Food Sci. Biotechnol. 25(2): 581-587 (2016)
DOI 10.1007/s10068-016-0081-z

Food Science
and
Biotechnology

Insect Tea Extract Attenuates CCl_4-induced Hepatic Damage Through Its Antioxidant Capacities in ICR Mice

Yuanjiang Liao[1], Xiaochuan Zou[2,3,4,5], Cun Wang[2,3,4,5], and Xin Zhao[2,3,4,5,*]

[1]*Department of Nephrology, The Ninth People's Hospital of Chongqing, Chongqing 400700, China*
[2]*Department of Biological and Chemical Engineering, Chongqing University of Education, Chongqing 400067, China*
[3]*Chongqing Collaborative Innovation Center of Functional Food, Chongqing University of Education, Chongqing 400067, China*
[4]*Chongqing Engineering Technology Research Center for Functional Food, Chongqing University of Education, Chongqing 400067, China*
[5]*Chongqing Engineering Laboratory of Functional Food, Chongqing University of Education, Chongqing 400067, China*

Received July 11, 2015
Revised December 22, 2015
Accepted January 13, 2016
Published online April 30, 2016

*Corresponding Author
*Tel/Fax: +86-23-62658256
E-mail: foods@live.cn

pISSN 1226-7708
eISSN 2092-6456

Abstract The Insect tea extract (ITE) contained many polyphenols, the aim of the present study was to determine the preventive effects of ITE on CCl_4-induced hepatic damage in mice. ITE treated mice could reduce hepatic injury compared to the control mice. The 200 mg/kg ITE increased TC, ALB, SOD, CAT, GSH-Px serum levels, and decreased ALT, AST, ALP, TG, BUN, NO, MDA levels compared to the control group. By histological observation, ITE reduced injury to hepatic cells, and these effects were close to that seen with the drug silymarin. The antioxidant related mRNA and protein expressions of Mn SOD, Gu/Zn SOD, CAT, and GSH-Px increased with ITE treatment in hepatic damage mice. ITE treated mice also showed higher IκB-α mRNA and protein expression, and lower NF-κB-p65, iNOS, COX-2 expressions than those of control mice. These results proved ITE has a prophylactic effect in protecting against hepatic injury through the antioxidant capacities.

Keywords: insect tea, polyphenol, hepatic damage, antioxidant, mice

Introduction

Insect tea, a particular kind of solid brew drink with a special brewing method and strong ethnic characteristics, is native mainly to certain provinces in China such as Guizhou, Hunan, Guangxi, Sichuan, and Yunan (1). Locals gather fresh leaves from a wide variety of wild plants, such as wild Japanese *ligusticum* leaf and *dyetree* leaf. The leaves are layered in wooden barrels after removing astringency by stewing and drying naturally. Then rice water is poured evenly in each layer. The early stages of fermentation give off an aroma, which attracts insects like *hydrillodes repugnalis* to oviposit on the leaves. When the eggs hatch, the larvae eat the leaves and defecate pellets. The pellets are separated from the leaves through processes like solarization and stir-frying and become Insect tea (2,3).

$CC1_4$ (carbon tetrachloride) produces free radicals in the process of reduction and activation in the liver, which leads to hepatic injury. This mechanism is used in an animal model of hepatic injury in order to test a compound's inhibitory effects on functional hepatic injury (4). In addition, silymarin, which has strong antioxidative function, can protect hepatic cells from free radicals and contribute to protein synthesis so as to speed up production of new hepatic cells and promote injured hepatic cell repair (5). Therefore, silymarin maintains the function of protecting hepatic cells from invasion of toxic substances, especially those from alcohol and environmental pollutants like pesticides and heavy metals; moreover, silymarin can inhibit lipoxygenase and peroxidase. Clinically, it can be used in the treatment of hepatotoxicity and hepatosis (6). It serves as a positive drug control in this project so that the efficacy of tea polyphenols in Insect tea can be better evaluated.

Insect tea contains various functional substances, including rich tea polyphenols (7). Tea polyphenols are powerful in scavenging radicals and increasing enzymatic activity inside the body by protecting lipids from peroxidation as an anti-inflammatory action (8). As research showed, tea polyphenols in Insect tea played a significant role in its functional action (7). Further studies indicated that Insect tea crude extract has a profound protective effect on the liver and could inhibit hepatic injury and hepatoma formation (9). This preventative effect of Insect tea was possibly related to the high polyphenols. The causes for hepatic injury are varied, and if measures are not taken in time, may lead to severe and lethal consequences (10). This research focused on the inhibitory effect of polyphenol substances extracted from Insect tea on $CC1_4$-induced mice hepatic injury. The research also studied the antioxidative mechanism of Insect tea polyphenols on hepatic injury. The data presented here can provide a new direction for the development of this valuable food resource, Insect tea, and put forward new plans for hepatic injury prevention and treatment by using functional foods.

Materials and Methods

Extraction of Insect tea polyphenols Insect tea was collected from the manufacturing location (Zunyi, China) in a dried form and was further freeze-dried. One kilogram of Insect tea was extracted twice with 80% ethanol (20 L) at 75°C using a sonication protocol (20 min, 600 W, extract system; Hangzhou Success Ultrasonic Equipment Co., Ltd., Hangzhou, China). Then the extract was filtered and put into diethyl ether (2 L) for chlorophyll removal. After, the Insect tea polyphenols were isolated by rotary evaporation (RE- 52A; Shanghai Yarong Biochemical Instrument Factory, Shanghai, China) at 60°C.

Determination of the polyphenol content of Insect tea The content of polyphenols in Insect tea was determined with ferrous tartrate colourimetry. Gallic acid was dissolved in distilled water. 5 mL ferrous tartrate solution was added to 1 mL gallic acid and diluted with sulphuric acid of pH 7.5 to 25 mL. Calibration was drawn at 540 nm absorbance (UV-5100 ultraviolet spectrophotometer; Shanghai Metash Instruments Co., Ltd., Shanghai, China) (11).

Animal experiment ICR mice (male, 7 weeks old, Chongqing Medical University, Chongqing, China) of similar weight were grouped into 5 groups randomly: normal, control, Insect tea polyphenols (dosage of 100 and 200 mg/kg) and drug positive control silymarin (dosage of 100 mg/kg) with 10 mice in each group. Mice in normal and control groups were fed normally for 14 day without any experimental treatment; mice in Insect tea polyphenols group were perfused each day with 0.2 mL Insect tea polyphenols with either 100 or 200 mg/kg for 14 day; mice in the silymarin group were perfused each day with 100 mg/kg for 14 day. After fourteen days, mice from the control, Insect tea polyphenols and silymarin group were induced with hepatic injury. Having perfusion on the last day of the experiment, mice were fasted for 12 h, and 0.2 mL/kg $CC1_4$ (Tianjin Zhiyuan Chemical Reagent Co., Ltd., Tianjin, China) was injected into the abdomen. Twenty-four hours later, the mice were euthanized. Blood plasma and livers were collected, and hepatic index was determined by the formula: hepatic index (hepatic injury indicator)=(mouse' s hepatic weight/mouse's weight)×100 (12). Experiments followed a protocol approved by the Animal Ethics Committee of Chongqing Medical University (Chongqing, China).

Blood plasma experiments Blood plasma was centrifuged for 15 min at 5,000x*g* at 4°C. According to the manufacturer protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the upper layer of the serum was drawn to determine the level of ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), TG (triglyceride), TC (total cholesterol), BUN (blood urea nitrogen), ALB (albumin), SOD (superoxide dismutase), NO (nitric oxide), CAT (catalase), MDA (malondialdehyde) and GSH-Px (glutathione peroxidase).

Liver histological observation The sectioned hepatic tissue was fixed in 10% formalin and dehydrated in high concentration alcohol. The alcohol in the tissue was exchanged by dimethylbenzene. The tissue was then embedded in paraffin and sectioned after cooling (12). After H&E staining, the tissue was sealed by gum and observed under a light microscope (BX41; Olympus, Tokyo, Japan).

Reverse transcription polymerase chain reaction (RT-PCR) assay RNAzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from hepatic tissue. The concentration of the extracted total RNA was adjusted to 1 μg/μL. RNA (2 μL) was added with Oligo(dT)18, RNase, dNT and MLV enzyme (Roche, Basel, Switzerland) of 1 μL, 5×buffer 10 μL, respectively, and cDNA was synthesized with cycling conditions of 37°C for 120 min, 99°C for 4 min, 4°C for 3 min. Then the primers (Thermo Fisher Scientific, Waltham, MA, USA) of Mn SOD (forward: 5'-TTCAATAAGGAGCA GGGAC-3'; reverse: 5'-CAGTGTAAGGCTGACGGTTT-3'), Cu/Zn SOD (forward: 5'-GAAGAGAGGCATGTTGGAGA-3'; reverse: 5'-CCAATTACA CCACGAGCCAA-3'), GSH-Px (forward: 5'-GCGGGAGCAGGACTTCTA CGA-3'; reverse: 5'-CCCGATAGTGCTGGTCTGTGAA-3'), CAT (forward: 5'-AGATACTCCAAGGCGAAGGTG-3'; reverse: 5'-AAAGCCACGAGGGTC ACGAAC-3'), NF-κB-p65 (nuclear factor kappa-B-p65) (forward: 5'-CACTTATGGACAACTATGAGGTCTCGG-3'; reverse: 5'-CTGTCTTGTGGA CAACGCAGTGGAATTTTAGG-3'), IκB-α (inhibitor of nuclear factor kappa-B alpha) (forward: 5'-GCTGAAGAAGGAGCGGCTACT-3'; reverse: 5'-TCGTACTCCTCGTCTTTCATGGA-3'), iNOS (inducible nitric oxide synthase) (forward: 5'-AGAGAGATCGGGTTCACA-3'; reverse: 5'-CACA GAACTGAGGGTACA-3') and COX-2 (cyclooxygenase-2) (forward: 5'-TTAAAATGAGATTGTCCGAA-3'; reverse: 5'-AGATCACCTCTGCCTGAGTA-3') were amplified through RT-PCR (Eppendorf, Hamburg, Germany), along with GAPDH (forward: 5'-CGGAGTCAACGGATTTGGTC-3'; reverse: 5'-AGCCTTCTCCATGGTCGTGA-3') as a housekeeping gene. Finally, the PCR amplified product was visualized by the electrophoresis of agaragar with ethidium bromide of 1% (12), and the expression was quantitatively analyzed with ImageJ 1.44 (National Institute of Mental Health, Bethesda, Maryland, MD, USA).

Western blot analysis After tissue protein was extracted with RIPA lysis buffer, the total protein concentration of each group was determined with UV spectrophotometer, and the concentrations were adjusted to the same level. Samples were electrophoresed in 10%-12% SDS-PAGE gels (Schleicher & Schuell Bioscience, Inc., Keene, NH, USA) for 4 h and transferred to nitrocellulose filters. The nitrocellulose filters were sealed for 3 h in sealing fluid and then washed with TBS 3 times and incubated in primary antibody working solution for 2 h; then washed with TBS for 3 times and incubated in secondary antibody working solution marked by horseradish peroxidase for 2 h. After being washed with TBS for three times, super ECL plus was used for chromogenic reaction. The protein levels of SOD, GSH, CAT, NF-κB, IκB-α, iNOS and COX-2 (Santa Cruz Biotechnology Inc.,

Dallas, TX, USA) were determined, and β-actin was used as loading control (12).

Statistical analysis Experimental data are presented as the mean± SD. Differences between the mean values for individual groups were assessed by a one-way ANOVA with Duncan's multiple range tests. $p<0.05$ was considered to indicate a statistically significant difference. SAS v9.2 statistical software package (SAS Institute Inc., Cary, NC, USA) was used for the analysis.

Results and Discussion

Content of Insect tea polyphenols Gallic acid was used as polyphenol standard reference substance in this study, and the polyphenols content standard curve was y=0.655x+0.002 (R^2= 0.999). By this standard curve, the ITE extract contained 40.85% of polyphenol materials. Insect tea and green tea both contained the functional materials of polyphenols, but their component contents had difference. Insect tea contained 26.28% tea polyphenol, included EGCG (epigallocatechin gallate), EGC (epigallocatechin), ECG (epicatechin gallate), EC (epicatechin), gallocatechin, and catechin (13).

Hepatic injury index in mice Reactive oxygen species (ROS) were the main causes of CCl_4-induced acute liver damage. After metabolic activation of hepatic cytochrome P450, CCl_4 could generate CCl_3 free radical, CCl_3 free radical could cause the liver cell membrane lipid peroxidation, this process led to liver damage (14). After inducing liver injury by CCl_4, the body weights of reduced, and the liver weight of hepatic damage mice was highest in all groups (Table 1). The hepatic index (hepatic injury indicator) of the normal group was lowest, and the silymarin and 200 mg/kg ITE groups also had low hepatic injury indicators. The hepatic injury indicator of control mice increased compared to other groups.

Obviously, injured liver had increased weight and was swollen in mice, which caused the increase in Hepatic Index (hepatic injury indicator). This index is a key parameter for hepatic injury (12). ITE could reduce the liver weight and hepatic injury index in hepatic injury of mice, and ITE could also ease the CCl_4-induced liver injury by decreasing the hepatic injury index.

Effect of MALP on serum levels of ALT, AST, ALP, TG, TC, BUN and ALB in mice The ALT, AST and ALP serum levels in control mice were the highest, and the levels in normal mice were the lowest (Table 2). Silymarin and 200 mg/kg ITE treated mice drastically reduced ALT, AST and ALP serum levels compared to the control mice, but still were a little higher than normal mice. The TG and BUN levels of control mice also were highest, 100 and 200 mg/kg ITE could reduce these levels compared to the control mice, and 200 mg/kg ITE showed a more noticeable reduction (Table 2). After treatment of the drug silymarin, the mice showed similar TG and BUN levels to normal mice, and lower than that of the mice in other groups. In the TC and ALB levels results, the levels of normal, silymarin, 200 mg/kg ITE, 100 mg/kg ITE and control groups were found to have significant increased ($p<0.05$).

AST and ALT in blood serum is the key biochemical index to diagnose hepatic injury. ALT is mainly distributed in hepatic cytosol, and AST in hepatic cytosol and hepatic cell's chondriosome (15). When hepatic cell gets injured, AST and ALT are released to blood (12). ALP is widely distributed in bone, liver, intestine, muscles etc. Injured hepatic cell produces excess ALP, and ALP enters blood through lymphogenous tract and sinus hepaticas (16). Meanwhile, due to bile acatharsia in hepatic biliary passage, ALP flows back into the blood, causing elevation of ALP in serum (17). The hepatic injury in mice showed the increased levels of AST, ALT, and ALP compared to the normal mice (15). In this case study, CCl_4-induced hepatic injured mice also had the highest AST, ALT, and ALP levels in all groups mice, ITE decreased these levels, and the higher concentration of ITE

Table 1. Body weight, liver weight and liver index of CCl_4-induced hepatic damage mice

Group		Body weight (g)	Liver weight (g)	Liver index
Normal		38.42±2.46$^{a1)}$	1.70±0.04^{e}	4.42±0.02^{e}
Control		30.12±2.09^{e}	2.78±0.09^{a}	9.23±0.04^{a}
Silymarin (100 mg/kg)		36.71±2.26^{b}	1.79±0.05^{d}	4.88±0.02^{d}
Insect tea extract (mg/kg)	100	33.34±1.03^{d}	2.26±0.06^{b}	6.78±0.03^{b}
	200	35.03±1.68^{c}	1.93±0.05^{c}	5.51±0.03^{c}

$^{1)a-e}$Mean values with different letters in the same column are significantly different ($p<0.05$) according to Duncan's multiple range test.

Table 2. AST, ALT, ALP, TG, TC, BUN, and ALB serum levels of CCl_4-induced hepatic damage mice

Group		AST (Karmen/mL)	ALT (Karmen/mL)	ALP (K-A)	TG (mg/dL)	TC (mg/dL)	BUN (mg/dL)	ALB (g/dL)
Normal		55.23±3.26^{e}	54.20±2.15^{e}	7.62±0.59^{e}	47.52±2.06^{e}	94.52±5.24^{a}	25.29±1.87^{e}	3.81±0.52^{a}
Control		149.71±10.22$^{a1)}$	139.87±8.39^{a}	15.68±1.57^{a}	162.31±18.52^{a}	42.18±3.21^{e}	41.28±2.87^{a}	3.02±0.32^{d}
Silymarin (100 mg/kg)		78.39±3.75^{d}	70.52±3.28^{d}	9.82±0.88^{d}	82.65±3.22^{d}	80.36±3.50^{b}	27.31±1.39^{d}	3.58±0.59^{ab}
Insect tea extract (mg/kg)	100	120.62±8.21^{b}	104.52±5.97^{b}	13.82±0.68^{b}	131.23±8.38^{b}	61.36±2.85^{d}	34.98±2.10^{b}	3.16±0.38^{c}
	200	90.61±4.69^{c}	86.52±3.08^{c}	11.03±0.75^{c}	107.59±6.82^{c}	75.31±2.03^{c}	29.52±1.32^{c}	3.47±0.29^{b}

$^{1)a-e}$Mean values with different letters in the same column are significantly different ($p<0.05$) according to Duncan's multiple range test.

Table 3. SOD, NO, CAT, MDA, and GSH-Px serum levels of CCl_4-induced hepatic damage mice

Group		SOD (U/mL)	NO (μmol/L)	CAT (U/mL)	MDA (μmol/L)	GSH-Px (U/mL)
Normal		131.25±11.21$^{a1)}$	72.15±3.59^{e}	35.08±2.33^{a}	9.56±0.41^{e}	225.79±25.47^{a}
Control		75.36±5.52^{e}	110.35±8.91^{a}	22.18±1.98^{e}	12.69±0.32^{a}	167.20±18.72^{e}
Silymarin (100 mg/kg)		108.28±7.19^{b}	78.86±2.28^{d}	31.33±1.21^{b}	10.28±0.37^{d}	210.39±15.62^{b}
Insect tea extract (mg/kg)	100	83.11±3.65^{d}	96.31±2.25^{b}	25.36±0.97^{d}	11.68±0.29^{b}	189.31±10.55^{d}
	200	97.50±4.05^{c}	86.17±3.57^{c}	28.39±1.08^{c}	10.98±0.23^{c}	198.32±11.28^{c}

$^{1)a-e}$Mean values with different letters in the same column are significantly different (p<0.05) according to Duncan's multiple range test.

could decrease more AST, ALT, and ALP levels compared to the lower concentration of ITE. ITE could attenuate the liver injury by these changes.

Liver is an important organ for human lipid metabolism, and TC is mainly synthesized in hepatic cell's microsome. When hepatic injury appears hepatic cells degenerates and becomes necrotized, and cell organelles suffer damage (16). As a result, the synthesis of rate-limiting enzyme which contributes to cholesterol synthesis decreases and glucagon, which inhibits rate-limiting enzyme synthesis, increases. Thus, obstruction occurs in the whole process from cholesterol synthesis to plasma lipoproteins processing and assembling, and the level of TC is much lower than that of normal condition (18,19). Meanwhile, liver is also the main place for human protein metabolism, and can synthesize amino acid into protein that human body needs, such as ALB (20). Once ALB is synthesized in rough surfaced endoplasmic reticulum of hepatic cells, it is secreted to sinus hepaticas by Golgi body. When hepatic injury occurs, it blocks ALB's synthesis, transportation in cell and gets released, which further decrease the level of ALB in serum (21). BUN is the main end product of protein metabolism. If hepatic function gets damaged, especially liver cirrhosis happens, renal failure may come with and urea excretion in urine is reduced, which increases the level of BUN in blood (22). ITE increases TC, ALB and decrease TG, BUN serum levels in the liver injury in mice, ITE could make the serum levels of liver injury mice close enough to the levels in normal mice.

Effect of MALP on antioxidant related serum levels of SOD, NO, CAT, MDA and GSH-Px in mice Treatment with ITE (200 mg/kg) significantly (p<0.05) increased the antioxidant related serum levels of SOD, CAT and GSH-Px compared to the control mice (Table 3). The 100 mg/kg ITE treatment group also showed high SOD, CAT, and GSH-Px levels, but lower than the 200 mg/kg treatment group. ITE treatments showed similar SOD, CAT, and GSH-Px levels to silymarin treatment, and close to normal mice. The NO and MDA levels of control were higher than that of other groups, ITE could significantly reduce these levels (p<0.05), and these levels of ITE treated mice were close to silymarin and normal groups.

SOD, an active substance of life, can eliminate other harmful substances produced in the process of metabolism. As after entering the body, it is activated by hepatocyte pigment P450, $\cdot ECCl_3$ and $\cdot EOOCCl_3$, which are two kinds of free radicals are generated, which leads to hepatic injury (23). When pathogen invades liver, endotoxin causes liver or blood vessel endothelium to release massive NO. As a free radical, NO can interact with O_2^- and produce $ONOO^-$ and with other free radicals and triggers the lipid peroxidation, which eventually causes hepatic injury (24). In the hepatic injury caused by $CC1_4$, $CC1_4$ was activated by P450, produces many free radicals, causing hepatic injury (25). As research shows, CAT can effectively inhibit free radical H_2O_2.

Research also shows that when the symptoms of animal with liver injury alleviates, then the same time content of CAT increase significantly. Thus, CAT plays a protective and detoxified role in body (26). If there is disorder and imbalance of the coordination and dynamic balance between free radical reaction and lipid peroxidation, lipid peroxidation occurs and it destroys cells and tissues (27). Once hepatic injury occurs, lipid per-oxidative products MDA in tissue, which can change the mobility and permeability of cell membrane, which eventually changes the structure and function of cell and make hepatic injury worsen (28). Therefore, the content of MDA in serum and tissue can indicate the degree of lipid per-oxidative, and it can be used to determine degree of $CC1_4$-induced hepatic injury (12). One of the most important clastic enzymes for peroxide widely existing in organism, GSH-Px can block the peroxidation of superoxide anion cellular lipids which damages histiocyte. Besides, it can also prevent the second-degree reaction of free radical triggered by ROOH and thus reduce ROOH's harm to visceral tissues. GSH-Px can clean out ROOH produced by hepatic injury and thus protect liver (29). Again, ITE increased SOD, CAT, GSH-Px, and decreased NO, MDA serum levels.

Histological observation in liver tissue As shown in Fig. 1, in normal group, seen from light microscope, the hepatic color of the mouse liver was normal, the cytoplasm of hepatic cells was abundant and the cell shape was unchanged. In the control group, there were massive pale watery cytoplasm in hepatic cells, and obvious meronecrobiosis and infiltration of inflammatory cells could be found around the central vein. Compared to the control group, ITE experimental groups have improved evidently, among which, the hepatic cells of ITE high-dose (200 mg/kg) group was the closest to normal and drug (100 mg/kg silymarin) treated hepatic tissue for the cell's normal color and almost unchanged shape. The high-dose group had a preferable effect when compared with the low-dose (100 mg/kg) group.

Liver biopsy is one of the important lab exam to determine the

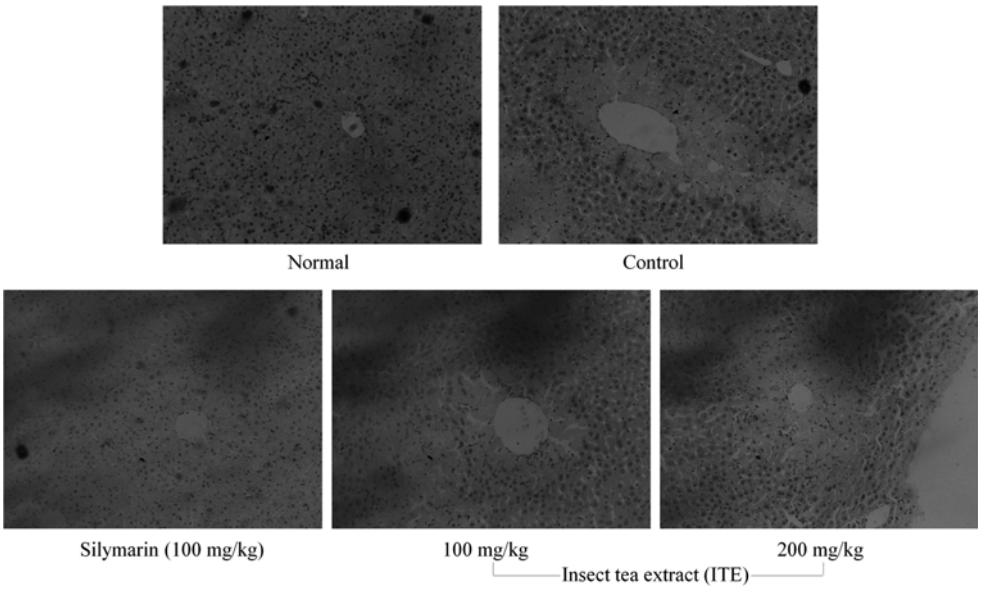

Fig. 1. Histopathology observation of liver in CCl_4-induced hepatic damage mice.

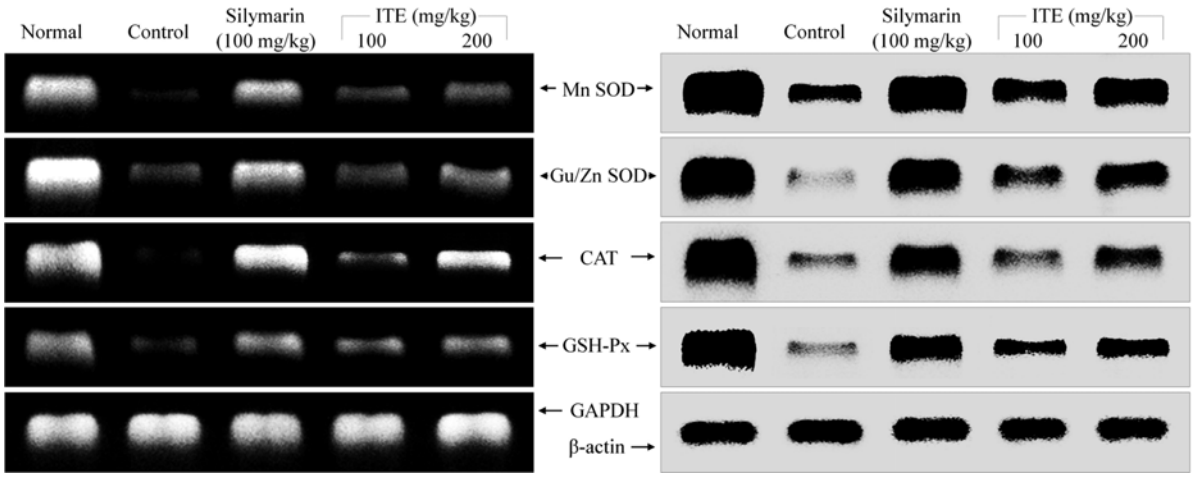

Fig. 2. Effects of insect tea extract (ITE) on the mRNA and protein expressions of Mn SOD, Gu/Zn SOD, CAT and GSH-Px (GSH) in liver of CCl_4-induced hepatic damage mice. Fold-ratio: gene expression/ GAPDH (β-actin)×control numerical value (control fold ratio: 1).

degree of liver tissue injury. The normal structure of liver which includes, central vein passing radially, hepatocytes, hepatic vessels and hepatic sinusoid, all are regularly arranged. And high concentration of ITE could make hepatic lobule a complete structure. Injured hepatic tissue shows itself frequently in the form of swollen hepatocytes and pale watery cytoplasm, and moreover, around central vein there are necrotized hepatocytes and inflammatory cells invasion (30). From this observation, ITE could prevent the hepatocytes damaged liver in mice.

Antioxidant related gene and protein expression in liver tissue

By RT-PCR and western blot assay, CCl_4 reduced Mn SOD, Gu/Zn SOD, CAT and GSH-Px mRNA and protein expression (Fig. 2). ITE treatment helped increase these levels, and high dose (200 mg/kg) treatment had more "obvious" (change word) effects. The 200 mg/kg ITE treated mice showed 3.2, 2.6, and 3.1 times stronger expression compared to the control mice, and were similar to silymarin (3.5, 3.2, and 4.2 times increase over the control group) and normal groups (3.8, 3.9, and 4.6 times increase over the control group).

The Mn SOD and Gu/Zn SOD are the form of SOD. SOD can clean out O_2^- at some extent and decrease hepatic injury. More researches have testified that hepatic injury induced by $CC1_4$ can be decreased and even recovered by SOD (31). CAT, GSH-Px, and GSH are important antioxidant related genes, the antioxidant effects of tea could be determined by these genes checking. ITE caused these levels of CCl_4-induced hepatic injury in mice close to normal and drug (silymarin) treatment groups of mice. By the RT-PCR and western blot assay, SOD, CAT, GSH-Px mRNA and SOD, CAT, GSH protein expressions of ITE treated mice also higher liver injury control mice. ITE could help to attenuate liver injury in mice by the antioxidant abilities of ITE.

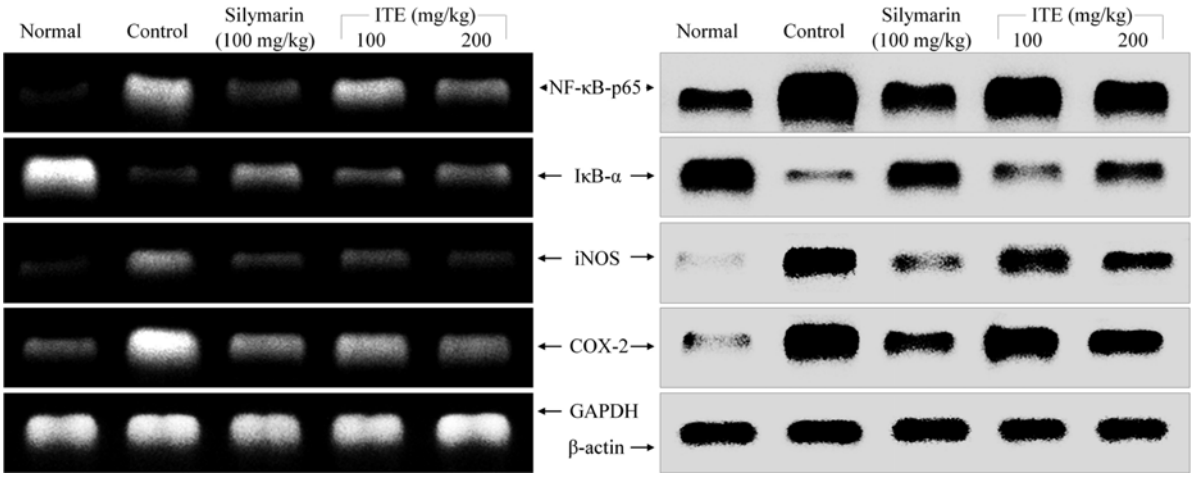

Fig. 3. Effects of insect tea extract (ITE) on the mRNA and protein expressions of NF-κB-p65, IκB-α, iNOS and COX-2 in liver of CCl_4-induced hepatic damage mice. Fold-ratio: gene expression/GAPDH (β-actin)×control numerical value (control fold ratio: 1).

Inflammation related gene and protein expression in liver tissue

As shown in Fig. 3, NF-κB-p65, iNOS and COX-2 mRNA and protein expressions of control mice were highest, and normal mice showed lower levels than the other mice. ITE treatment significantly (p<0.05) reduced the NF-κB-p65, iNOS and COX-2 mRNA and protein levels compared to the control mice and these effects were similar to treatment with silymarin. 200 mg/kg of ITE showed stronger effects than 100 mg/kg ITE. Mice showed the reverse trend of IκB-α mRNA and protein expression compared to NF-κB-p65, iNOS and COX-2.

NF-κB maintains crucial physiological function in body. And the inflammatory reaction it's been involved is an important mechanism for organism's normal defense and recovery. As a nuclear transcription factor sensitive to oxidation, NF-κB can be activated by regular stimulation to oxidation. The over-expression of NF-κB exerts great impact on liver and exacerbates hepatic injury (32). IκB-α is an important protein for meditating the activity of NF-κB. When histiocyte was activated by oxygen free radical, IκB-α is degrades. This process can promote the phosphorylation and nuclear transcription of NF-κB, which then translocate nucleus for its functioning. Not only, IκB-α can inhibit the activation of NF-κB, but also it can regulate NF-κB system to reduce inflammation and eases hepatic injury (33). NF-κB protein family is comprised of 5 different subunits, and p50/p65 heterodimers were most important factors of NF-κB family in human body. The positive signal of p50 and p65 mainly shows in cytoplasm and nucleus. When the immune response is abnormal, NF-κB can't restore to the original state and stay at nucleus, this course causes the normal response signal of human body change, than the tissue cells get injury (34,35). Oxidation could influence NF-κB-p65 and make the liver damage (36), NF-κB-p65 might be an important oxidation related factor of liver damage. COX-2 plays an important role in inflammatory action formation. NF-κB can facilitate COX-2 gene's transcription and regulate its expression, which magnify inflammatory reaction and aggravate hepatic injury (37). Meanwhile, hepatic inflammatory cells can generate hyper-oxide and triggers oxidative stress, which produces massive ROS. And ROS can cause damage of nucleic acid, protein and lipid significantly. And it can also result in inflammatory reaction by regulating and controlling COX-2 and induce hepatic injury. Moreover, ROS can increase NF-κB's activity, which further trigger the expression of COX-2 and aggravates hepatic injury. COX-2 inhibitor may play a role in preventing hepatic injury and reducing inflammation (38). Distributed mainly in hepatic cells and kupffer cells, iNOS is not expressed normally. While under pathological state of hepatitis and hepatic injury, iNOS's activity increases significantly and its expression enhances (39). In this state, it continually produces massive NO, which can trigger the generation of a series of inflammatory factors (cytokines) and thus increases inflammatory reaction. Besides this, as a kind of mutagen, NO can itself cause mutation and disruption to DNA, accompanying massive NO releasing (40). The mRNA and protein expressions of NF-κB-p65, iNOS, COX-2 in liver of ITE treated liver injured mice were stronger than controlled mice, these changes means that ITE is a good material for liver.

In this case study, model of the CCl_4-induced hepatic injured mice was used for prophylactic effects of ITE determination. After two concentrations of ITE treatment, the serum and liver tissue had many changes compared to the hepatic damage mice (controlled mice), these changes were determined by the Kits and Molecular biology tests. The high concentration of ITE showed strong inhibitory effects on hepatic damage by decreasing of hepatic injury indicator, AST, ALT, ALP, TG, BUN and increasing of TC, ALB. The low concentration of ITE also had the preventive effects, but weaker than high concentration. ITE also reduced the liver injury cells by the pathological observation of H&E secretions. These serum and tissue changes were related with the antioxidant mechanisms, ITE treated mice had the higher SOD, CAT, GSH-Px serum levels than controlled mice, but it had lower NO, MDA levels than controlled mice. Further results show that ITE decreased the inflammatory related mRNA and protein expressions of NF-κB-p65, iNOS, COX-2, and increased the IκB-α expressions.

These inflammatory related expressions were also changed through anti-oxidation of ITE on Mn SOD, Gu/Zn SOD, CAT and GSH-Px (GSH) raise. These results indicated that Insect tea extract has the strong preventive effects on CCl_4-induced hepatic damage *in vivo*.

Acknowledgments Y.J. Liao and X.C. Zou contributed equally to this work. This work is supported by the Scientific and Technological Research Program of Chongqing Municipal Education Commission (Grant No. KJ1401402) and the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (Jiaowaisiliu(2014)1685), China.

Disclosure The authors declare no conflict of interest.

References

1. Qian Y, Li GJ, Wang R, Zhou YL, Sun P, Zhao X. *In vitro* anticancer effects of insect tea in TCA8113 cells. J. Canc. Res. Ther. 10: 1045-1051 (2014)
2. Zhao X, Wang R, Qian Y, Li G, Zhou Y, Sun P. *In vivo* preventive effects of insect tea on buccal mucosa cancer in ICR mice. J. Canc. Res. Ther. 10: 651-657 (2014)
3. Zhou YL, Wang R, Feng X, Zhao X. Preventive effect of insect tea against reserpine-induced gastric ulcers in mice. Exp. Ther. Med. 8: 1318-1324 (2014)
4. Cheng X, Xie CL, Wang J. Changes of serum nitric oxide level after acute hepatic injury in mice and the effect of yugan capsule on it. Chin. J. Integr. Tradit. West Med. 26: 58-60 (2006)
5. Sharifi R, Pasalar P, Kamalinejad M, Dehpour AR, Tavangar SM, Paknejad M, Mehrabani Natanzi M, Nourbakhsh M, Ahmadi Ashtiani HR, Akbari M, Rastegar H. The effect of silymarin (*Silybum marianum*) on human skin fibroblasts in an *in vitro* wound healing model. Pharm. Biol. 51: 298-303 (2013)
6. Gound SS, Thakare VN, Khan S, Wadekar RR, Naik SR. Ameliorative effects of Tricholepis glaberrima in experimentally induced hepatic damage in rats: Modulation of cytokines functions. J. Ethnopharmacol. 160: 164-172 (2015)
7. Zhao X, Wang Q. Antioxidant, antimutagenic and *in vitro* anticancer effects of crude polyphenols in Insect tea made from Kuding tea leaves. Mod. Food Sci. Technol. 31: 24-28 (2015)
8. Zhou YL, Feng X, Zhu K, Zhao X. Apoptosis inducing effects of crude polyphenols of Insect tea made by Kuding tea leavses in HepG2 human hepatoma cells. Sci. Technol. Food Ind. 9: 339-342 (2015)
9. Fu S, Wang F, Cao Y, Huang Q, Xiao J, Yang C, Popescu LM. Telocytes in human liver fibrosis. J. Cell Mol. Med. 19: 676-683 (2015)
10. Xu GM, Peng XJ, Wang CJ, Wen LZ, Liu WL, Jiang XM. Extraction of polyphenols in aglossa dimidiata with ions Al^{3+} and Zn^{2+} sedimentation. J. TCM Univ. Hunan 2: 46-46 (2007)
11. Zhao X, Kim SY, Park KY. Bamboo salt has *in vitro* anticancer activity in HCT-116 cells and exerts anti-metastatic effects *in vivo*. J. Med. Food 16: 9-19 (2013)
12. Zhao X, Song JL, Kil JH, Park KY. Bamboo salt attenuates CCl_4-induced hepatic damage in Sprague-Dawley rats. Nutr. Res. Pract. 7: 273-280 (2013)
13. Guo SY, Xu WX, Wen LZ, Huang YM, Wang F. The nutrient analysis and evaluation of Sanye insect-fermented tea. Chinese Bull. Entimol. 45: 128-132 (2008)
14. Wang R, Sun P, Zhao X. Protective effects of crude polysaccharide in Insect tea against carbon tetrachloride-induced hepatic damage in mice. Mod. Food Sci. Technol. 31:6-11 (2015)
15. Lu B, Xu Y, Xu L, Cong X, Yin L, Li H, Peng J. Mechanism investigation of dioscin against CCl_4-induced acute liver damage in mice. Environ. Toxicol. Phar. 34: 127-135 (2012)
16. Xin X, Yang W, Yasen M, Zhao H, Aisa H. The mechanism of hepatoprotective effect of sesquiterpene rich fraction from *Cichorum glandulosum* Boiss. et Huet on immune reaction-induced liver injury in mice. J. Ethnopharmacol. 155: 1068-1075 (2014)
17. Wang T, Zhou ZX, Sun LX, Li X, Xu ZM, Chen M, Zhao GL, Jiang ZZ, Zhang LY. Resveratrol effectively attenuates α-naphthyl-isothiocyanate-induced acute cholestasis and liver injury through choleretic and anti-inflammatory mechanisms. Acta Pharmacol. Sin. 35: 1527-1536 (2014)
18. Wang Z, Su B, Fan S, Fei H, Zhao W. Protective effect of oligomeric proanthocyanidins against alcohol-induced liver steatosis and injury in mice. Biochem. Bioph. Res. Co. 458: 757-762 (2015)
19. Patankar JV, Chandak PG, Obrowsky S, Pfeifer T, Diwoky C, Uellen A, Sattler W, Stollberger R, Hoefler G, Heinemann A, Battle M, Duncan S, Kratky D, Levak-Frank S. Loss of intestinal GATA4 prevents diet-induced obesity and promotes insulin sensitivity in mice. Am. J. Physiol. Endocrinol. Metab. 300: 478-488 (2011)
20. Takahashi H, Shigefuku R, Yoshida Y, Ikeda H, Matsunaga K, Matsumoto N, Okuse C, Sase S, Itoh F, Suzuki M. Correlation between hepatic blood flow and liver function in alcoholic liver cirrhosis. World J. Gastroenterol. 20: 17065-17074 (2014)
21. Araki H, Ueda H, Fujimoto S. Immunocytochemical localization of alpha-fetoprotein in the developing and carbon tetrachloride-treated rat liver. Acta Anat. 143: 169-177 (1992)
22. Dabiri H, Azimi Rad M, Tavafzadeh R, Taheri E, Safakar S, Nazemalhosseini Mojarad E, Farzaneh N, Zali MR. Bacteriologic study of cirrhotic patients with non-neutrocytic ascites. Gastroenterol. Hepatol. Bed Bench. 7: 224-229 (2014)
23. Lee KJ, Jeong HG. Protective effect of Platycodi radix on carbon tetrachloride-induced hepatotoxicity. Food Chem. Toxicol. 40: 517-525 (2002)
24. Ioannidis I, de Groot H. Cytotoxicity of nitric oxide in Fu5 rat hepatoma cells: Evidence for co-operative action with hydrogen peroxide. Biochem. J. 296: 341-345 (1993)
25. Thakore KN, Mehendale HM. Role of hepatocellular regeneration in CCl_4 autoprotection. Toxicol. Pathol. 19: 47-58 (1991)
26. Krithika R, Verma RJ, Shrivastav PS, Suguna L. Phyllanthin of standardized phyllanthus amarus extract attenuates liver oxidative stress in mice and exerts cytoprotective activity on human hepatoma cell line. J. Clin. Exp. Hepatol. 1: 57-67 (2011)
27. Remmer H, Hintze T, Frańk H, Müh-Zange M. Cytochrome P-450 oxidation of alkanes originating as scission products during lipid peroxidation. Xenobiotica 14: 207-219 (1984)
28. Wang B, Bai M, Bai Y, Li Q. Liver injury following renal ischemia reperfusion in rats. Transplant. P. 42: 3422-3426 (2010)
29. Li W, Qu XN, Han Y, Zheng SW, Wang J, Wang YP. Ameliorative effects of 5-hydroxymethyl-2-furfural (5-HMF) from Schisandra chinensis on alcoholic liver oxidative injury in mice. Int. J. Mol. Sci. 16: 2446-2457 (2015)
30. Semenov DE, Zhukova NA, Ivanova EP, Sorokina IV, Baiev DS, Nepomnyashchikh GI, Tolstikova TG, Biryukova MS. Hepatoprotective properties of betulonic acid amide and heptral in toxic liver injury induced by carbon tetrachloride in combination with ethanol. B. Exp. Biol. Med. 158: 336-341 (2015)
31. Curry-McCoy TV, Osna NA, Nanji AA, Donohue TM Jr. Chronic ethanol consumption results in atypical liver injury in copper/zinc superoxide dismutase deficient mice. Alcohol. Clin. Exp. Res. 34: 251-261 (2010)
32. Pan H, Chen J, Shen K, Wang X, Wang P, Fu G, Meng H, Wang Y, Jin B. Mitochondrial modulation by epigallocatechin 3-gallate ameliorates cisplatin induced renal injury through decreasing oxidative/nitrative stress, inflammation and NF-κB in mice. PLoS ONE 10: e0124775 (2015)
33. Kim HR, Shin DY, Chung KH. The role of NF-κB signaling pathway in polyhexamethylene guanidine phosphate induced inflammatory response in mouse macrophage RAW264.7 cells. Toxicol. Lett. 233: 148-155 (2015)
34. Ma XY, Wang H, Ding B, Zhong H, Ghosh S, Lengyel P. The interferon-inducible p202a protein modulates NF-kappaB activity by inhibiting the binding to DNA of p50/p65 heterodimers and p65 homodimers while enhancing the binding of p50 homodimers. J. Biol. Chem. 278: 23008-23019 (2003)
35. Murley JS, Kataoka Y, Hallahan DE, Roberts JC, Grdina DJ. Activation of NFκB and *MnSOD* gene expression by free radical scavengers in human microvascular endothelial cells. Free Radical Bio. Med. 30: 1426-1439 (2001)
36. Kaur J, Shalini S, Bansal MP. Influence of vitamin E on alcohol-induced changes in antioxidant defenses in mice liver. Toxicol. Mech. Method. 20: 82-89 (2010)
37. Xu D, Hu L, Su C, Xia X, Zhang P, Fu J, Wang W, Xu D, Du H, Hu Q, Song E, Song Y. Tetrachloro-p-benzoquinone induces hepatic oxidative damage and inflammatory response, but not apoptosis in mouse: The prevention of curcumin. Toxicol. Appl. Pharm. 280: 305-313 (2014)
38. Das P, De T, Chakraborti T. Leishmania donovani secretory serine protease alters macrophage inflammatory response via COX-2 mediated PGE-2 production. Indian J. Biochem. Biophys. 51: 542-551 (2014)
39. Lin X, Liu X, Huang Q, Zhang S, Zheng L, Wei L, He M, Jiao Y, Huang J, Fu S, Chen Z, Li Y, Zhuo L, Huang R. Hepatoprotective effects of the polysaccharide isolated from *Tarphochlamys affinis* (Acanthaceae) against CCl_4-induced hepatic injury. Biol. Pharm. Bull. 35: 1574-1580 (2012)
40. Chen TH, Liao FT, Yang YC, Wang JJ. Inhibition of inducible nitric oxide synthesis ameliorates liver ischemia and reperfusion injury induced transient increase in arterial stiffness. Transplant. P. 46: 1112-1116 (2014)