

Antioxidant characteristics and hepatoprotective effects of a formula derived from *Maydis stigma*, *Nelumbo nucifera* and *Taraxacum officinale* against carbon tetrachloride-induced hepatic damage in rats

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Abstract: The hepatoprotective effects of a water extract formula (WEF) derived from three selected TCM herbs (i.e. Corn silk (*Maydis stigma*), lotus leaf (*Nelumbo nucifera* Gaertn) and dandelion (*Taraxacum officinale*)) were appraised by the antioxidant activities and by the decay of carbon tetrachloride (CCl₄)-induced rats. The results indicated that the WEF had higher contents of total phenolic and flavonoids, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability, ferric reducing antioxidant potential (FRAP) and equivalent antioxidant capacity (TEAC). The animal experiments revealed that the WEF administration could lower malondialdehyde (MDA) level, serum alanine aminotransferase (ALT), aspartate amino transferase (AST) activities, resume triglyceride (TG) and decreased glutathione (GSH) levels, and reform or resume super oxide dismutase (SOD) content as well as improve peroxidase (GPx), glutathione reductase (GRd) and catalase (CAT) activities in CCl₄-induced rats. The histological inspections of liver demonstrated that CCl₄ enlarged the extents of bile duct proliferation, necrosis, fibrosis, steatosis and fatty vacuolation all round liver, but the first three can be ameliorated by WEF. Based on these evidences from this study, the WEF by regulating the related antioxidant enzymes of liver to exhibit hepatoprotective effects was confirmed and it can be use as a substitute for the silymarin.

Keywords: Hepatoprotective effect, *Maydis stigma*, *Nelumbo nucifera* Gaertn, *Taraxacum officinale*, carbon tetrachloride.

INTRODUCTION

It was well-known that perennial exposure under alcohol, some drugs, toxic chemicals or viral infection could result in chronic liver diseases. The characteristics of liver diseases are evolutionary progress and may finally cause hepatocellular cancer consequence (Loguercio and Fredico, 2003), thus liver diseases are relevant to critical morbidity and high mortality rate.

Tetrachloromethane, as known as carbon tetrachloride (CCl₄), is one of highly toxic chemical for animals a highly toxic chemical for animals and could come into human body by skin contact, digestive and respiratory (Jiang *et al.*, 1992). The CCl₄ can form some highly reactive free radicals, i.e. trichloromethyl free radical and trichloromethylperoxy free radical, bind with cellular molecules including carbohydrates, lipids, proteins and nucleic acids (Weber *et al.*, 2003) and urge lipid peroxidation, injure organelles and liver cells, result in the hepatocytes swelling and necrosis of and further cause the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) release into the bloodstream (Singh *et al.*, 1998). Fatty liver, cirrhosis and necrosis are the most outstanding pathological features in CCl₄-

induced hepatotoxicity (Recknagel *et al.*, 1989). Experimentally, researchers diffusely employed CCl₄ in animal testing to survey chemically induced hepatic injury since the induction of cirrhotic response in animals by CCl₄ are very similar to those of human liver cirrhosis (Rudnicki *et al.*, 2007).

Because both the reactive oxygen species and free radicals play a critical role, thus it is suggested that supplement or uptake antioxidants from dietary as an effective strategy to avoid oxidative stress-related liver disorders (Loguercio and Fredico, 2003). Antioxidant demonstrates not only common hydrogen-donator, but, more significantly, exhibit adjusting consequence in cells through multiple actions of repairing enzymes, drug-metabolizing and antioxidant (Chang *et al.*, 2002). Many reports (Weber *et al.*, 2003; Yang *et al.*, 2013) revealed that complex mixtures may possess various natural antioxidants and are further effective than those of pure chemical substances for remitting oxidative stress-related disorders as the results of specific interplays and collaboration.

In traditional Chinese medicine (TCM), many herbs have constantly been used to remedy hepatic defect. For example, *Maydis stigma* (Gramineae) used to cure liver and kidney diseases (Hasanudin *et al.*, 2012) and most of

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its activity was concentrated in phenolic acids and flavonoids (Maksimovic and Kovacevic, 2003). *Nelumbo nucifera* Gaertn (Nymphaeaceae) a medicinal herb for blood clotting, dysentery and dizziness (Liu *et al.*, 2015) and its leaf extracts possess powerful antioxidant effects (Huang *et al.*, 2013). *Taraxacum officinale* (Asteraceae) has traditionally been used for the therapy of liver illnesses in Asia (Gulfraz *et al.*, 2014) and as an important commercial source of natural drugs (Martinez *et al.*, 2015) as well as its polysaccharides seem as an antioxidant agents (Park *et al.*, 2014). Silymarin, a well known medicine which reduces hepatic wound caused by CCl₄ was also reported (Abenavoli *et al.*, 2010), which display as antioxidant, antiinflammatory, anticarcinogenic and growth adjusting effects (Flora *et al.*, 1998).

Above-mentioned pharmacological effects of *M. stigma*, *N. nucifera* Gaertn and *T. officinale* have been reported; however, lack of scientific proof regarding the effect of these herbal combination products until now. Therefore, the objectives of the research were to determine antioxidant characteristics and hepatoprotective capabilities of the water extract formula (WEF) originated from the combination of these three herbs against CCl₄-induced hepatic injury in rat.

MATERIALS AND METHODS

Preparations of herb materials and WEF

M. stigma, *N. nucifera* Gaertn and *T. officinale* were purchased from a TCM herb store in Taipei. The voucher specimens were deposited in the herbarium of Department of Food Science, National Pingtung University of Science and Technology (NPUST) (Pingtung, Taiwan). Each of these herbs was boiled in twenty-fold volume of deionized water for 2h, heated at 70°C for 2.5h continually, and then centrifuged at 4,000×g for 10min to obtain the suspension. After filtration under vacuum, the suspension was evaporated at 37°C and then lyophilized, fine-ground and stored at 4°C for further employ. Mixed three herb extracts and in the ratio of 1:1:1 to obtain the water extract formula (WEF). The yield of *M. stigma*, *N. nucifera* Gaertn, *T. officinale* and WEF were 22.2, 10.2, 10.3 and 15.1%, respectively.

Analyses of antioxidant characteristics of three herbs and WEF

The assessments of antioxidant characteristics of three herbs and the WEF were assessed according to determining the contents of total phenolic and flavonoid, scavenging activities of superoxide anion radical and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, as well as analyses of trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant potential (FRAP). Total contents of phenolic and flavonoid were separately appraised according to the methods of Kujala *et al.* (2000) and Chang *et al.* (2002) and their determination results

were presented as mg of gallic acid equivalent and as mg of quercetin per gram of dry herb weight for phenolic and flavonoid contents, respectively. DPPH assay was executed using the method of Hsu *et al.* (Hsu *et al.*, 2007) and the DPPH radical scavenging activity (%) was counted as (1-A Sample/A Control) × 100. FRAP assay was demonstrated according to the introduction of Benzie and Strain (Benzie and Strain, 1996) with some modification and showed as mg of ascorbic acid equivalent per gram of dry herb weight. TEAC assay was done according to the scavenging of the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical as Re *et al.* reported (Re *et al.*, 1999) and presented as trolox equivalent per gram of dry herb weight.

Animal experimentation

Sprague Dawley rats (fifty, 6-weeks-old, male, 200-250g of body weight, BW) were purchased from Bio LASCO Co., Ltd. (Taipei, Taiwan) and housed in makrolon cages with stainless steel grid covers and sterilized wood shavings as bedding material and a controlled environment (temperature 25±2°C, relative humidity 60±5% and artificial lighting was sequenced at 12-h light/dark cycles) was adopted. Rats were fed with standard rat chow (Laboratory Rodent Diet 5001, Lab Diet, USA) and ad lib tap water. The rats were subjected to further hepatotoxicity testing after assimilating in laboratory conditions for 7 days. Fifty subject rats were randomly divided into 5 groups of 10 in each group as follows: normal control group (NCG), negative control group (i.e. CCl₄ group), positive control group (i.e. silymarin group), low dose and high dose of WEF groups (L- and H-WEF group). Semiweekly, the NCG accepted olive oil (0.1ml/100g BW) while other groups treated with CCl₄ dissolved in olive oil (20% v/v, 0.1ml/kg BW). The normal control and CCl₄ groups were given with distilled water, and the silymarin group was fed with silymarin (200mg/kg BW) and the L- and H-WEF groups were accepted with WEF (0.15 and 0.75g/kg BW, respectively) four times a week. Under ether anesthesia, the tested rats were sacrificed at the end of experiment (8 weeks). The liver and blood samples were gathered for further biochemical analyses and histological examinations. All animal experimental procedures were reviewed and agreed by the Institutional Animal Care and Use Committee (IACUC) of NPUST.

Biochemical analyses

Collected blood samples from rats' tail vein and allowed to clump at room temperature for 1h and then centrifuged (4,000×g at 4°C for 10 min) to gain the serum. The amounts of total cholesterol (TC), triglyceride (TG), AST, and ALT were determined using assay kits (Formosa Biomedical Technology Corp., Taipei, Taiwan).

Table 1: The total phenolic and flavonoids content as well as IC₅₀ value in DPPH radical scavenging activity, TEAC and FRAP of *M. stigma*, *N. nucifera* Gaertn, *T. officinale* and WEF

Herb	Total phenolic (mg/g)	Flavonoids (mg/g)	DPPH radical scavenging effect, IC ₅₀ (mg/mL)	TEAC (mM/mg)	FRAP (mM/mg)
<i>M. stigma</i>	150.7 ± 1.5 ^b	41.6 ± 1.7 ^c	0.39 ± 0.03 ^a	394.2 ± 17.3 ^c	295.5 ± 39.1 ^c
<i>N. nucifera</i> Gaertn	195.4 ± 4.0 ^a	194.1 ± 1.2 ^a	0.21 ± 0.01 ^b	553.7 ± 24.7 ^a	603.4 ± 50.7 ^a
<i>T. officinale</i>	57.6 ± 2.7 ^d	34.4 ± 0.6 ^d	0.41 ± 0.02 ^a	276.7 ± 45.8 ^d	536.1 ± 49.0 ^{ab}
WEF	134.6 ± 2.7 ^c	90.0 ± 1.2 ^b	0.39 ± 0.02 ^a	454.2 ± 15.7 ^b	485.1 ± 50.9 ^b

^{abcd} Numbers are significantly different from one another ($P < 0.05$). Data are expressed as mean ± S.D. (n = 3). WEF: water extract of formula.

Table 2: The effect of WEF on the relative weights of liver and kidney of rats treated with CCl₄

Group	Relative organ weight (g/100 g BW)	
	Liver	Kidney
A	2.68 ± 0.40 ^b	0.31 ± 0.06 ^b
B	4.92 ± 0.68 ^a	0.38 ± 0.06 ^a
C	2.91 ± 0.42 ^b	0.30 ± 0.03 ^b
D	3.34 ± 0.57 ^b	0.33 ± 0.06 ^b
E	2.94 ± 0.46 ^b	0.28 ± 0.03 ^b

^{ab} Numbers are significantly different from one another ($P < 0.05$). Data are expressed as mean ± S.D. (n = 8). A: normal control. B: negative control (20% CCl₄). C: positive control (20% CCl₄ + 0.2 mg/g BW silymarin). D: low dose (20% CCl₄ + 0.15 mg/g BW WEF). E: high dose (20% CCl₄ + 0.75 mg/g BW WEF). WEF: water extract formula.

Table 3: The effect of WEF on the serum ALT, AST, TG and TC contents rats treated with CCl₄

Group	ALT (U/L)	AST (U/L)	TG (mg/dL)	TC (mg/dL)
A	35.64 ± 4.81 ^d	106.34 ± 27.19 ^c	107.04 ± 28.79 ^{cd}	105.33 ± 19.57 ^a
B	234.07 ± 38.39 ^a	261.88 ± 32.41 ^a	219.74 ± 16.72 ^a	111.63 ± 16.74 ^a
C	86.33 ± 36.41 ^c	214.62 ± 45.79 ^b	161.53 ± 19.94 ^b	121.70 ± 43.23 ^a
D	113.43 ± 24.88 ^{bc}	223.25 ± 37.07 ^{ab}	137.76 ± 39.41 ^{bc}	113.72 ± 19.32 ^a
E	146.29 ± 29.50 ^b	205.26 ± 41.58 ^b	95.07 ± 26.85 ^d	109.74 ± 23.96 ^a

^{abcd} Numbers are significantly different from one another ($P < 0.05$). Data are expressed as mean ± S.D. (n = 8). The groups A to E are the same as footnotes in table 2.

Table 4: The effect of WEF on the liver GSH, GPx, GRd, SOD and CAT levels and MDA content of rats treated with CCl₄

Group	GSH (μmol/mg protein)	GPx (U/mg protein)	GRd (U/mg protein)	SOD (U/mg protein)	CAT (K/mg protein)	MDA (nmol/mg protein)
A	42.08 ± 1.52 ^a	795.87 ± 90.80 ^c	234.42 ± 26.74 ^c	91.39 ± 37.84 ^{bc}	274.90 ± 68.53 ^a	0.26 ± 0.07 ^{bc}
B	38.36 ± 4.52 ^b	390.56 ± 40.35 ^d	203.09 ± 20.98 ^c	67.64 ± 19.33 ^c	98.64 ± 35.60 ^c	0.43 ± 0.14 ^a
C	41.10 ± 4.07 ^{ab}	823.77 ± 150.10 ^c	344.00 ± 62.68 ^b	146.30 ± 28.11 ^a	231.94 ± 60.98 ^{ab}	0.35 ± 0.10 ^{ab}
D	42.40 ± 2.45 ^a	1072.93 ± 182.23 ^b	478.53 ± 81.28 ^a	101.01 ± 26.77 ^b	192.92 ± 70.19 ^b	0.25 ± 0.03 ^c
E	42.60 ± 2.39 ^a	1194.42 ± 123.04 ^a	367.57 ± 37.86 ^b	96.14 ± 37.28 ^{bc}	204.37 ± 76.54 ^b	0.20 ± 0.06 ^c

^{abcd} Numbers are significantly different from one another ($P < 0.05$). Data are expressed as mean ± S.D. (n = 8). The groups A to E are the same as footnotes in table 2.

Table 5: The effect of WEF on the histology injury of rats treated with CCl₄

Group	Bile duct proliferation	Steatosis	Portal inflammation	Lobular inflammation	Necrosis	Fibrosis
A	0 ^c	0 ^c	0 ^c	0 ^c	0 ^c	0 ^c
B	2 ^a	2 ^a	1 ^b	1 ^b	2 ^a	2 ^a
C	2 ^a	1 ^b	1 ^b	1 ^b	1 ^b	1 ^b
D	1 ^b	1 ^b	1 ^b	1 ^b	1 ^b	1 ^b
E	1 ^b	2 ^a	1 ^b	1 ^b	1 ^b	1 ^b

^{abc} Numbers are significantly different from one another ($P < 0.05$). Data are expressed as mean ± S.D. (n = 8). Livers were scored for hepatic injury via light microscopy with score 0 = no visible cell damage; score 1 = focal hepatocyte damage on less than 25% of the tissue; score 2 = focal hepatocyte damage on 25-50% of the tissue; score 3 = extensive, but focal, hepatocyte lesions; score 4 = global hepatocyte necrosis. The groups A to E are the same as footnotes in table 2.

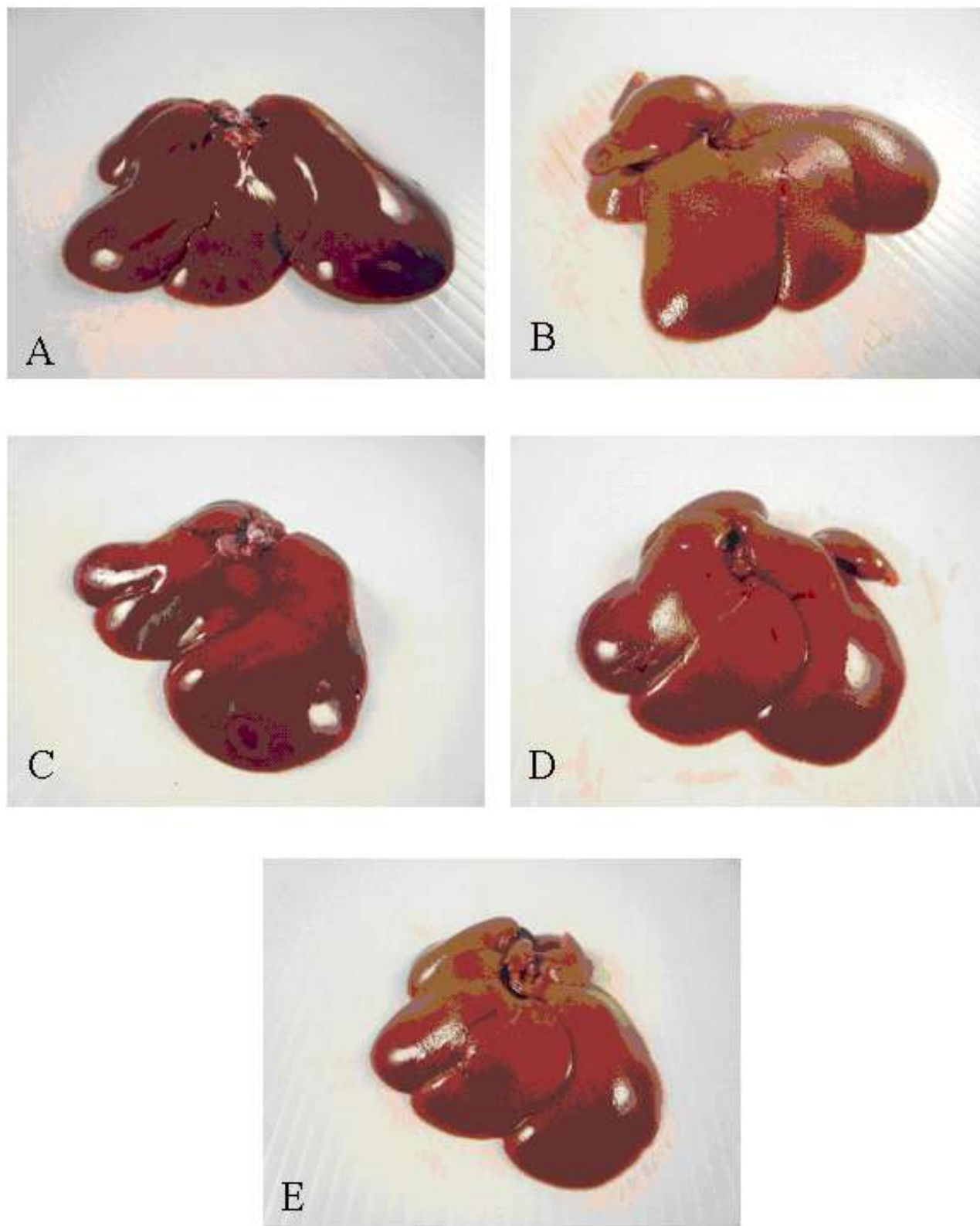


Fig. 1: The effect of WEF on the liver appearance of rats treated with CCl_4 . The groups A to E are the same as footnotes in table 2.

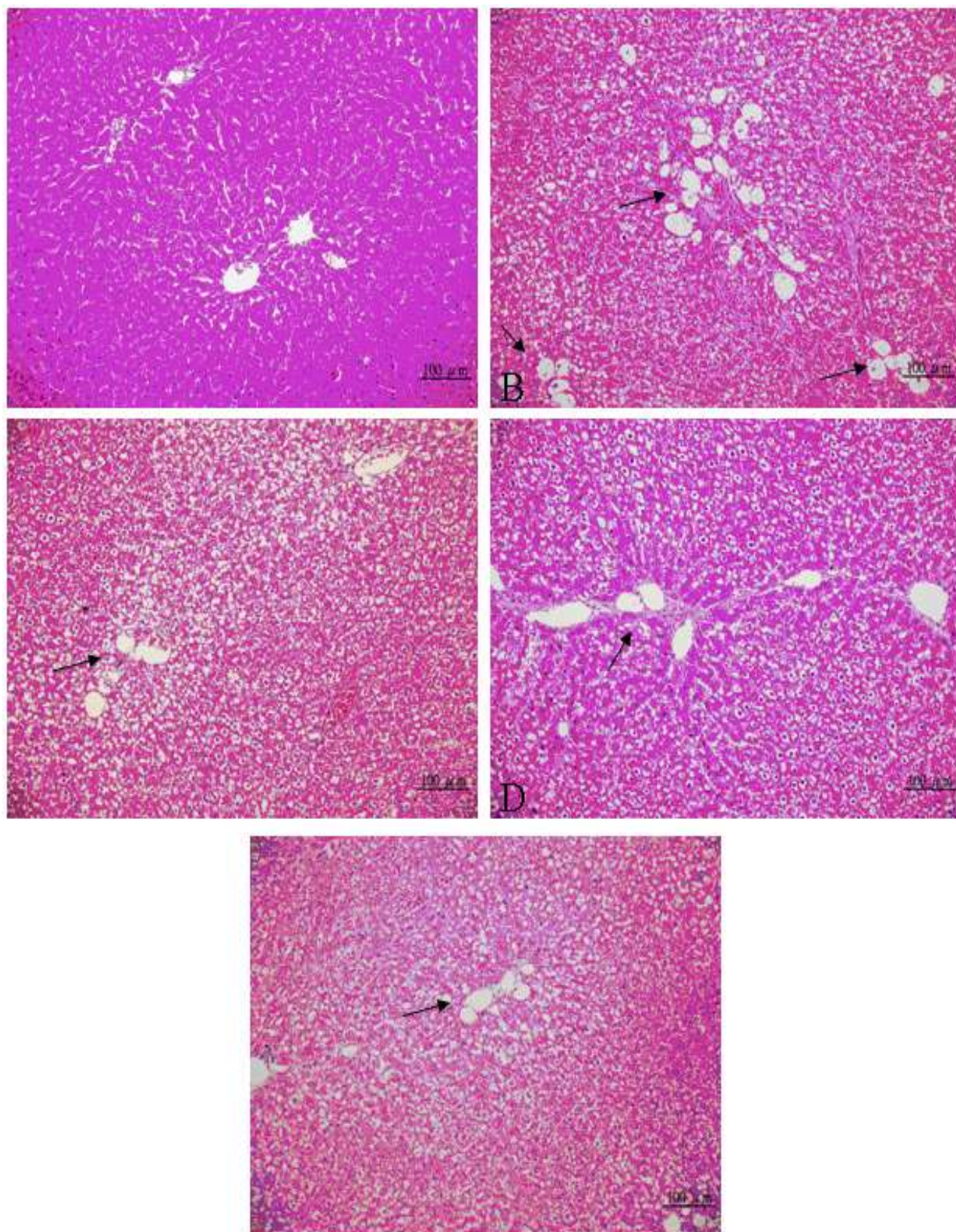


Fig. 2: The effect of WEF on the liver tissue sections (by H&E staining) of rats treated with CCl_4 . Arrow: steatosis. The groups A to E are the same as footnotes in table 2.

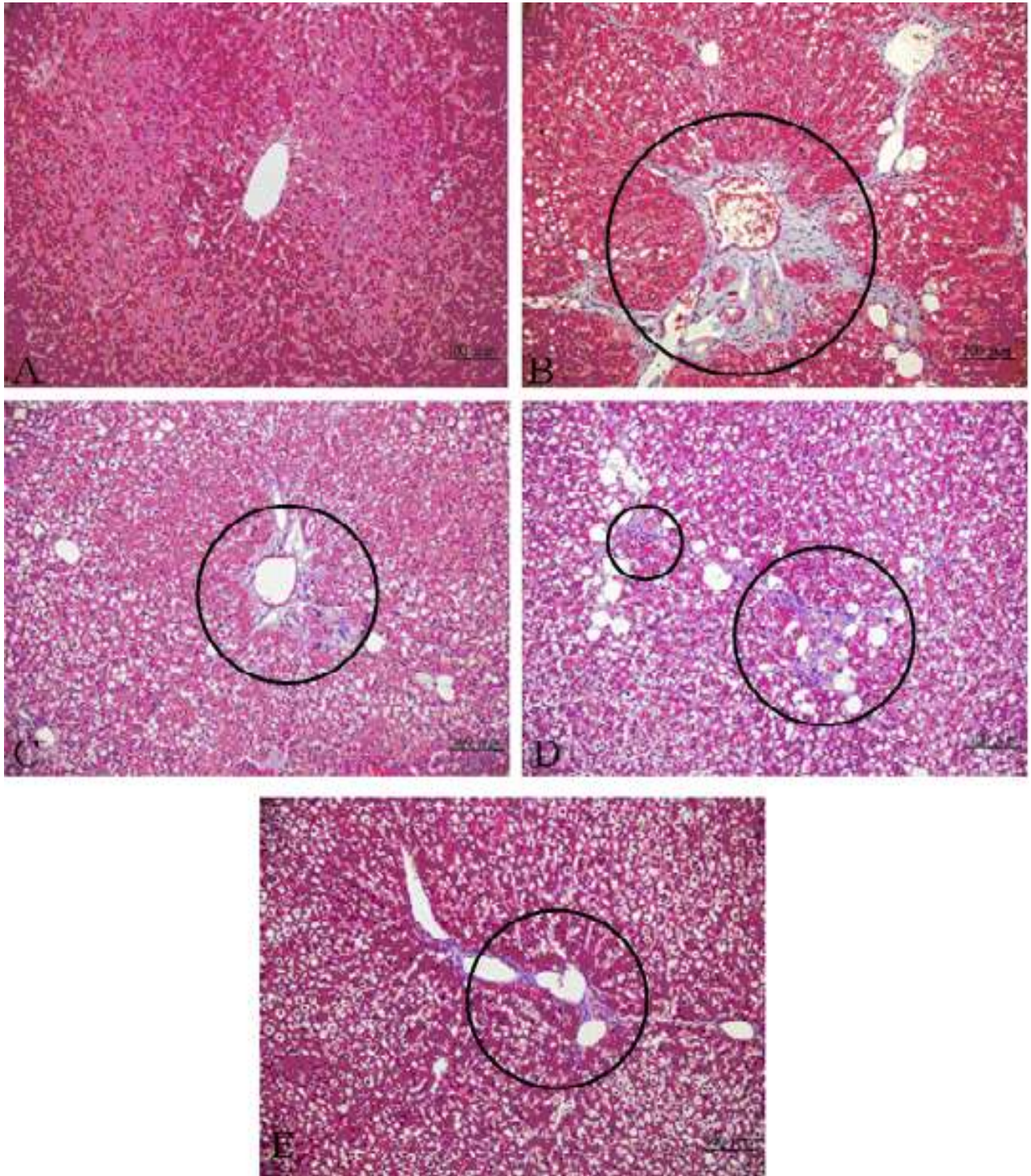


Fig. 3: The effect of WEF on the liver tissue sections (by Masson's trichrome staining) of rats treated with CCl_4 . Circle: fibrosis. The groups A to E are the same as footnotes in table 2.

Antioxidant characteristic analyses of the hepatic cells

The content of glutathione (γ -glutamyl cysteinyl glycine, GSH) was determined by a commercial kit obtained from Calbiochem Co. (Merck, Germany). The activities of glutathione peroxidase (GPx) and glutathione reductase (GRd) were analyzed separately according to Lawrence

and Burk (Lawrence and Burk., 1976) and Bellomo *et al.* (Bellomo *et al.*, 1987) and their determination results were presented as U/mg protein, respectively. Protein concentration of liver homogenates was determined according to Bradford (Bradford, 1976). Super oxide dismutase (SOD) and catalase (CAT) assays were

executed separately according to Nandi and Chatterjee (Nandi and Chatterjee, 1988) and Aebi (Aebi, 1984) and one unit of enzyme activity was presented as 50% inhibition of nitroblue tetrazolium reduction per mg of protein for SOD and decomposition 1.0 μ mole of H_2O_2 /min mg of protein for CAT, respectively. Thiobarbituric acid reactive substances (TBARS) was estimated according to Ohkawa *et al.* (Ohkawa *et al.*, 1979) to assess the lipid peroxidation and the TBARS amount and the results were showed as μ mol malondialdehyde (MDA) mg/protein.

Histopathological assessment of hepatic damage

A volume of 1cm³ liver tissue was adopted from right lobe and following formalin-fixed (10%) for 1 week and then paraffin-embedded. The liver tissue was desiccate by a series of xylene and ethanol (100, 90 and 70%, consecutively) and sliced to a thickness of 5 μ m and dyeing by hematoxylin and eosin (H&E) stain and then the bile duct steatosis, proliferation, necrosis and lobular inflammation were observed under the microscope. It is commonly employed Masson's trichrome (MT) stain technology to observe liver fibrosis. Five grades from 0 to 4 (i.e. absent, trace (less than 25%), weak (between 25-50%), moderate (between 50-75%) and strong (more than 75%), respectively) were employed to assess the bile duct steatosis, proliferation, necrosis and lobular inflammation according to Jonker *et al.* (Jonker *et al.*, 1992). Also, five grades from 0 to 4 were used to evaluate hepatic fibrosis based on the methods of Ruwart *et al.* (Ruwart *et al.*, 1989) and Gabriele (Gabriele, 1997) as follows: Grade 0 means absent (normal liver), grade 1 means few (formation of collagen hyperplasia without septa (radial fibrous hyperplasia in the central vein or portal region), grade 2 means mild (formation of incomplete septa between central vein and portal area but without rendezvous among these septum), grade 3 means moderate (formation of thin and complete septum and with rendezvous and divide liver parenchyma into many segments) and grade 4 means strong (formation of thick and complete septum, i.e. complete cirrhosis), respectively.

STATISTICAL ANALYSIS

The experiments were replicated at least three times and all the experimental data were analyzed by one-way analysis of variance (ANOVA) using SAS (Cary, NC) software (version 9.2) as well as the Duncan's New Multiple Range test ($P < 0.05$) was conducted to compare the treatment means.

RESULTS

Antioxidant characteristics

The antioxidant characteristics of three herbs and WEF are shown in table 1. The greatest content of the phenolic was observed in *N. nucifera* Gaertn (i.e. 195.4mg/g),

followed by *M. stigma* (i.e. 150.7mg/g), WEF (i.e. 134.6 mg/g) and *T. officinale* (i.e. 57.6mg/g). The greatest flavonoids content was observed in *N. nucifera* Gaertn (i.e. 194.1mg/g), followed by WEF (i.e. 90.0mg/g), *M. stigma* (i.e. 41.6mg/g) and *T. officinale* (i.e. 34.4 mg/g). For the DPPH scavenging activity, the *N. nucifera* Gaertn exhibited the highest IC₅₀ (i.e. 0.21mg/mL), means its DPPH radical scavenging activity was stronger than three others which showed same scavenging activities. The levels sequence of TEAC same order as the flavonoids content for *N. nucifera* Gaertn, WEF and *M. stigma* order and the superiority of WEF showed a second highest TEAC (i.e. 454.2mM/mg) thus it was lower than *N. nucifera* Gaertn (i.e. 553.7mM/mg). In terms of FRAP, the *N. nucifera* Gaertn and *T. officinale* showed the higher potential (i.e. 603.4 and 536.1mM/mg, respectively), which both had the equivalent level, followed by WEF and *M. stigma* (i.e. 485.1 and 295.5mM/mg, respectively), and the FRAP of *M. stigma* was significantly lower than three others.

Weights of body, relative liver and kidney

No significant difference of body weights of rats were observed among different groups 8 weeks later. The rat body weights were from 200-250g in initial to 400-450g in final. The changes of rat relative liver and kidney weight following 8 weeks of different treatments are shown in table 2. A noticeable relative liver and kidney weight increasing was recorded in CCl₄ group; however, no difference among other four groups.

Biochemical analysis of blood serum

The ALT and AST activities and TG and TC contents in rats' serum are outlined in table 3. As seen in the table, the CCl₄ group remarkably stimulated the levels of ALT (i.e. 234.07U/L) and AST (i.e. 261.88U/L) contrasted with those of NCG (i.e. 35.64 and 106.34U/L, respectively). Feeding with L- and H-WEF well reduced the levels of ALT (i.e. 113.43-146.29U/L) and AST (i.e. 223.24-205.26U/L). The silymarin group exhibited a remarkably inferior ALT level (i.e. 86.33U/L) than those of the L- and H-WEF groups; however, for AST level no significant difference among silymarin (i.e. 214.62U/L) and L- and H-WEF groups. For TG, the CCl₄ group displayed a significantly higher TG level (i.e. 219.74 mg/dL) compared with silymarin (i.e., 161.53mg/dL), normal control (i.e. 107.04mg/dL), and L- and H-WEF (i.e. 137.76 and 95.07mg/dL, respectively) treatments which both were able to restore the TG to normal level. No significant difference of the TC level among the different groups.

Antioxidant characteristic of hepatic cells

Antioxidant characteristics of the hepatic cells 8 weeks following different treatments are shown in table 4. The CCl₄ group represented a remarkably lower GSH concentration (i.e. 38.36 μ mol/mg protein) compared with

NCG (i.e. 42.08 μ mol/mg protein), while silymarin group, and L- and H-WEF groups displayed a similar GSH concentration (i.e. 41.10, 42.40 and 42.60 μ mol/mg protein, respectively) to those of NCG. Also, the CCl₄ group exhibited a remarkably lower GPx activity (i.e. 390.56U/mg protein), while those of silymarin, L- and H-WEF groups were able to elevate the enzyme level but only silymarin group (i.e. 823.77U/mg protein) could bring the enzyme activity back to a similar level with NCG (i.e. 795.87U/mg protein). The L-WEF group illustrated a superior GRd activity (i.e. 478.53U/mg protein) compared with all other groups, while no significant difference were observed between normal group and CCl₄ group as well as between silymarin group and H-WEF group.

For the SOD activity, silymarin group exhibited a comparatively superior SOD activity (i.e. 146.30U/mg protein) than those of CCl₄ group (i.e. 67.64U/mg protein), normal group (i.e. 91.39U/mg protein), L- and H-WEF groups (i.e. 101.01 and 96.14U/mg protein, respectively) as well as no significant difference among last three groups.

In the CCl₄ group, the lowest CAT activity (i.e. 98.64 U/mg protein) was found. In silymarin group the CAT activity was raised (i.e. 231.94U/mg protein) and was fully regained the similar level to NCG (i.e. 274.90U/mg protein). Both L- and H-WEF raised the CAT activity (i.e. 204.37 and 192.92U/mg protein, respectively); nevertheless, they are lower than those of silymarin and NCGs. Finally, the CCl₄ group showed the highest MDA level (i.e. 0.43nmol/mg protein), followed by silymarin group (i.e. 0.35nmol/mg protein); however, both L- and H-WEF (i.e. 0.25 and 0.20nmol/mg protein, respectively) resumed the similar level to NCG (i.e. 0.26nmol/mg protein) while no significant difference was found between both.

Histopathological examination of hepatic injury

The hepatic morphologies following 8 weeks of different treatments are shown in fig. 1. Red and shiny liver could be observed in NCG and the surface was smooth and flexible to the feel (fig. 1A). On the contrary, the livers color of CCl₄ treatment turned to xanthous and their periphery was blunt and firm to the feel (fig. 1B). Also, the livers of CCl₄ group were swelling and with lump protuberances on the coarse surface contrast with those of NCG. Though the livers of silymarin (fig. 1C) and WEF (fig. 1D and 1E) groups could not be resume the deep red color of NCG, the entire semblance was more shiny, reddish and smooth compared with those of CCl₄ group.

The exterior of H&E-stained liver following 8 weeks of different treatments are illustrated in fig. 2. In NCG, regular tissue structure, tidy hepatic plate, obvious margin for liver sinusoids, integrated hepatic cells and no fatty

infiltration (vacuolation) were found (fig. 2A). The vacuolated hepatocytes was found and accompanied with liver normal construction lost following the CCl₄ treatment (fig. 2B). The distinct levels of vacuolation dispersed all over the liver tissue demonstrated that CCl₄ had induced hepatic injury successfully. In silymarin group, lobular inflammation and necrosis was extremely diminished, as well as some vacuolations were observed (fig. 2C) but it was less obvious than that of CCl₄ group. Also, the bile duct proliferation and necrosis was greatly reduced (fig. 2D and 2E) in liver administered with WEF.

When stained by MT the collagen of fibrous tissues should be illustrated a blue color. The typical histological micrographs of MT stained-liver following 8 weeks of different treatments are summarized in fig. 3. The hepatic sections demonstrated normal cells without fibrosis in NCG (fig. 3A). Once treated with CCl₄ the rat livers showed many hepatic lobules encompassed by massive fibrous tissue and radioactively spread from the portal and vein to the surrounding (fig. 3B). The much gentle degree of pathologies in silymarin group (fig. 3C) and WHF groups (fig. 3D and 3E) than those of CCl₄ group were noticed and these revealed that incomplete formation and broken of septa and moderate fibrosis.

Degrees of proliferation of bile duct, steatosis, portal and lobular inflammation and scores of necrosis and fibrosis are provided in table 5. No liver injuries were observed in NCG; however, the greatest degrees of bile duct proliferation were noticed in CCl₄ and silymarin groups (i.e. 2 and 2, respectively) followed by the L- and H-WEF groups (i.e. 1 and 1, respectively). The coordinate degree of steatosis were found in all the treatment groups, which CCl₄ and H-WEF groups had higher degree (i.e. 2 and 2, respectively) than those of silymarin and L-WEF groups. A similar degree of portal inflammation and lobular inflammation (i.e. 1 and 1, respectively) was remarked in all the treatment groups. Regarding hepatic necrosis and fibrosis, the CCl₄ group exhibited the supreme level of necrosis (i.e. 2 and 2, respectively) than other treatment groups (i.e. 1).

DISCUSSION

Antioxidants and Antioxidant activities

Recently, for curing many diseases by using traditional Chinese medicinal herbs has become more and more popular in the world. As it is known, 'A unilateral medicine is not a good prescription', this is one of the TCM principles. Additionally, natural medicinal herbs contain extensive chemical compounds be able to act particularly, additionally or synergically to enhance human health. Thus the hunt for effective antioxidants from plant community is suggested. Phenolics usually exist in plant tissues and organs, such as leaves, flowers, stems and barks in vary kinds of plant (Larson, 1988).

Frequently, the antioxidant activity (AA) of the plant is closely related with the total phenolic compounds. The phenolics' AA is dominantly in consequence of their redox attributes and thus allows them to perform as hydrogen donors, singlet oxygen quenchers and reducing agents (Hsu *et al.*, 2007). Scientists commonly employ FRAP and TEAC assay to estimate the antioxidant components and use the DPPH, one of free radicals, to assess the antioxidant activity of plant extracts and drugs (Jiang *et al.*, 2011). Our results revealed that higher total phenolic and total flavonoid contents were observed in *N. nucifera* Gaertn alone, followed by WEF and followed by *M. stigma*, respectively. In addition, the *N. nucifera* Gaertn also exhibited the highest DPPH scavenging activity, FRAP and TEAC capabilities, followed by WEF, were all superior to each of *M. stigma* and *T. officinale*, suggesting the fundamental antioxidant characteristics of WEF may contributed from *N. nucifera* Gaertn.

It should be considering the chemical compounds, practical application and metabolic as well as combination effects etc. (Weber *et al.*, 2003) though many reports (Yan *et al.*, 2009; Kim *et al.*, 2010) demonstrated that the multi-herbal formula predominate protective effect against CCl₄-treated hepatotoxicity in rats. The results from this present research are in agreement with our previously report (Yang *et al.*, 2013) where it has been discovered that when the some selected TCM herbs were combinatively employed they can exert their utilities and a synergistic efficacy.

Relative liver and kidney weight

The relative liver and kidney weight significantly increased in CCl₄ control group compared with other four groups suggesting that the hepatic injury occurred in CCl₄-intoxicated rats. Both silymarin and WEF treatments could resume the relative liver and kidney weight to NCG. It means that WEF provided potential protective effect on rats in this study.

Clinical chemistry

Both biochemical markers ALT and AST are often hire to evaluate hepatic damage. The ALT and AST are divulged into the blood flux when liver injured and then both of them increased in plasma. This is why the increment of the activities of ALT and AST in plasma could be considered as indices of injury of the hepatocytes. Results from this research revealed that the CCl₄ treatment remarkably respectively raised ALT and AST activities by 6.5 and 2.4 times than those of NCG. These results are in accordance with those discoveries of Opoku *et al.* (Opoku *et al.*, 2007) and Mitra *et al.* (Mitra *et al.*, 1998). Interestingly, compared with the CCl₄ group the feeding with L- and H-WEF during CCl₄ treatment outstandingly reduced the activities of ALT by 51.5 and 37.5% and of AST by 14.7 and 21.6% respectively, meaning the latent hepatoprotective effect of the WEF. The WEF capability

for reducing AST activity was analogous to silymarin but reducing the ALT activity was lower than silymarin. These phenomena are similar to our previous research (Yang *et al.*, 2013). Ordinarily, plasma transaminase levels come back to normal after regeneration of hepatocytes and recovery of hepatic parenchyma. In this research; nevertheless, neither silymarin nor WEF were adequate to resume the ALT and AST activities to NCG levels.

Both of plasma and liver lipid concentrations can be raised from the decrease of the plasma-LDL (low-density lipoprotein) protein moiety in the liver. Our results demonstrated that CCl₄ administration increased the TG level above 2 times than NCG; however, only the administration of WEF resumed the TG level compared with NCG. It were reported that the TG level can be reduced by administration of *N. nucifera* Gaertn and *T. officinale* water extracts respectively (Rao *et al.*, 2005). In terms of TC level no significant difference was found among all groups suggesting that administration of CCl₄, silymarin or WEF were not affected the rat TC levels.

Campo *et al.* reported (Campo *et al.*, 2001) the momentous paths including obstruction of free-radical generation and the activities of antioxidants and antioxidant enzymes for preventing liver cells from CCl₄-induced injury. GSH plays like an antioxidant both extracellularly and intracellularly and participate in different enzymatic processes to decrease hydro peroxide and hydrogen peroxide by way of oxidizing GSH itself to glutathione disulfide (GSSG) and other mixed disulfides (Hung *et al.*, 2006). The lipid per oxidation in liver tissue can be elevated when organ impairment (Mogulkoc *et al.*, 2006a) or harvesting (Oztekin *et al.*, 2007) and the GSH content can be decreased. The plant extracts have inhibited antioxidant activities such as raise GSH content in animal test (Ionita *et al.*, 2017; Tabassum and Khan, 2017; Uthaya Kumar *et al.*, 2016). GPx can metabolize hydrogen peroxide (H₂O₂) and hydroperoxides to nontoxic products and terminate lipid peroxidation chain reaction thereupon stabilize polyunsaturated membrane lipids (Christophersen, 1968). By conjugating with GSH the GRd participate in the detoxification of all sorts of xenobiotic compounds (Baudrimont *et al.*, 1997). Regarding super oxide anion (O₂⁻), it acts as a critical role in the primary and chain reaction of lipid peroxidation (Okamoto *et al.*, 1992). Super oxide anions can be dismutate by SOD into H₂O₂ (Reiter *et al.*, 2000) and CAT decomposes H₂O₂ to oxygen and water (Jiang *et al.*, 2011). By means of the participation of GPx the adequate quantity of GSH was reported be able to improve the detoxification of active metabolites (Bhandarkar and Khan, 2004). The contents of GPx and GSH and the activities of SOD and CAT were remarkably reduced in CCl₄-treated rat livers compared with those of NCG, implying that CCl₄ enlarged oxidative stress and damaged

the rat liver. Nevertheless, administration with both L- and H-WEF can raise the contents of GPx and GSH as well as the activity of GRd, and resume the levels of GSH and SOD to NCG. Though the oxidative damages cannot be adequately prevented by increasing antioxidant activity (Mogulkoc *et al.*, 2006b), for GSH content, the L- and H-WEF (i.e. 42.40 and 42.60 μ mol/mg protein, respectively) treatments were able to restore the GSH content to normal level and the effect like or better than silymarin (i.e. 41.10 μ mol/mg protein). Silymarin-administrated rats were found to resume the content of GSH and the activities of GPx and CAT. Compare with NCG, silymarin also increased the activities of GRd and SOD to superior levels. Notably, for GRd, GSH and CAT levels the hepatoprotective effects of L- and H-WEF were remarked to be as efficient as silymarin.

MDA, a reactive aldehyde, is diffusely employed as an indicator of lipid peroxidation (Mansour, 2000). For MDA level, no significant discrepancy was noticed among NCG, silymarin and L- and H-WEF groups. It hints that WEF could bring the MDA back and close to NCG and furtherly ameliorate the peroxidation in the rats. It is in agreement with the report by Hung *et al.* (Hung *et al.*, 2006) who revealed that water extracts of *Du-Zhong* (*Eucommia ulmoides* Oliv.) leaves can reduce MDA level of CCl₄-treated rats.

Histopathology

According microscopic examinations, serious liver injury leaded by CCl₄ can be remarkably decreased by WEF administration. This is in strongly correlated with the results of the liver antioxidant enzyme activities and hepatic functional parameters of the serum. The liver sections of NCG represented normal liver cells while the livers of CCl₄-treated rats exhibited from medium to serious swelling vacuolation, fibrosis and necrosis. The liver pathologies of the rats treated with silymarin exhibited a highly gentler level compared with the CCl₄ group and displayed slight to medium vacuolations and small to moderate suffered necrosis of hepatocytes. The same was inspected in administration of WEF rats. In the CCl₄ group all of the evaluation scores were remarkably greater than those of NCG, representing CCl₄ induced serious liver cells damage. Contrast to silymarin, L- and H-WEF remarkably reduced the scores of bile duct proliferation and necrosis as well as favorably lowered the portal inflammation, lobular inflammation and fibrosis to the same degree as the silymarin.

CONCLUSION

Remarkable hepatotoxicity caused by CCl₄ administration in rats was demonstrated based on the activities increment of AST and ALT, liver tissues oxidation and elevated histopathological evidences of liver damage. The CCl₄-induced hepatotoxicity can be inhibited by both silymarin

and WEF were evidenced in this research. Both the silymarin and WEF exhibited similar hepatoprotective effects on the content of GSH, activities of AST and CAT, as well as the level of portal inflammation, lobular inflammation, necrosis and fibrosis. The WEF was advantageous than silymarin for the content of SOD, while silymarin emerged more efficacious than WEF for the activities GPx and GRd. Regardless of difficulty for the WEF to entirely recover the CCl₄-induced liver injury, the supplementation of WEF has elevated the healing of liver injury and the abilities of hepatic regenerative.

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