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Improvement for High Fat Diet-Induced Hepatic Injuries and Oxidative Stress by Flavonoid-Enriched Extract from *Nelumbo nucifera* Leaf

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Nelumbo nucifera Gaertn is widespread and a popular food in central and southern Taiwan. It has also been reported to possess different therapeutic effects, but the effects of *N. nucifera* leaf on lipid metabolism and liver function remain unclear. In this study, a high fat diet was used to induce hyperlipidemia, hypercholesterolemia, and fatty liver in hamster. The effects of flavonoid-enriched *N. nucifera* leaf extract supplement and two lipid-lowing drugs, silymarin and simvastatin, on the disorders induced by high fat diet were investigated. The results showed that a 10-week application of a high fat diet to hamsters led to significant increases of body weight, plasma lipid derivatives (triglyceride, total cholesterol, and lipoproteins), lipid peroxidation, and liver damage markers (plasma aspartate aminotransferase and alanine aminotransferase). Interestingly, flavonoid-enriched *N. nucifera* leaf extract supplement effectively ameliorated the high fat diet-induced lipid metabolic disorders as significantly as silymarin and simvastatin did. Moreover, the flavonoid-enriched supplement alleviated the high fat diet-induced accumulation of lipids in liver, the findings showing distinguishing mechanisms from the effects of silymarin and simvastatin. These results suggested that the flavonoid-enriched *N. nucifera* leaf extract supplement may significantly improve the high fat diet-induced abnormal blood lipids and liver damage as significantly as the common drugs. Consequently, it is suggested that the flavonoid-enriched *N. nucifera* leaf extract supplement is beneficial for the improvement of lipid metabolisms and the alleviation of liver damage in high fat diet treatment.

KEYWORDS: ***Nelumbo nucifera*; hyperlipidemia; hypercholesterolemia; fatty liver**

INTRODUCTION

Lotus (*Nelumbo nucifera* Gaertn) is a widely distributed and distinguishing crop in central and southern Taiwan. In Southeastern Asia, lotus seed and lotus root are usually regarded as a popular food, and lotus leaf is also used as a common relish in traditional Taiwanese foods. Besides, lotus is extensively used as a folk remedy and has been shown to possess different therapeutic effects, and its pharmacological properties have been increasingly reported: for example, suppression of cytokine expression and hepatoprotective effects of *N. nucifera* seed extract (*1*, *2*); reduction of blood sugar level (*3*), antiinflammation (*4*), and antipyretic (*5*) effects of *N. nucifera* rhizome extracts; and antiobesity activities of *N. nucifera* leaf extract (NLE) (*6*, *7*).

In today's modern lifestyle, an increase in dietary fat intake, particularly saturated fat, not only increases the prevalence of obesity, diseases caused by insulin resistance, and metabolic syndrome but also may lead to atherosclerosis, diabetes mellitus, and steatosis (*8*, *9*). Atherosclerosis, induced by a complex pathophysiological process, is one of the major risk factors of cardiovascular diseases (*10*). It is believed that circulating concentrations of lipids and free fatty acids are increased after high-fat feeding, and the hyperlipidemia as well as the elevated plasma low-density lipoprotein (LDL) may result in initiation of atherosclerosis (*11*−*13*). Excess dietary fat consumption associated with an increase in the prevalence of insulin resistance and type 2 diabetes mellitus has also been reported (*14*−*16*). Additionally, the risk for cardiovascular diseases has been found to rise up to 10-fold higher than normal in people with diabetes mellitus, largely because of underlying atherosclerosis (*17*). A high-fat diet (HFD) may also lead to steatohepatitis (*18*). Steatosis is regarded as an initiating factor for chronic liver diseases, which may result in a spectrum of hepatic bases for morbidity including simple steatosis, nonalcoholic steatohepatitis, fibrosis, and cirrhosis. Moreover,

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steatosis is likely a common cause of cryptogenic cirrhosis (*19*); once the cirrhosis is present, hepatocellular carcinoma may also develop (*20*, *21*).

Although different parts of *N. nucifera* have shown therapeutic effects on various ailments, studies for the effects of *N. nucifera* leaf are rare. To further investigate the effects of *N. nucifera* leaf on HFD-fed animals, we used a flavonoid-enriched NLE as a supplement to HFD-fed hamsters. After 10 weeks, the hamsters fed general meal, HFD, HFD supplemented with the NLE, and HFD individually supplemented with two lipid-lowing drugs (silymarin and simvastatin) were sacrificed, and their plasma and liver samples were analyzed for lipid metabolism and liver damage. Our results showed that the NLE may effectively reduce both the triglycerides and total cholesterol in plasma and liver in HFD-fed hamsters. Additionally, the flavonoid-enriched NLE supplement also alleviated the liver damage by lowering the levels of two common markers for liver damage, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which were increased by HFD treatment.

MATERIALS AND METHODS

Materials. *N. nucifera* leaves were purchased from an organic certification farm in central Taiwan. Female Syrian hamsters were purchased from National Laboratory Animal Center, Taipei, Taiwan. Aluminum (III) chloride ($AlCl_3$), trichloroacetic acid (TCA), potassium dihydrogen phosphate (KH_2PO_4), potassium hydroxide (KOH), potassium acetate (CH_3COOK), 2-thiobarbituric acid, 1,1,3,3-tetraethoxypropane (TEP), and silymarin were purchased from Sigma (St. Louis, MO). Simvastatin (Zocor) and Folin−Ciocalteu reagent were purchased from Merck & Co., Inc. (Whitehouse Station, NJ).

Preparation of Aqueous NLE. The purchased *N. nucifera* leaves were washed with distilled water and air-dried. Twenty grams dried leaves was resuspended in 100 mL of distilled water at 4 °C for overnight. The supernatant was filtered through two layers of gauze to remove the debris and then lyophilized. The dry weight yield of NLE is approximately 15%. The NLE supplement was prepared by mixing the lyophilized NLE powder with HFD mash in 1 or 2% (w/w). Polyphenol composition in NLE has been analyzed in our previous study by using HPLC (*22*). Briefly, the HPLC analysis was performed with a Hitachi liquid chromatograph (Hitachi, Ltd., Tokyo, Japan) consisting of a model L-6200 pump and a model L-3000 photodiode array detector set at 280 nm. A reverse-phase LiChrosphere RP-18 column (Merck, 4.6 × 1506 mm, 5 μm) was used for HPLC analysis. Elution was carried out at room temperature and used solvent A (2% v/v acetic acid) and solvent B (0.5% v/v acetic acid and 50% v/v acetonitrile). The solvent B elution gradient was performed as 5−10% (0−10 min), 10−20% (10−20 min), 20−30% (20−30 min), 30−40% (30−40 min), 40−55% (40−55 min), 55−80% (55−60 min), 80−100% (60−65 min), 100−50% (65−70 min), 50−30% (70−75 min), and 30−10% (75−80 min). The flow rate was kept at 1 mL/min. Polyphenols were identified by comparison of their retention time values and UV−vis spectra with those of known standards and were quantitated by peak areas from the chromatograms. Eight polyphenols were used as standard, and the quantitative results revealed that NLE contained 20.47% gallic acid, 1.78% protocatechuic acid, 2.63% catechin, 1.39% epigallocatechin gallate, 0.99% caffeic acid, 1.41% epicatechin, 6.08% rutin, and 4.60% quercetin.

Determination of Total Phenolic Acids, Flavonoids, Polysaccharides, and Proteins in NLE. Total phenolic acid was analyzed according to the colorimetric Folin−Ciocalteu assay. Briefly, NLE was dissolved in 1 mL of distilled water and then mixed with 0.5 mL of Folin−Ciocalteu reagent. After 3 min, 3 mL of Na_2CO_3 (2%, w/v) was added to the mixture and reacted for 15 min with gentle mixing. The content of phenolic acids was determined by measuring absorbance at 750 nm (Hitachi U-3210 spectrophotometer) and using gallic acid as standard. The total flavonoid content was analyzed by using $AlCl_3$. Briefly, 0.5 mL of NLE was mixed with 0.1 mL of $AlCl_3$ (10%, w/v), 1.5 mL of ethanol, and 0.1 mL of 1 M CH_3COOK. The total volume of the mixture was adjusted to 5 mL with distilled water and then mixed well. After 40 min, absorbance at 415 nm was measured and the flavonoid content was estimated by calibration curves using rutin. The percentage of flavonoid was expressed as rutin equivalents in milligrams per 100 g of fresh weight. The contents of polysaccharide and protein in NLE were determined using the phenol−sulfuric acid method and Bio-Rad protein assay kit, respectively.

Animals and Diets. A total of 48 Syrian hamsters, aged 6−7 weeks and weighing 100−120 g, were kept at a constant temperature of 24 °C and illuminated for 12 h daily (lights on from 6:00 a.m.to 6:00 p.m.). After a 2-week maintenance for adaptation to the environment, the hamsters were randomly grouped by body weight into six groups; each group was fed a unique diet for 10 weeks and weighed every 2 weeks. The six groups and their corresponding meals were (1) control, normal meals; (2) HFD, normal meals containing 0.2% cholesterol and 10% coconut oil; (3) + 1% NLE, HFD supplemented with 1% NLE as described; (4) + 2% NLE, HFD supplemented with 2% NLE as described; (5) + silymarin, HFD supplemented with silymarin (100 mg/kg); and (6) + simvastatin, HFD supplemented with simvastatin (1 mg/kg). After a 10-week application of different diets, whole blood and livers were collected from hamsters that had been fasted for 12−14 h and then were sacrificed. The blood was collected by EDTA tubes and centrifuged at 1500*g* for 10 min at 4 °C. The supernatant plasma was transferred into new tubes for determination of triglycerides, total cholesterol, low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), AST, ALT, blood urea nitrogen, and plasma creatinine. After hamsters had been sacrificed, the livers were quickly frozen in liquid nitrogen for the extraction of liver lipids or freshly cut into pieces for fixation, embedding, and hematoxylin and eosin (H&E) staining.

Quantitation of Triglycerides, Total Cholesterol, and Lipoproteins. The quantitative analyses for triglycerides and total cholesterol were performed by using clinical chemistry reagent kits (Randox Laboratories Ltd., Antrim, U.K.) according to the manufacturer's institutions. Briefly, the plasma was analyzed by enzyme-coupling reactions and colorimetric measurement. Quantitation of the analytes was performed by standard curve and the calibrating factors.

For quantitation of total cholesterol, the plasma samples were treated with cholesterol esterase to release cholesterol prior to enzyme-coupling reactions and colorimetric measurement. For quantitation of HDL-C, phosphotungstic acid and magnesium chloride were added to the plasma to precipitate LDL-C, very low-density lipoprotein (VLDL), and chylomicrometer. After centrifugation (1500*g* for 10 min), HDL-C in the clear supernatant was determined. For quantitation of LDL-C, heparin was added to the plasma to precipitate the LDL-C. After centrifugation (1500*g* for 10 min), HDL-C and VLDL in the supernatant were determined, and the LDL-C was quantitated by using the total cholesterol minus the HDL-C and VLDL.

Extraction of Liver Lipids. Liver lipids were extracted as described previously (*23*). Briefly, liver (1.25 g) was homogenized with chloroform/methanol (1:2, 3.75 mL), and then chloroform (1.25 mL) and distilled water (1.25 mL) were added to the homogenate and mixed well. After centrifugation (1500*g* for 10 min), the lower clear organic phase solution was transferred into a new glass tube and then lyophilized. The lyophilized powder was dissolved in chloroform/methanol (1:2) as the liver lipid extract and stored at −20 °C for < 3 days. The lipid extracts were used for quantitation of liver triglycerides and liver cholesterol.

Lipid Peroxidation Assay. Lipid peroxidation was determined on the basis of the level of thiobarituric acid-reactive substances (TBARS) (*24*). One hundred microliters of plasma or standard (series diluted TEP) was mixed with an equal volume of TCA (25%) and centrifuged at 8000*g* for 30 min. The supernatant (150 μL) was transferred into a 1.5-mL screw-cap Eppendorf tube and mixed with an equal volume of 2-thiobarbituric acid (1%) vigorously. The Eppendorf tube was placed in a heating cabinet at 95 °C for 40 min in the dark. After cooling at room temperature for 15 min, the TBARS concentration was determined by fluorescence spectrophotometer (excitation at 532 nm and emission at 600 nm) and TEP standard curve.

Assessment of Liver and Kidney Damage. Plasma samples were collected for the following biochemical tests. AST and ALT were assayed by using commercial diagnostic kits (Randox Laboratories) as previously

Table 1. Components of NLE

component of NLE	percentage (%)
polyphenolic content	14.8
flavanone content	56
protein content	4.55
polysaccharide content	16.6

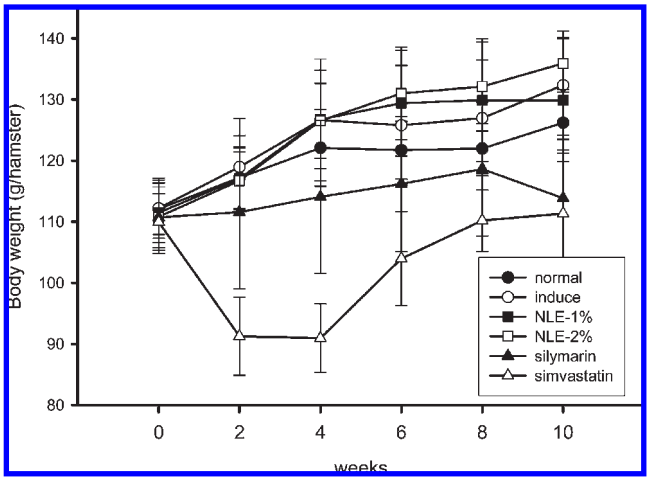

Figure 1. Body weights of hamsters fed different diets during the experiment. Control, normal diet; HFD, high-fat diet; + 1% NLE, HFD containing 1% (w/w) NLE; + 2% NLE, HFD containing 2% (w/w) NLE; + silymarin, HFD containing silymarin (1 mg/kg); +simvastatin, HFD containing simvastatin (100 mg/kg). Data are shown as means ± SD.

described (*25*). Blood urea nitrogen and plasma creatinine were assayed by Roche Diagnostics according to the previous studies (*26*, *27*).

Histopathological Examination of Liver. Small pieces of liver were fixed in 10% buffered neutral formalin and embedded in paraffin as described (*28*). Sections were cut at a thickness of 3−5 μm and stained with H&E. The histopathological changes including cell morphology and cellular lipid vesicles were examined by light microscopy (200×).

RESULTS

Flavonoid Is the Major Component of NLE. To evaluate the plausible functional elements of NLE, the component analysis of NLE was performed. As shown in **Table 1**, HPLC analysis revealed that NLE consisted of 14.8% total phenolic acids, 56% flavonoids, 16.6% polysaccharides, 4.55% protein, and some lipids. On the basis of the results, flavonoids were the major polyphenols in NLE.

NLE Supplement Was Not Affected by the Increase of Body Weight (BW) Induced by HFD. The variation of body weights during the 10-week experiment is shown in **Figure 1**. The ratios of average BWs on week 10 to week 0 for each group were 1.126 (normal), 1.179 (HFD), 1.165 (+1% NLE), 1.227 (+ 2% NLE), 1.029 (+ silymarin), and 1.012 (+ simvastatin). The increase of average BW of hamsters by HFD supplemented with 1% or 2% NLE was as much as by HFD. Interestingly, the average BW of the simvastatin group was decreased by 16.4 and 17.2% at week-2 and week-4 as compared to week-0. These results showed that NLE supplement may not affect the increase of BW raised by HFD. However, the silymarin and the simvastatin treatments significantly suppressed the increase of BW raised by HFD. Together, these results implied that silymarin and simvastatin, but not NLE supplement, may interfere with fat metabolism or body growth.

NLE Supplement Decreased Plasma Lipids in Hamsters Fed HFD. After the 10-week feeding with different meals, six groups of hamsters were sacrificed and the variations of plasma lipids

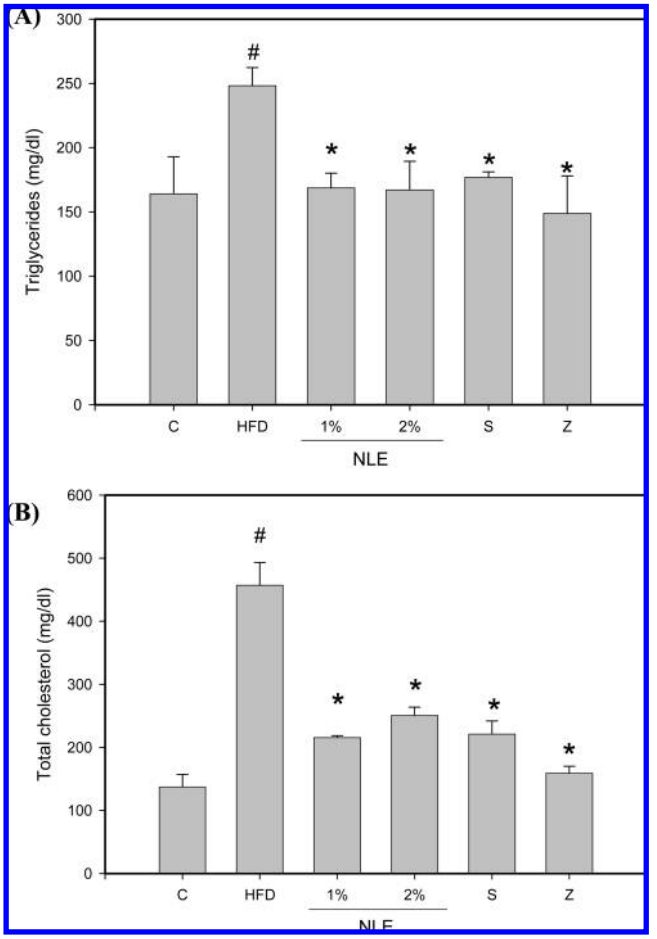

Figure 2. Effects of NLE supplement on plasma triglycerides and total cholesterol: (**A**) plasma triglyceride and (**B**) total cholesterol levels in hamsters fed HFD, and HFD containing 1% NLE, 2% NLE, silymarin (S) and simvastatin (Z); corresponding levels in hamsters fed normal diet (C) were used as control. Data are shown as means ± SD; #, $p < 0.05$ compared with normal group; ∗, $p < 0.05$ compared with HFD group.

examined. As shown in **Figure 2**, the HFD increased concentrations of both plasma triglycerides and total cholesterol, but the 1% or 2% NLE supplement significantly decreased levels of both plasma triglycerides and total cholesterol. Moreover, the level of triglycerides in hamsters fed HFD with NLE supplements was similar to that of hamsters fed normal meal and HFD with silymarin or simvastatin supplements. These findings suggest that the NLE supplement, as well as the silymarin and the simvastatin treatments, effectively inhibited the increase of triglycerides and total cholesterol resulting from HFD.

NLE Supplement Decreased Plasma Lipoproteins in HFD-Fed Hamsters. To further investigate the effects of NLE supplement on the high level of plasma lipids induced by HFD, concentrations of LDL-C and HDL-C were determined and compared. As shown in **Figure 3**, HFD increased the concentrations of LDL-C, HDL-C, and the ratio of LDL-C to HDL-C. The increase of LDL-C, HDL-C, and the ratio was significantly decreased by 1% and 2% NLE supplement, as well as by the silymarin and the simvastatin treatments. Although both the LDL-C and the HDL-C decreased, the ratio of LDL-C to HDL-C was significantly decreased by the NLE supplement. These results suggested that NLE supplement not only decreased the levels of LDL-C and HDL-C but also decreased the ratio of LDL-C to HDL-C. Similar results were also shown in the silymarin and the simvastatin treatments (**Figure 3**).

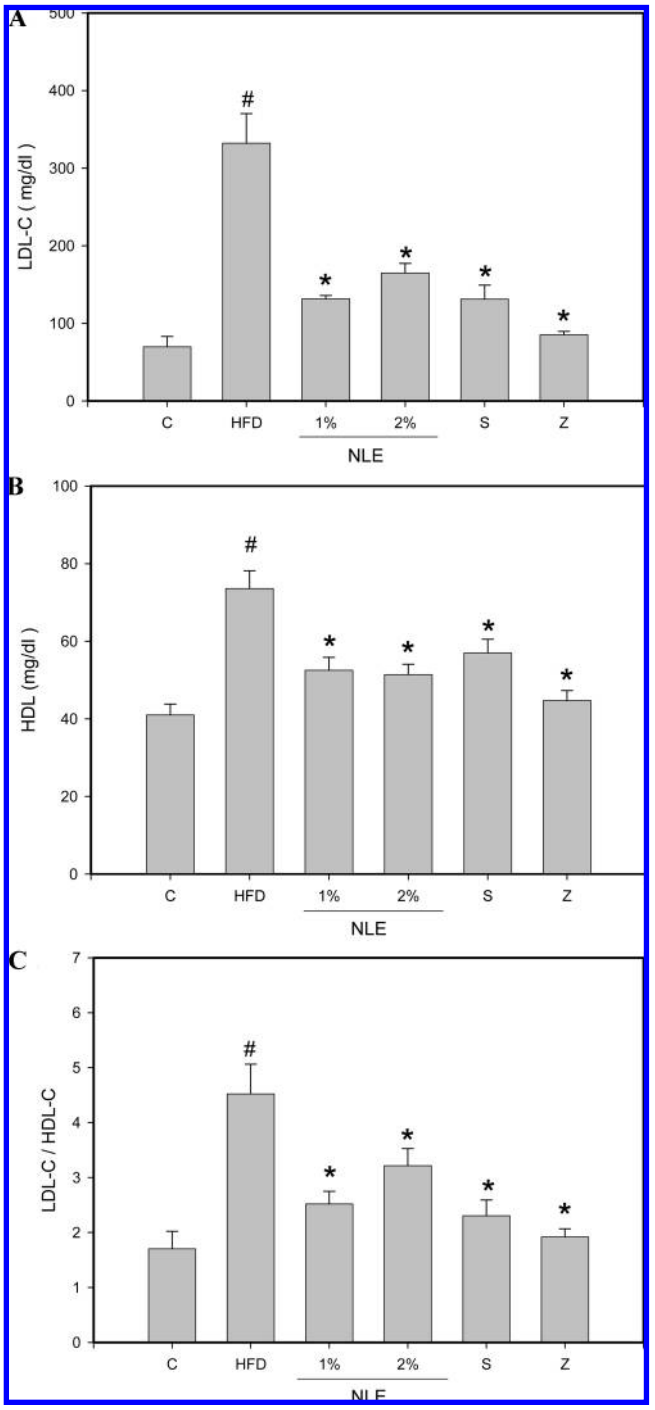

Figure 3. Effects of HFD without and with NLE supplement on plasma lipoproteins: levels of (**A**) LDL-C and (**B**) HDL-C and (**C**) the ratio of LDL-C to HDL-C in plasma of hamsters fed HFD and HFD containing 1% NLE, 2% NLE, silymarin (S), and simvastatin (Z). Corresponding levels in hamsters fed normal diet (C) were used as control. Data are shown as means ± SD; #, $p < 0.05$ compared with normal group; ∗, $p < 0.05$ compared with HFD group.

NLE Supplement Reduced Lipid Peroxidation in HFD-Fed Hamsters. The effects of NLE supplement on lipid peroxidation were examined by quantitation of TBARS in plasma. As shown in **Figure 4**, HFD greatly increased the level of plasma TBARS up to triple the control. Interestingly, both 1% and 2% NLE supplement significantly reduced the level of TBARS induced by HFD. Similar effects on a decrease of TBARS were also shown by silymarin and simvastatin treatments. These results suggested

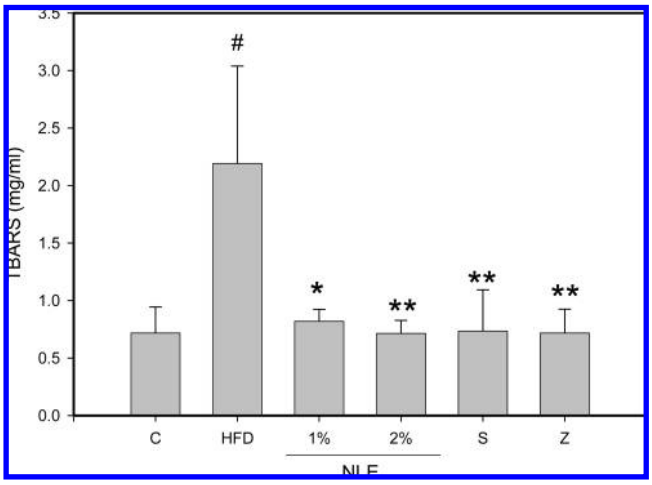

Figure 4. Effects of NLE supplement on plasma lipid peroxidation: TBARS in plasma from hamsters fed HFD and HFD containing 1% NLE, 2% NLE, silymarin (S), and simvastatin (Z). Corresponding levels in hamsters fed normal diet (C) were used as control. Data are shown as means ± SD; #, $p < 0.05$ compared with normal group; ∗, $p < 0.05$ compared with HFD group; ∗∗, $p < 0.01$ compared with HFD group.

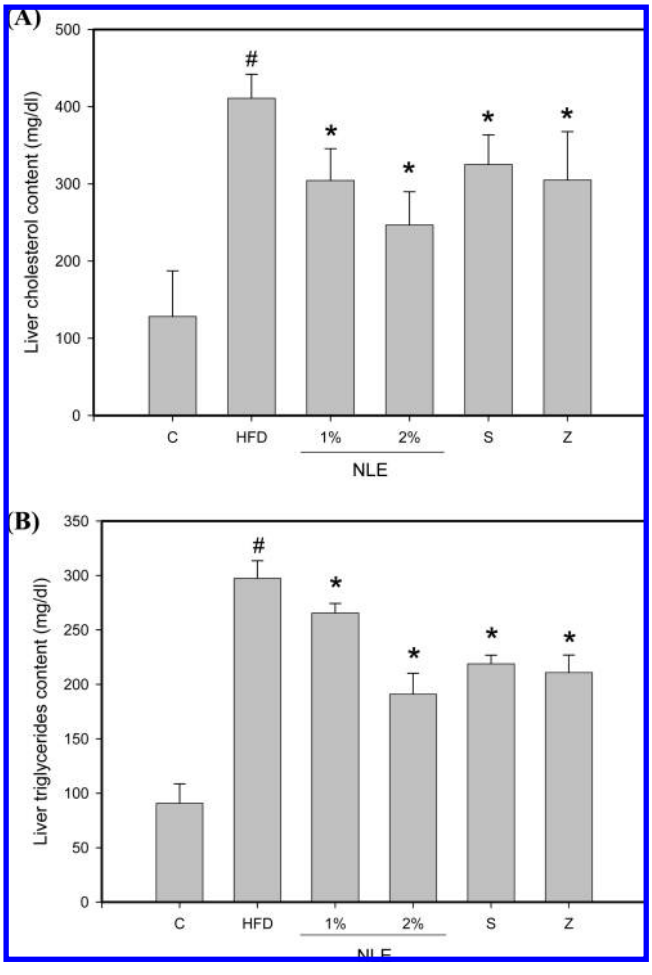

Figure 5. Effects of NLE supplement on the levels of liver cholesterol and liver triglyceride: (**A**) liver cholesterol and (**B**) liver triglyceride in hamsters fed HFD and HFD containing 1% NLE, 2% NLE, silymarin (S), and simvastatin (Z). Corresponding levels in hamsters fed normal diet (C) were used as control. Data are shown as means ± SD; #, $p < 0.05$ compared with normal group; ∗, $p < 0.05$ compared with HFD group.

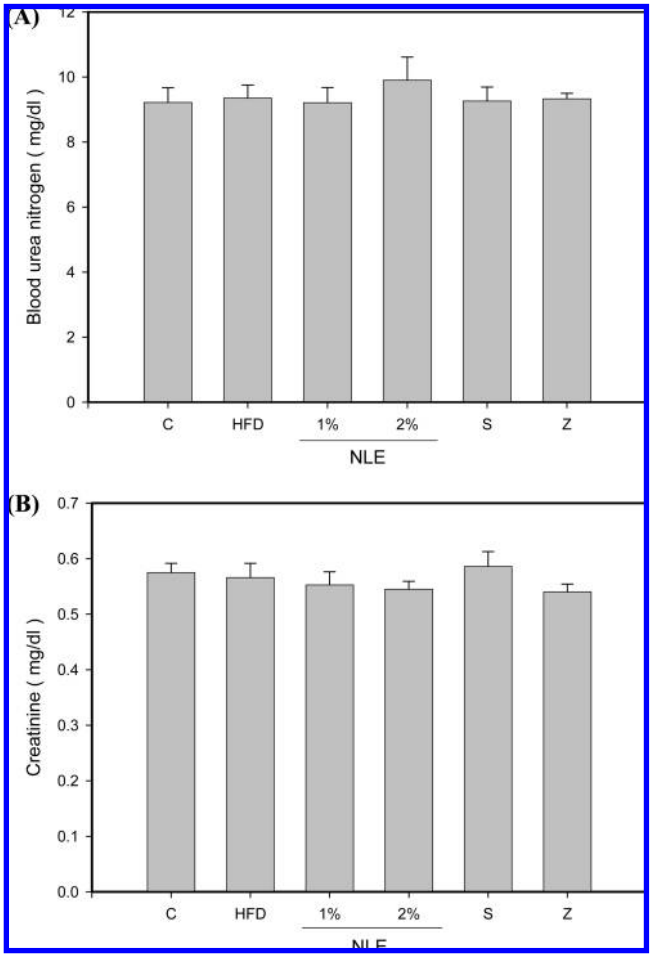

Figure 7. Effects of NLE supplement on blood urea nitrogen and plasma creatinine: (**A**) blood urea nitrogen and (**B**) plasma creatinine in hamsters fed HFD and HFD containing 1% NLE, 2% NLE, silymarin (S), and simvastatin (Z). Corresponding levels in hamsters fed normal diet (C) were used as control. Data are shown as means ± SD. No statistical significances are shown between the experiments.

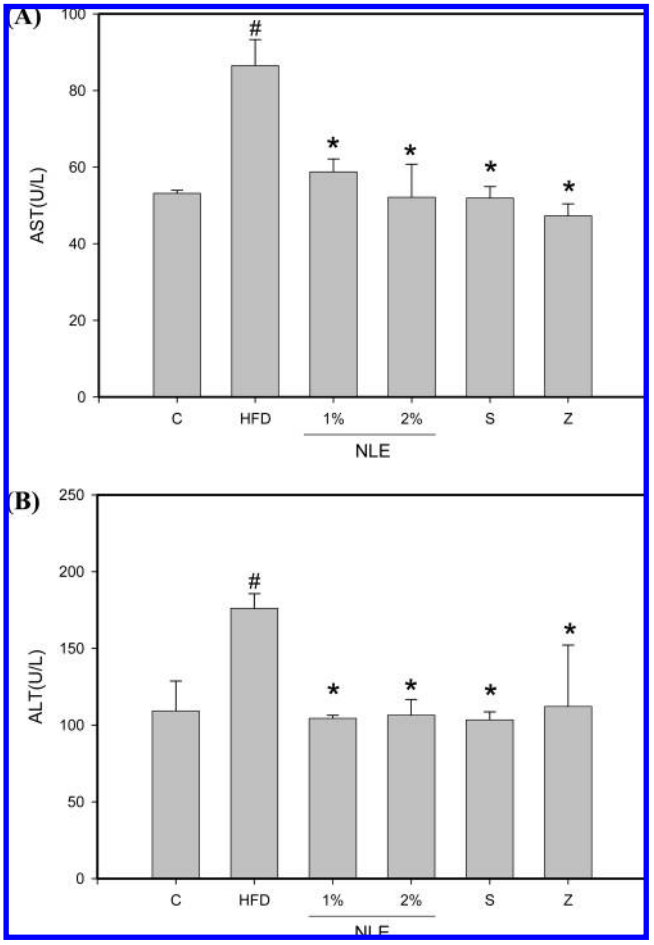

Figure 6. Effects of NLE supplement on the activities of plasma AST and ALT: (**A**) plasma AST and (**B**) plasma ALT in hamsters fed HFD and HFD containing 1% NLE, 2% NLE, silymarin (S), and simvastatin (Z). Corresponding levels in hamsters fed normal diet (C) were used as control. Data are shown as means ± SD; #, $p < 0.05$ compared with normal group; ∗, $p < 0.01$ compared with HFD group.

that the NLE supplement, as well as silymarin and simvastatin treatments, may effectively reduce lipid peroxidation in HFD-fed hamsters.

NLE Supplement Alleviated Liver Triglycerides and Cholesterol in HFD-Fed Hamsters. HFD has been demonstrated to lead to the accumulation of cholesterol and triglycerides in liver. Therefore, effects of NLE supplement on the liver cholesterol and triglycerides were investigated. HFD increased levels of liver cholesterol and triglycerides, but the NLE supplement significantly and dose-dependently lowered the level of liver cholesterol and triglycerides induced by HFD (**Figure 5**). Furthermore, the effect of 2% NLE supplement on the decrease of liver cholesterol and triglycerides was more significant than the effects of silymarin and simvastatin treatments. These findings suggest that the NLE supplement may more effectively alleviate the increase of liver cholesterol and triglycerides induced by HFD than the silymarin and the simvastatin treatments.

NLE Supplement Showed Potential Hepatoprotective Functions in HFD-Fed Hamsters. The NLE supplement played important roles in decreasing plasma triglycerides and total cholesterol, improvement of lipid peroxidation, and alleviation of liver cholesterol and triglycerides, and it was suggested that NLE supplement may alleviate liver damage in HFD-fed hamster. As shown in **Figure 6**, HFD increased the AST and ALT in plasma as high as 1.63× and 1.66× to the control, respectively. However, the 1% and 2% NLE supplements significantly decreased the levels of plasma AST and ALT. Furthermore, the effect of NLE supplement on the decrease of plasma AST and ALT was similar to the effects of silymarin and simvastatin treatments. These results showed that the NLE supplement presented a potential hepatoprotective function alleviating liver damage, similar to the silymarin and the simvastatin treatments.

NLE Supplement Negligibly Affected Blood Urea Nitrogen and Creatinine in HFD-Fed Hamsters. To further investigate the effects of HFD and NLE supplement on kidney function in hamster, two common plasma markers for kidney disorder (blood urea nitrogen and creatinine) were determined. As shown in **Figure 7**, neither HFD nor HFD with NLE supplement significantly influenced the levels of blood urea nitrogen and creatinine. Similar results were obtained in HFD with silymarin and with simvastatin treatments (**Figure 7**).

NLE Supplement Reduced the Accumulation of Lipids in Liver in HFD-Fed Hamsters. Investigation for the effects of NLE supplement on lipid accumulation in liver was performed by H&E staining. As shown in **Figure 8**, the presence of numerous large lipid vesicles that compressed and displaced the nucleus to the periphery of hepatocytes was presented in the liver of HFD-fed hamsters. Interestingly, NLE supplement significantly and dose-dependently decreased the number of lipid vesicles increased by

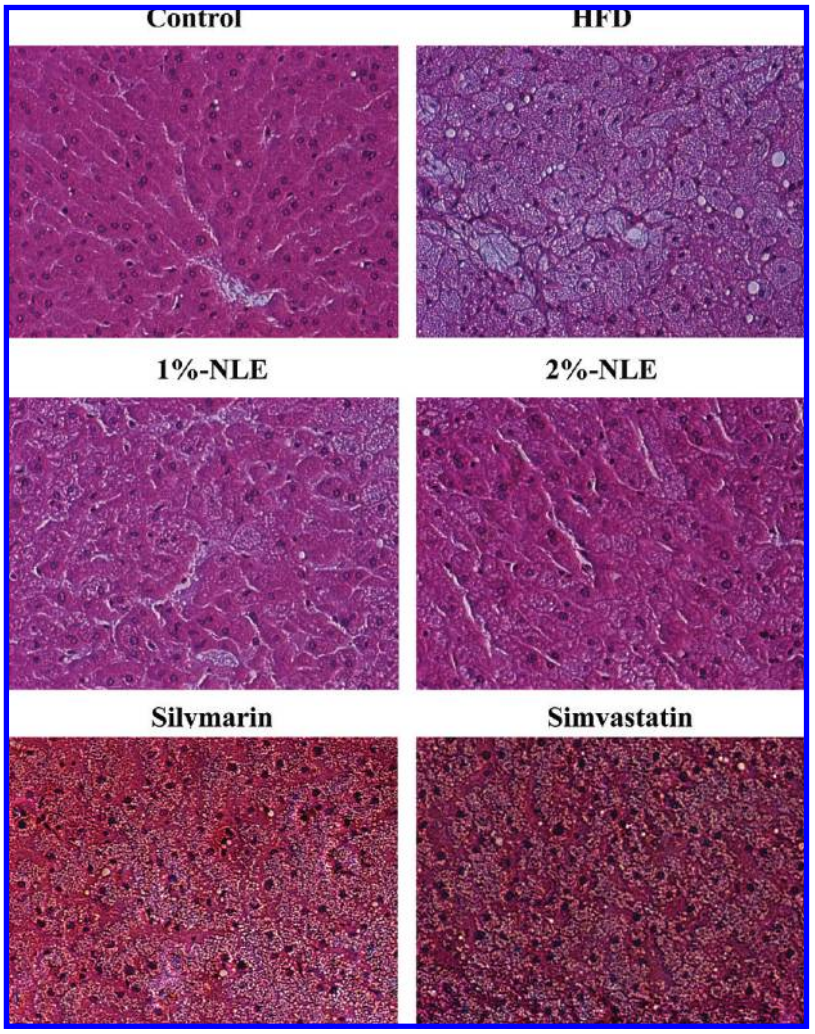

Figure 8. H&E staining of liver tissue in hamsters fed control diet and HFD: Control, normal diet; HFD, HFD without NLE; 1%-NLE, HFD with 1% NLE; 2%-NLE, HFD with 2% NLE; S, HFD with silymarin; Z, HFD with simvastatin. Hamsters in all groups were sacrificed after 10-week experiments. Liver tissues were obtained immediately after sacrifice and stained with H&E.

HFD in liver. The number of lipid vesicles increased by HFD in liver was also decreased by silymarin and simvastatin treatments. Moreover, the H&E stain of liver in hamsters by the silymarin and the simvastatin treatments showed a different morphological pattern from the 1% or 2% NLE supplement. These results indicate that NLE may decrease the accumulation of lipids in liver induced by HFD through a different mechanism from silymarin and simvastatin.

DISCUSSION

The increase of cardiovascular diseases, insulin resistance, and fatty liver has been reported to correlate closely to obesity resulting from excess intake of energy (*29*−*31*). Therefore, alleviating excess energy intake and lowering blood lipids have become urgent issues in modern life. NLE has been reported to reduce body weight and to inhibit absorption of lipids and carbohydrates in high-fat fed rats (*6*). The present study found that the HFD feed led to an increase in body weight of the hamsters, but the NLE supplement did not show inhibitory effects on the increase of body weight induced by HFD. The inconsistent results may result from the different extraction approach (alcoholic extract), the dose of NLE supplement (5% w/w), the duration of high-fat feeding (5 weeks), and the species (female ICR mice and male Wistar rats) that were used in the previous study (*6*). Accordingly, effects of the aqueous NLE on BW in high-fat fed animals will need further investigation.

According to the results, the effects of 2% NLE supplement on the decrease of TBARS, liver cholesterol, and liver triglycerides are better than the effects of 1% NLE supplement. However, 2% NLE supplement shows almost the same effects as 1% NLE supplement on the decrease of plasma triglycerides, plasma total cholesterol, LDL-C, and ratio of LDL-C to HDL-C. The findings suggest that the dose of NLE supplement used in this study is incompletely proportional to the effects on decreasing blood lipids and liver functions. Nevertheless, the higher NLE supplement shows significantly more effect on alleviating lipid accumulation in the liver.

To further evaluate the effects of aqueous NLE on hepatoprotection and reduction of blood lipids, two well-known drugs are used as standard reference in this study. Silymarin, a well-characterized bioactive complex from *Silybum marianum* L. Gaertn, is regarded as a hepatoprotective drug and used as standard reference in this study. Among the well-defined components in silymarin, the polyphenol silibinin, a flavolignane representing approximately 60% of silymarin, has been identified as the major active moiety and has been proposed as an antihepatotoxic agent for the treatment of various liver diseases (*32*, *33*). These positive effects have been ascribed to the putative antioxidant, anti-inflammatory, and antiproliferative

properties of silibinin based on the modulation of specific signaling pathway, transcription factor, and gene expression (*34*, *35*). Recently, silymarin has also been revealed to possess the ability to inhibit oxidation of LDL and to prevent the cardiotoxicity and nephrotoxicity that were induced by a potent anticancer agent, Adriamycin (*36*, *37*). In this study, NLE supplement showed similar effects on the alleviation of lipid peroxidation and liver damage in HFD-fed animals as compared to silymarin treatment. Furthermore, the level of decrease in liver cholesterol and liver triglycerides by NLE supplement was greater than by silymarin treatment. The findings suggest that NLE may become a potent hepatoprotective pharmaceutical. Simvastatin, a competitive inhibitor to 3-hydroxy-3-methylglutaryl-coenzyme A reductase, is generally used in the treatment of hyperlipidemia and hypercholesterolemia. Additionally, simvastatin also presented antimicrobial effects (*38*) and inhibition of iNOS-mediated NO production in endothelial cells (*39*). The present study shows that the NLE supplement decreased the blood lipids increased by HFD as significantly as simvastatin. Interestingly, the increase of HDL-C by NLE supplement is greater than with simvastatin treatment. Therefore, NLE supplement should be beneficial to the control of hyperlipidemia and hypercholesterolemia.

Antioxidant activity in botanical leaf extracts has been demonstrated to play an important role in preventing the liver injury induced by alcohol, acetaminophen, carbon tetrachloride, and D-galactosamine. It is also reported that botanical leaf extracts may achieve hepatoprotective activity through restoration of glutathione and increase of superoxide dismutase activity (*40*−*43*). Most therapeutic mechanisms of *N. nucifera* have been demonstrated as associating with the antioxidant activity to scavenge various free radicals. In *N. nucifera* seed, several important antioxidants have been identified and characterized, for example, oligomeric procyanidins, nelumboroside A, and nelumboroside B. These compounds revealed the ability to significantly reduce the free radicals 2,2′-diphenyl-1-picrylhydrazyl and authentic peroxynitrite, which are harmful to cells (*2*, *44*). Methanol extract of *N. nucifera* leaf also presents scavenging activities on free radicals and hydroxyl radicals (*22*). There are few studies demonstrating therapeutic effects of polysaccharide, protein, and lipid in *N. nucifera* extract. For this reason, we suggest that the antioxidants may play an important role in alleviating lipid accumulation and liver damage by HFD. However, the mechanisms of antioxidant activity of the flavonoid-enriched *N. nucifera* leaf extract for hepatoprotection and reduction of blood lipids will need further investigation.

In conclusion, the present study using a high-fat-fed animal model shows that the flavonoid-enriched NLE supplement significantly decreases blood lipids, liver lipids, lipid peroxidation, and the release of AST and ALT. Furthermore, the flavonoid-enriched NLE supplement also reduces the LDL-C to HDL-C ratio and lipid accumulation in the liver. These findings strongly indicate that the flavonoid-enriched NLE may provide novel and potent cardiovascular and hepatocellular protection from HFD-induced disorders.

LITERATURE CITED

(1) Liu, C. P.; Tsai, W. J.; Lin, Y. L.; Liao, J. F.; Chen, C. F.; Kuo, Y. C. The extracts from *Nelumbo nucifera* suppress cell cycle progression, cytokine genes expression, and cell proliferation in human peripheral blood mononuclear cells. *Life Sci.* **2004**, *75*, 699–716.

(2) Sohn, D. H.; Kim, Y. C.; Oh, S. H.; Park, E. J.; Li, X.; Lee, B. H. Hepatoprotective and free radical scavenging effects of *Nelumbo nucifera*. *Phytomedicine* **2003**, *10*, 165–169.

(3) Mukherjee, P. K.; Saha, K.; Pal, M.; Saha, B. P. Effect of *Nelumbo nucifera* rhizome extract on blood sugar level in rats. *J. Ethnopharmacol.* **1997**, *58*, 207–213.

(4) Mukherjee, P. K.; Saha, K.; Das, J.; Pal, M.; Saha, B. P. Studies on the anti-inflammatory activity of rhizomes of *Nelumbo nucifera*. *Planta Med* **1997**, *63*, 367–369.

(5) Mukherjee, P. K.; Das, J.; Saha, K.; Giri, S. N.; Pal, M.; Saha, B. P. Antipyretic activity of *Nelumbo nucifera* rhizome extract. *Indian J. Exp. Biol.* **1996**, *34*, 275–276.

(6) Ono, Y.; Hattori, E.; Fukaya, Y.; Imai, S.; Ohizumi, Y. Anti-obesity effect of *Nelumbo nucifera* leaves extract in mice and rats. *J. Ethnopharmacol.* **2006**, *106*, 238–244.

(7) Ohkoshi, E.; Miyazaki, H.; Shindo, K.; Watanabe, H.; Yoshida, A.; Yajima, H. Constituents from the leaves of *Nelumbo nucifera* stimulate lipolysis in the white adipose tissue of mice. *Planta Med.* **2007**, *73*, 1255–1259.

(8) Damjanovic, M.; Barton, M. Fat intake and cardiovascular res ponse. *Curr. Hypertens. Rep.* **2008**, *10*, 25–31.

(9) Cerf, M. E. High fat diet modulation of glucose sensing in the beta-cell. *Med. Sci. Monit.* **2007**, *13*, RA12–RA17.

(10) Glass, C. K.; Witztum, J. L. Atherosclerosis the road ahead. *Cell* **2001**, *104*, 503–516.

(11) Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **1989**, *320*, 915–924.

(12) Steinberg, D. Role of oxidized LDL and antioxidants in atherosclerosis. *Adv. Exp. Med. Biol.* **1995**, *369*, 39–48.

(13) Rusinol, A. E.; Yang, L.; Thewke, D.; Panini, S. R.; Kramer, M. F.; Sinensky, M. S. Isolation of a somatic cell mutant resistant to the induction of apoptosis by oxidized low density lipoprotein. *J. Biol. Chem.* **2000**, *275*, 7296–7303.

(14) Lovejoy, J.; DiGirolamo, M. Habitual dietary intake and insulin sensitivity in lean and obese adults. *Am. J. Clin. Nutr.* **1992**, *55*, 1174–1179.

(15) Marshall, J. A.; Hoag, S.; Shetterly, S.; Hamman, R. F. Dietary fat predicts conversion from impaired glucose tolerance to NIDDM. The San Luis Valley Diabetes Study. *Diabetes Care* **1994**, *17*, 50–56.

(16) Vessby, B.; Aro, A.; Skarfors, E.; Berglund, L.; Salminen, I.; Lithell, H. The risk to develop NIDDM is related to the fatty acid composition of the serum cholesterol esters. *Diabetes* **1994**, *43*, 1353–1357.

(17) Laing, S. P.; Swerdlow, A. J.; Slater, S. D.; Burden, A. C.; Morris, A.; Waugh, N. R.; Gatling, W.; Bingley, P. J.; Patterson, C. C. Mortality from heart disease in a cohort of 23,000 patients with insulin-treated diabetes. *Diabetologia* **2003**, *46*, 760–765.

(18) Cong, W. N.; Tao, R. Y.; Tian, J. Y.; Liu, G. T.; Ye, F. The establishment of a novel non-alcoholic steatohepatitis model accompanied with obesity and insulin resistance in mice. *Life Sci.* **2008**, *82*, 983–990.

(19) Caldwell, S. H.; Oelsner, D. H.; Iezzoni, J. C.; Hespenheide, E. E.; Battle, E. H.; Driscoll, C. J. Cryptogenic cirrhosis: clinical characterization and risk factors for underlying disease. *Hepatology* **1999**, *29*, 664–669.

(20) Bugianesi, E.; Leone, N.; Vanni, E.; Marchesini, G.; Brunello, F.; Carucci, P.; Musso, A.; De Paolis, P.; Capussotti, L.; Salizzoni, M.; Rizzetto, M. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology* **2002**, *123*, 134–140.

(21) Ratziu, V.; Bonyhay, L.; Di Martino, V.; Charlotte, F.; Cavallaro, L.; Sayegh-Tainturier, M. H.; Giral, P.; Grimaldi, A.; Opolon, P.; Poynard, T. Survival, liver failure, and hepatocellular carcinoma in obesity-related cryptogenic cirrhosis. *Hepatology* **2002**, *35*, 1485–1493.

(22) Lee HJC, C. C.; Chou, F. P.; Wu, C. H.; Lai, F. S.; Yang, M. Y.; Wang, C. J. Water extracts from *Nelumbo nucifera* leaf reduced plasma lipids and atherosclerosis in cholesterol fed rabbits. *J. Food Biochem.* **2008**, in press.

(23) Folch, J.; Lees, M.; Sloane Stanley, G. H. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509.

(24) Ohkawa, H; Ohishi, N; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **1979**, *95*, 351–358.

(25) Bergmeyer, H. U.; Bowers, G. N.Jr.; horder, M.; Moss, D. W. Provisional recommendations on IFCC methods for the measurement of catalytic concentrations of enzymes. Part 2. IFCC method for aspartate aminotransferase. *Clin. Chim. Acta* **1976**, *70*, F19–29.
(26) Mazzachi, B. C.; Peake, M. J.; Ehrhardt, V. Reference range and method comparison studies for enzymatic and Jaffe creatinine assays in plasma and serum and early morning urine. *Clin. Lab. (Heidelberg, Ger.)* **2000**, *46*, 53–55.
(27) Tietz, N. W.; Thompson, J. Possible concentration-dependent suppression of immune response by verapamil. *Arch. Fam. Med.* **1995**, *4*, 368–369.
(28) Lin, C. C.; Shieh, D. E.; Yen, M. H. Hepatoprotective effect of the fractions of Ban-zhi-lian on experimental liver injuries in rats. *J. Ethnopharmacol.* **1997**, *56*, 193–200.
(29) Hill, J. O.; Wyatt, H. R.; Reed, G. W.; Peters, J. C. Obesity and the environment: where do we go from here?. *Science* **2003**, *299*, 853–855.
(30) Klein, S.; Burke, L. E.; Bray, G. A.; Blair, S.; Allison, D. B.; Pi-Sunyer, X.; Hong, Y.; Eckel, R. H. Clinical implications of obesity with specific focus on cardiovascular disease: a statement for professionals from the American Heart Association Council on Nutrition, Physical Activity, and Metabolism: endorsed by the American College of Cardiology Foundation. *Circulation* **2004**, *110*, 2952–2967.
(31) Larsson, B.; Bjorntorp, P.; Tibblin, G. The health consequences of moderate obesity. *Int. J. Obes.* **1981**, *5*, 97–116.
(32) Wen, Z.; Dumas, T. E.; Schrieber, S. J.; Hawke, R. L.; Fried, M. W.; Smith, P. C. Pharmacokinetics and metabolic profile of free, conjugated, and total silymarin flavonolignans in human plasma after oral administration of milk thistle extract. *Drug Metab. Dispos.* **2008**, *36*, 65–72.
(33) Schümann, J.; Prockl, J.; Kiemer, A. K.; Vollmar, A. M.; Bang, R.; Tiegs, G. Silibinin protects mice from T cell-dependent liver injury. *J. Hepatol.* **2003**, *39*, 333–340.
(34) Katiyar, S. K.; Roy, A. M.; Baliga, M. S. Silymarin induces apoptosis primarily through a p53-dependent pathway involving Bcl-2/Bax, cytochrome *c* release, and caspase activation. *Mol. Cancer Ther.* **2005**, *4*, 207–216.
(35) Roy, S.; Kaur, M.; Agarwal, C.; Tecklenburg, M.; Sclafani, R. A.; Agarwal, R. p21 and p27 induction by silibinin is essential for its cell cycle arrest effect in prostate carcinoma cells. *Mol. Cancer Ther.* **2007**, *6*, 2696–2707.
(36) Wallace, S.; Vaughn, K.; Stewart, B. W.; Viswanathan, T.; Clausen, E.; Nagarajan, S.; Carrier, D. J. Milk thistle extracts inhibit the oxidation of low-density lipoprotein (LDL) and subsequent scavenger receptor-dependent monocyte adhesion. *J. Agric. Food Chem.* **2008**, *56*, 3966–3972.
(37) El-Shitany, N. A.; El-Haggar, S.; El-Desoky, K. Silymarin prevents adriamycin-induced cardiotoxicity and nephrotoxicity in rats. *Food Chem. Toxicol.* **2008**, *46*, 2422–2428.
(38) Jerwood, S.; Cohen, J. Unexpected antimicrobial effect of statins. *J. Antimicrob. Chemother.* **2008**, *61*, 362–364.
(39) Alvarez de Sotomayor, M.; Vega, S.; Mingorance, C.; Marhuenda, E.; Herrera, M. D. Effects of HMG-CoA reductase inhibition by simvastatin on vascular dysfunction induced by lipopolysaccharide in rats. *Pharmacology* **2008**, *82*, 89–96.
(40) Faremi, T. Y.; Suru, S. M.; Fafunso, M. A.; Obioha, U. E. Hepatoprotective potentials of *Phyllanthusamarus* against ethanol-induced oxidative stress in rats. *Food Chem. Toxicol.* **2008**.
(41) Fakurazi, S.; Hairuszah, I.; Nanthini, U. *Moringa oleifera* Lam prevents acetaminophen induced liver injury through restoration of glutathione level. *Food Chem. Toxicol.* **2008**.
(42) Jain, A.; Soni, M.; Deb, L.; Rout, S. P.; Gupta, V. B.; Krishna, K. L. Antioxidant and hepatoprotective activity of ethanolic and aqueous extracts of *Momordica dioica* Roxb. leaves. *J. Ethnopharmacol.* **2008**, *115*, 61–66.
(43) Yoo, Y. M.; Nam, J. H.; Kim, M. Y.; Choi, J.; Park, H. J. Pectolinarin and pectolinarigenin of *Cirsium setidens* prevent the hepatic injury in rats caused by D-galactosamine via an antioxidant mechanism. *Biol. Pharm. Bull.* **2008**, *31*, 760–764.
(44) Jung, H. A.; Kim, J. E.; Chung, H. Y.; Choi, J. S. Antioxidant principles of *Nelumbo nucifera* stamens. *Arch. Pharm. Res.* **2003**, *26*, 279–285.

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