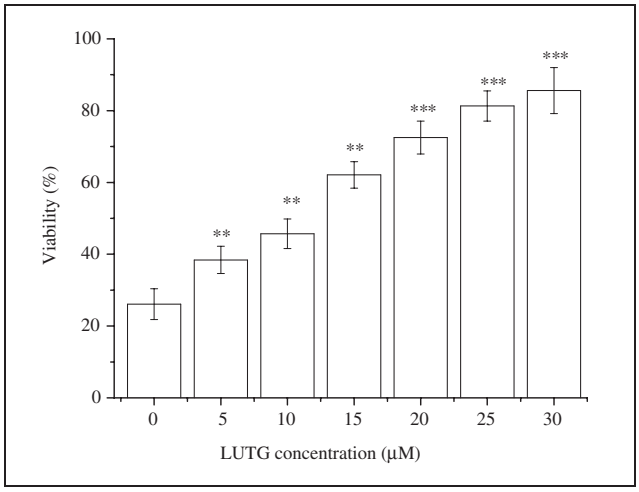


Fig. 4: Effects of LUTG on viability of hepatocytes. Hepatocytes were pre-incubated with various concentrations of LUTG 30 min before CCl_4 administration. The initial viability of hepatocytes was 90% (control). $^{**}P < 0.01$; $^{***}P < 0.001$ vs control. Results are expressed as mean ± S.E.M. of 3 experiments

The values of GPT and GOT serum enzymes in the LUTG-treated group were found to be: 1421 ± 67.4 IU/l and 1629 ± 160.7 IU/l for 10 mg/kg group; 1137 ± 76.1 IU/l and 1445 ± 74.1 IU/l for 20 mg/kg group; and 874.9 ± 67.3 IU/l and 1151 ± 77.8 IU/l for 30 mg/kg group, respectively, which were lower ($P < 0.01$) than the values of the control (Figs. 1 and 2). When rats were treated with the highest dose (30 mg/kg) LUTG alone, no effects of hepatotoxicity were observed (data not shown).

2.2. Protection of LUTG on DNA damage induced by CCl_4

Pretreatment with LUTG was able to prevent damage to DNA caused by CCl_4. 8-Hydroxydeoxyguanosine (8-OHdG), which is an oxidative DNA product generated from active oxygen species, was detected in liver cells after administration of CCl_4. The 8-OHdG level in DNA ($223/10^5$ dG) in the animals treated with CCl_4 was 2.7 times higher than those of control ($82/10^5$ dG). Protective activity of LUTG evaluated at various doses showed that DNA could be almost fully protected by LUTG at the highest dose of 30 mg/kg (Fig. 3). The possibility of protecting DNA against oxidative damage is of great importance in terms of carcinogenesis (Ames and Gold 1991; Ames et al. 1995; Floyd 1990).

2.3. Protection of LUTG on lipid peroxidation caused by CCl_4

MDA is a marker of oxidative lipid damage. Pretreatment with LUTG reduced MDA concentration in the liver tissue in a dose-dependent manner. The effect of LUTG on MDA level indicated the inhibition of lipid peroxidation induced by CCl_4 (Table).

2.4. LUTG inhibited the reduction of GSH concentration induced by CCl_4

CCl_4 significantly reduced the GSH concentration in the liver tissue (14.3 ± 2.1 nmol/mg protein) when compared to control (41.2 ± 2.4 nmol/mg protein). The reduction of hepatic GSH could significantly influence the susceptibility of the liver to the effects of hepatotoxic agents (Skou-

Table: Effects of LUTG given to rats 6 h prior to CCl_4 administration on concentration of GSH and MDA in liver tissue

Group	GSH (nmol/mg pr)	MDA (nmol/mg pr)
Control	41.2 ± 4.6	0.94 ± 0.09
LUTG (alone, 30 mg/kg)	42.6 ± 3.8	1.01 ± 0.08
CCl_4 (alone, 1.25 ml/kg)	17.1 ± 3.3[a, b]	3.22 ± 0.14[a, b]
CCl_4 + LUTG 10 mg/kg	26.5 ± 2.9[c]	2.54 ± 0.11[c]
CCl_4 + LUTG 20 mg/kg	35.3 ± 3.2[c]	1.85 ± 0.11[c]
CCl_4 + LUTG 30 mg/kg	39.7 ± 3.2	1.14 ± 0.09

n = 10
[a] CCl_4 group vs Control, $P < 0.01$
[b] CCl_4 group vs LUTG group, $P < 0.01$
[c] LUTG group vs CCl_4 + LUTG group, $P < 0.01$

lis et al. 1989) and could lead to cell death. LUTG was able to significantly inhibit the reduction of GSH in a dose-dependent manner (Table).

2.5. LUTG reduced the damage of hepatocytes in vitro

Hepatocytes incubated with 10 mM CCl_4 demonstrated a loss of cell viability. The cytotoxicity of CCl_4 was significantly reduced by adding LUTG to the incubation media (Fig. 4). One of the possible mechanisms for the hepatoprotection by drugs is believed to be stabilization of the hepatocyte membranes through their antioxidant action when used for pretreatment (Davila et al. 1989).

2.6. Effects of LUTG on formation of reactive oxygen species (ROS)

The activity of LUTG on inhibiting ROS formation in hepatocytes was examined by determination of chemiluminescence. Hepatocytes were pretreated with various concentrations of LUTG for 30 min, and then were incubated at 37 °C with 10 mM of CCl_4. Spontaneous intensity of chemiluminescence was significantly inhibited by LUTG in a concentration dependent manner (Fig. 5).

The present study revealed that LUTG had protective effects against hepatic injury induced by CCl_4. The protective effects were evidenced by a blockage of the CCl_4-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 LUTG derived from *I. chinensis* has low toxicity. CCl_4 caused oxidative DNA damage in the target organ liver, which can be blocked by LUTG, it is well known that the oxidative DNA damage is involved in chemicals induced carcinogenesis, LUTG may have a protective effect against such carcinogenesis.

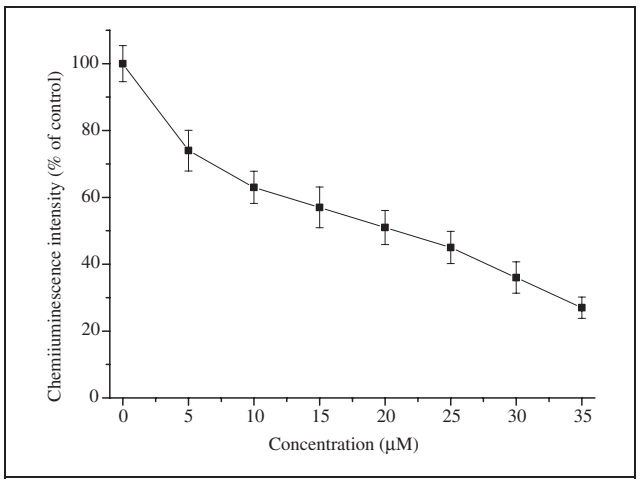


Fig. 5: Effect of LUTG on chemiluminescence of hepatocytes in the presence of CCl_4

The model of hepatotoxicity induced by CCl_4 is commonly used to evaluate the curative effects of drugs against hepatotoxicity (Slater 1966; Gilani and Janbazz 1995). The increases in GPT and GOT serum levels have been attributed to damage to the structural integrity of the liver (Chenoweth and Hake 1962). The mechanism of CCl_4–induced hepatotoxicity is considered to result from activation of CCl_4 by the respective specific isozyme of the cytochrome P-450 system in the hepatocyte's endoplasmic recticulum to the reactive metabolite, CCl_3^-, which can form covalent products with protein and lipid and interact with O_2 to generate CCl_3O_2, which in turn initiates lipid peroxidation of the endoplasmic reticulum (Slater 1966; Gilani and Janbazz 1995) and oxidative DNA damage. LUTG probably acted to preserve the structural integrity of the plasma cellular membrane of the hepatocytes to protect it from breakage by the reactive metabolites produced. Furthermore, the hepatoprotective activity of LUTG was also possibly due to its antioxidant properties, acting as scavengers of ROS. It must be noted, however, that the possible mechanisms of protection are rather speculative at this stage and more investigations are needed.

3. Experimental

3.1. Chemicals

5,5′-dithio-bis(2-nitrobenzoic) (DTNB), proteinase K, thiobarbituric acid, sodium dodecyl sulfate, were obtained from Sigma (St. Louis, MO). luteolin-7-glucoside was isolated from *Ixeris chinesis* (Thunb.) Ankai as described previously which has high purity (97.3%). All other reagents used were of analytical grade and were purchased from Shanghai Biochemical Co. (Shanghai, China), unless otherwise noted.

3.2. Animals

Male Wistar rats (150–180 g) obtained from the 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 12 h daylight.

3.3. 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 CCl_4 to induce chemical hepatitis followed 6 h later by oral saline administration; the third group which was treated similarly to group 2 except that LUTG was administered instead of saline to evaluate it's curative effects. Three dosages of LUTG (10, 20 and 30 mg/kg) dissolved in dimethyl sulphoxide (DMSO) (final concentration 0.1%) were used. Ten animals received one dose of LUTG. The CCl_4 was dissolved in corn oil and was administered orally to the stomach of the rat through an intragastric tube. All treated animals were sacrificed 24 h after receiving the administration of the LUTG or hepatotoxin. All experimentation was conducted in conformity with ethical and humane principles of research.

3.3.1. 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 $1100 \times g$ for 15 min, and then plasma was 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 (1957).

3.3.2. Analyses of 8-hydroxy-2′-deoxyguanosine (8-OHdG)

To minimize artificial 8-OHdG generation during sample preparation, DNA isolation from livers was achieved according to the pronase/ethanol method described by Kendall et al. (1991). Briefly, DNA was extracted from livers by homogenization in 1 ml of buffer containing 1% sodium dodecyl sulfate, 10 mM Tris, 1 mM EDTA (pH 7.4) and overnight incubation 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 M 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 l of 10 mM Tris/1 mM EDTA (pH 7.4) (Liang et al. 2000).
DNA digestion was performed as described by Kasai et al. (1986). The oxidative DNA adduct, 8-OHdG, was measured with (HPLC) equipped with electrochemical and UV detection (Kaneko and Tahara 1996) using a CoulArray system (Model 5600). Analytes were detected on two coulometric array cell modules, each containing four electrochemical sensors attached in series. UV detection was set at 260 nm. The HPLC was controlled and the data acquired and analysed using CoulArray software. The mobile phase was composed of 50 mM 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 for expressed as the numbers of 8-OHdG for every 10^5 dG in DNA.

3.3.3. Measurement of MDA

MDA, the marker of oxidative lipid damage, was measured to estimate the extent of lipid peroxidation. Liver tissue homogenate (prepared in 0.5 ml of PBS with 1% SDS) was 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 mM NaOH, and the tubes were 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 vortexd vigorously for 10 s, and were centrifuged at 5000 × 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 (Gutteridge 1975). Thus, TBARS are calculated as MDA equivalents and expressed as nmol/mg protein. TBARS formation was used as an index of lipid peroxidation (Gutteridge 1975).

3.3.4. Measurement of reduced glutathione (GSH)

GSH was assessed with DTNB based on the method as described by Jocelyn (1987). The liver tissue was homogenated in 300 µl 5% of 5-sulfosalicylic acid and centrifuged for 10 min at 1000 × 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 a spectrophotometer. Sample values were then calculated from a standard curve generated with known amounts of GSH.

3.3.5. Evaluation for in vitro hepatoprotective activity

Hepatocytes were prepared from liver of intact rats using the two-stage collagenase perfusion method described by Hung et al. (2002). The hapatocytes were suspended in Krebs-Henseleit buffer (pH 7.4), and cells were treated with 5% CO_2, at 37 °C and incubated in siliconised round-bottom flasks at a cell density of 1×10^6 cells/ml. Viability of hepatocytes was assessed by the Trypan blue exclusion method. LUTG were dissolved in dimethyl sulphoxide (DMSO) (final concentration 0.1%). To evaluate the protective activity of LUTG, cells were incubated with various concentrations of LUTG for 30 min before administration of CCl_4 (10 mM). Control group contained 0.1% of DMSO.

3.3.6. Measurement of ROS formation in hepatocytes

ROS formation in hepatocytes was determined at 37 °C using chemiluminescence single photon counting technology (Hung et al. 2002). 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 1 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.

3.4. Statistical analysis

Statistical analysis of data was carried out as previously described (Zheng 2001). Quantitative differences between groups values were statically analyzed using ANOVA (analysis of variance) with a multiple comparison post-test by the Bonferroni method. P values of < 0.05 were considered statistically significant. All values were expressed as mean ± SD, and each sample was analyzed three times.

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