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Improvement in High-Fat Diet-Induced Obesity and Body Fat Accumulation by a *Nelumbo nucifera* Leaf Flavonoid-Rich Extract in Mice

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Diets high in fat lead to excessive lipid accumulation in adipose tissue, which is a crucial factor in the development of obesity, hepatitis, and hyperlipidemia. In this study, we investigated the antiobesity effect of a flavonoid-enriched extract from *Nelumbo nucifera* leaf (NLFE) in vivo. C57BL/6 mice were fed with a high-fat diet (HFD) to induce obesity. NLFE reduced the body weight, body lipid accumulation, and activities of fatty acid synthase (FAS), glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase. NLFE also suppressed the expression of FAS, acetyl-CoA carboxylase, and HMGCoA reductase and increased the phosphorylation of AMP-activated protein kinase in the liver. Taken together, we demonstrated that NLFE targets lipid-regulated enzymes and may be effective in attenuating body lipid accumulation and preventing obesity.

KEYWORDS: Obesity; *Nelumbo nucifera* leaf flavonoid-enriched extract; fatty acid synthase; AMPK

INTRODUCTION

Obesity is characterized by an increase in fat mass and body weight (*1*). Obesity can induce all of the symptoms of metabolic syndrome, which is associated with many additional health problems, including an increased risk of insulin resistance, nonalcoholic fatty liver disease (NAFLD), atherosclerosis, degenerative disorders (e.g., dementia), immune-mediated disorders (e.g., asthma), and cancers (*2*, *3*). Therefore, prevention and treatment of obesity are relevant to health promotion.

The underlying cause of fat accumulation in patients with NAFLD is mostly due to the synthesis of fatty acids and inhibition of fatty acid oxidation (*4*). Differentiated adipocytes store fatty acids in the form of triglycerides (TGs) in the cytoplasm, with the involvement of various enzymes, such as fatty acid synthase (FAS (*5*)). Previous research has shown that the hepatic TG content is significantly correlated with the plasma TG level and fat mass in humans (*6*). TGs are synthesized in the liver, secreted into the bloodstream, and transported to the peripheral organs, including the adipose tissues (*7*, *8*). It is known that the availability of hepatic TG is controlled by the balance between fatty acid synthesis and oxidation in the liver (*9*). A high intake of saturated fatty acids is associated with a high level of serum cholesterol (*10*). It is believed that circulating concentrations of lipids and free fatty acids are increased after high-fat feeding, and hyperlipidemia, as well as elevated plasma low-density lipoprotein (LDL), may result in the initiation of atherosclerosis (*11*−*13*).

AMP-activated protein kinase (AMPK) monitors intracellular energy status and regulates the uptake and metabolism of glucose and fatty acids, as well as the synthesis and oxidation of fatty acids, cholesterol, glycogen, and protein, to meet energy demands (*14*, *15*). Once activated, AMPK phosphorylates and inactivates a number of metabolic enzymes involved in fatty acid and cholesterol synthesis, including FAS, acetyl-CoA carboxylase (ACC (*16*)), and HMGCoA reductase (HMGCR (*17*)). The activation of the AMPK pathway is necessary to prevent fat accumulation.

Nelumbo nucifera Gaertn, also known as the sacred lotus, is a flavonoid-rich plant widely distributed in Taiwan. In southeastern Asia, the lotus seed and lotus root are usually regarded as popular foods, and the lotus leaf is also used as a common relish in traditional Taiwanese foods. All parts of *N. nucifera*, including the leaves, flowers, stamens, embryos, and rhizomes, have been used as traditional medicines and have pharmacologic properties, including hepatoprotection (*18*), anti-HIV activity (*19*), antioxidant activity (*20*), antipyretic effects (*21*), and prevention of atherosclerosis (*22*, *23*) and fatty liver (*24*).

In this study, we evaluated the antiobesity activity of *N. nucifera* leaf (NLFE) in an animal model with high-fat diet (HFD)-induced obesity and elucidated the mechanisms underlying such an effect as exerted in visceral adipose tissues.

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MATERIALS AND METHODS

Preparation of Aqueous NLFE. The purchased *N. nucifera* leaves were washed with distilled water and air-dried. An amount of 20 g of dried leaves was resuspended in 100 mL of distilled water at 4 °C overnight. The supernatant was filtered through two layers of gauze to remove the debris and then lyophilized. The dry weight yield of NLFE was approximately 15%. The concentrations of total phenolic acids and the total flavonoids content were measured, as described previously (*25*). The polysaccharide and protein content in NLFE was determined using the phenolsulfuric acid method and a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA), respectively.

Animals and Experimental Design. All animal experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University in Taichung, Taiwan. Male C57BL/6 mice, aged 4−5 weeks and weighing 20 g, were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and housed in standard laboratory conditions (18−23 °C, humidity 55−60%, and 12 h light/dark cycle). After 1 week of adaptation to the environment, the mice were randomly divided into six groups (n=10/group) as follows: control (normal meals); HFD (normal meals containing 20% lard oil and 0.5% cholesterol); HFD supplemented with 0.5% NLFE, 1.5% NLFE, 1 mg/kg simvastatin (Z), or 100 mg/kg silymarin (S). After 6 weeks of feeding on the aforementioned diets, whole blood and livers were collected from mice that had fasted for 12−14 h and were then sacrificed.

Blood Sample Analysis. Blood samples were collected with EDTA tubes and immediately centrifuged at 1500*g* for 10 min at 4 °C. The serum was decanted and stored at 4 °C. Biochemical examinations were performed within 1 h after specimen collection. Serum levels of total cholesterol, TG, glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) were measured using clinical chemistry reagent kits (Randox Laboratories, Antrim, U.K.). Free fatty acids were assayed with a free fatty acid quantification kit (BioVision, Mountain View, CA) according to the manufacturer's protocol. Briefly, the free fatty acids in plasma were converted to their CoA derivatives, which were subsequently oxidized with the concomitant generation of color. Free fatty acids then can be easily quantified by colorimetric (spectrophotometry at λ = 570 nm) method.

Determination of Total Cholesterol and TGs in the Liver. After removal from the animals, a portion of the fresh liver was collected for liver lipid extraction. Liver lipids were extracted as described previously (*26*). Briefly, liver (1.25 g) was homogenized with chloroform/methanol (1:2, 3.75 mL). 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 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 liver TGs and liver cholesterol in the lipid extracts were measured by enzymatic colorimetric methods using commercial kits (HUMAN, Wiesbaden, Germany).

FAS Activity Assay. The FAS activity assay was performed as described by Goodridge et al. (*27*). The serum sample was used in the assays. FAS activity was measured by following the decrease in absorbance at 340 nm resulting from the oxidation of NADPH, which was dependent on added malonyl-CoA at 40 °C. Each cuvette contained 0.1 M potassium phosphate (pH 7.0), 3 mM EDTA, 0.1 mM NADPH, 25 mM acetyl-CoA, 1 mM DTT (all from Sigma-Aldrich, St. Louis, MO), and the sample. The reaction was initiated by adding malonyl-CoA (Sigma-Aldrich) to a final concentration of 0.1 mM. The doubly distilled H_2O was used for the blanks instead of the samples. Under these conditions, FAS activity was linear with respect to both time (for at least 10 min) and protein (0−200 mg/mL); 1 U of activity equals 1 nmol of palmitate formed per min (equivalent to the oxidation of 14 nmol of NADPH).

Preparation of Protein Extract of Liver Tissue. The protein from liver tissues was harvested in a cold RIPA buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, and 150 mM NaCl [pH 7.5]) containing leupeptin (17 μg/mL) and sodium orthovanadate (10 μg/mL). The cell mixtures were vortexed at 4 °C for 4 h, and the liver tissues were homogenized on ice for 3 min. All mixtures were then centrifuged at 12 000 rpm at 4 °C for 10 min, and the protein content of the supernatants was determined with Coomassie blue total protein reagent (Kenlor Industries, Santa Ana, CA) using bovine serum albumin as a standard.

Table 1. Components of NLFE

components of NLFE	percentage (%)
polyphenolic content	13.5 ± 0.28
flavanoids content	58.3 ± 0.03
protein content	3.9 ± 3.02
polysaccharide content	15.2 ± 4.05
others	9.1

Western Blot Analysis. Equal amounts of protein samples (80 μg) were subjected to SDS−polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat milk powder with 0.05% Tween-20 in PBS and incubated with the primary antibody at 4 °C overnight. The membranes were then washed three times with 0.05% Tween-20 in PBS and incubated with the secondary antibody conjugated to antimouse horseradish peroxidase (GE Healthcare, Buckinghamshire, U.K.). Bands were detected by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed ECL hyperfilm in FUJFILM Las-3000 (Tokyo, Japan). Protein quantity was determined by densitometry using FUJIFILM Multi Gauge, version 2.2, software.

Statistical Analysis. Data were analyzed using an unpaired t-test and represented as the mean ± SD. $p < 0.05$ was considered statistically significant.

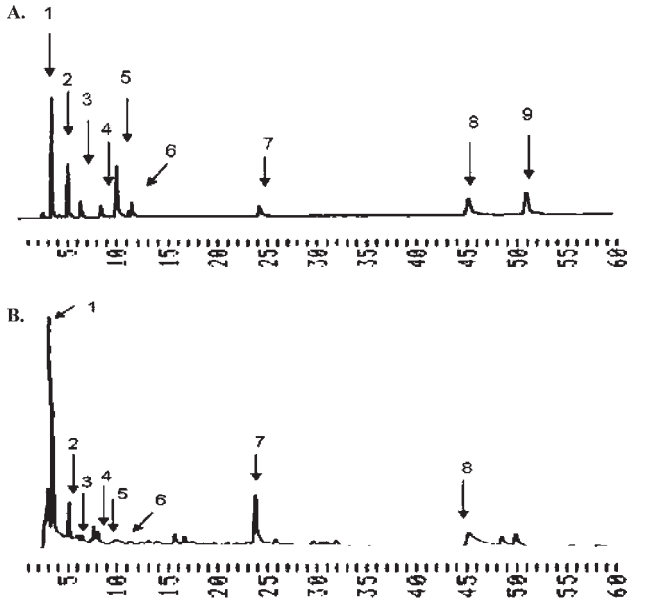
RESULTS

Components of NLFE. To evaluate the plausible functional elements of NLFE, a component analysis of NLFE was performed. **Table 1** shows that NLFE consisted of 13.5% total phenolic acids, 58.3% flavonoids, 3.9% protein, and 15.2% polysaccharides by spectrophotometric analysis. On the basis of the results, flavonoids were the major polyphenols in NLFE. **Figure 1** showed that the major polyphenols of NLFE by HPLC analysis were as follows: 20.1% gallic acid, 1.9% protocatechuic acid, 2.5% catechin, 1.4% gallocatechin gallate, 1.0% caffeic acid, 1.4% epicatechin, 6.8% rutin, 4.8% quercetin. This result was similar to those of Lee et al. (*23*).

Effect of NLFE, Simvastatin, and Silymarin on Body Weight. We compared the effect of simvastatin and silymarin with NLFE on lipid homeostasis. Statins (a HMGCR inhibitor) are a class of drugs that lower cholesterol levels in humans. By feeding mice (C57BL/6) a HFD and different concentrations of simvastatin, silymarin, and NLFE for 6 weeks, we compared relative body weight changes. As shown in **Table 2**, mice fed with HFD alone had a weight increase from 1.18% in week 2 to 21.00% in week 6. Mice fed with 1 mg/kg simvastatin had a weight gain of 1.25% in week 2 to 17.56% in week 6. Mice fed with 100 mg/kg silymarin had a weight gain of 2.54% in week 2 to 19.24% in week 6. Mice fed with 0.5% NLFE increased in weight from 1.93% in week 2 to 14.14% in week 6, while mice fed with 1.5% NLFE increased in weight from 0.73% in week 2 to 13.52% in week 6. The results showed that feeding with NLFE was better than simvastatin and silymarin for decreasing weight gain.

Effect of NLFE, Simvastatin, and Silymarin on Serum Biochemical Markers and FAS Activity. After feeding mice with both HFD and 1 mg/kg simvastatin, 100 mg/kg silymarin, 0.5% NLFE, or 1.5% NLFE for 6 weeks, animals were sacrificed and the serum content of GOT, GPT, cholesterol, TGs, free fatty acids, and FAS activity was analyzed. The results showed that the serum GOT of mice fed with HFD only was increased to 325.21 ± 43.33 U/L, but in mice fed with 1.5% NLFE, simvastatin, or silymarin, the serum GOT decreased to 242.23 ± 21.12 U/L ($p < 0.05$), 262.60 ± 23.86 U/L, and 258.80 ± 61.94 U/L, respectively (**Figure 2A**). The serum GPT level of mice fed with HFD alone increased to 72.25 ± 6.70 U/L, but in mice fed 1.5% NLFE, simvastatin, and silymarin the serum GPT decreased to 53.25 ± 8.09 U/L ($p < 0.01$), 62.43 ± 5.12 U/L

($p < 0.05$), and 56.80 ± 6.53 U/L ($p < 0.05$), respectively (**Figure 2B**). We also found that the serum cholesterol content in the HFD group increased to 154.20 ± 5.16 mg/dL, and in the 1.5% NLFE, simvastatin, and silymarin groups the serum cholesterol decreased to 117.20 ± 4.60 mg/dL ($p < 0.01$), 116.60 ± 7.33 mg/dL ($p < 0.05$), and 138.80 ± 5.89 mg/dL ($p < 0.01$), respectively (**Figure 3A**). The serum TG content of mice fed with HFD increased to 194.40 ± 7.53 mg/dL, and in mice fed with 1.5% NLFE, simvastatin, and silymarin, the serum TG decreased to 170.11 ± 19.67 mg/dL ($p < 0.05$), 116.80 ± 9.93 mg/dL ($p < 0.01$), and 68.80 ± 9.93 mg/dL ($p < 0.05$), respectively (**Figure 3B**). In addition, the FFA content of mice fed with HFD increased to 9.20 ± 2.18 nmol, but in groups of mice that were fed with 1.5% NLFE, simvastatin, and silymarin the FFA was decreased to 1.62 ± 0.20 nmol ($p < 0.01$), 2.72 ± 1.09 nmol ($p < 0.05$), and 1.94 ± 0.56 nmol ($p < 0.05$), respectively (**Figure 4A**). Finally, the FAS activity of mice fed with HFD increased to 0.023 ± 0.003 (U/min)/mg protein, but in mice treated with 1.5% NLFE, simvastatin, and silymarin, the FAS activity was decreased to 0.007 ± 0.002 (U/min)/mg protein ($p < 0.05$), 0.013 ± 0.001 (U/min)/mg protein ($p < 0.05$), and 0.018 ± 0.004 (U/min)/mg protein, respectively (**Figure 4B**). Thus, as



Peak No	Phenolic compound	%
1	Gallic acid	20.13 ± 0.97
2	Protocatechuic acid (PCA)	1.88 ± 1.09
3	Catechin	2.54 ± 2.32
4	Gallocatechin gallate (GCG)	1.39 ± 0.77
5	Caffeic acid	1.04 ± 1.20
6	Epicatechin	1.41 ± 0.22
7	Rutin	6.80 ± 3.32
8	Quercetin	4.79 ± 1.45

Figure 1. HPLC chromatogram of NLFE. (**A**) HPLC chromatogram of nine kinds of standard polyphenols (1 mg/mL, 10 μL): (1) gallic acid; (2) protocatechuic acid (PCA); (3) catechin; (4) gallocatechin (GC); (5) caffeic acid; (6) gallocatechin gallate (GCG); (7) rutin; (8) quercetin; (9) naringenin. (**B**) HPLC chromatogram of free polyphenols from NLFE (10 mg/mL, 10 μL).

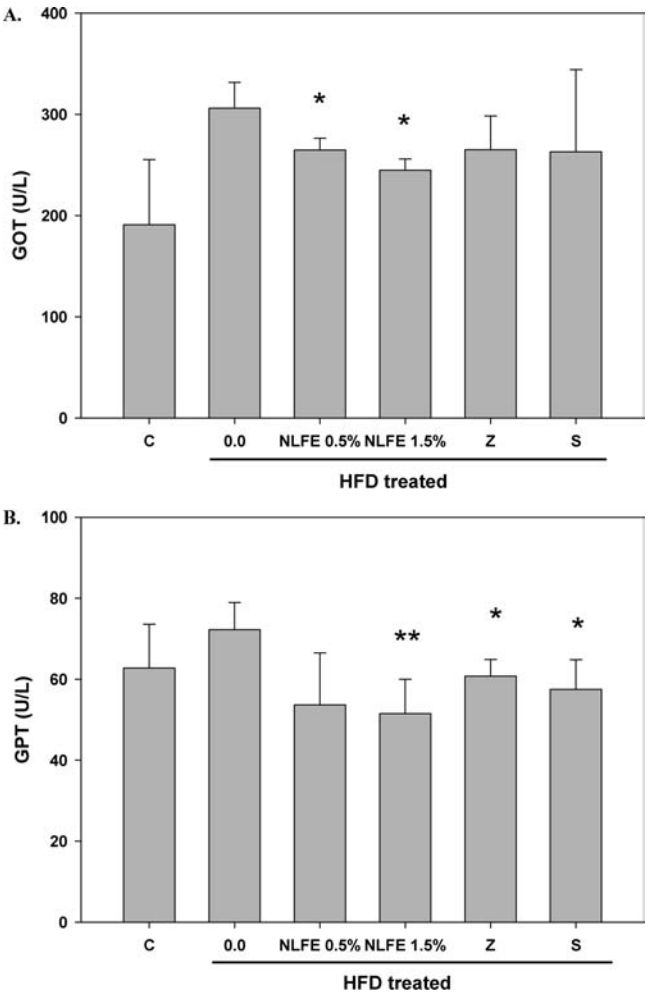


Figure 2. Effects of NLFE supplement on the activities of plasma GOT and GPT in C57BL/6 mice fed HFD. Plasma GOT and GPT in C57BL/6 mice fed HFD and HFD containing 0.5% NLFE, 1.5% NLFE, simvastatin (Z), and silymarin (S). Corresponding levels in C57BL/6 mice fed a normal diet (C) were used as a control. Data show (**A**) GOT and (**B**) GPT values from male mice ($n = 10$) on the indicated treatments for 6 weeks as the mean ± SD: (*) $p < 0.05$ compared with HFD group; (**) $p < 0.01$ compared with HFD group.

Table 2. Comparison of Relative Body Weight Change in Simvastatin, Silymarin, and NLFE Treated Mice[a]

		HFD treated				
		NLFE (%)				
	C	0	0.5	1.5	Z	S
2 week	0.92 ± 0.41	1.18 ± 0.36	1.93 ± 0.38[c]	0.73 ± 2.38	1.25 ± 1.96	2.54 ± 1.58
4 week	9.26 ± 1.13	19.82 ± 1.45	12.47 ± 2.43	13.18 ± 2.70[b]	15.55 ± 0.97[b]	18.64 ± 1.71[b]
6 week	12.39 ± 4.37	21.00 ± 2.99	14.14 ± 1.27[b]	13.52 ± 2.43[b]	17.56 ± 2.96[b]	19.24 ± 1.80[b]

[a] Animals were fed a normal diet (C), a high fat diet (HFD alone), or HFD pair feeding with a 0.5% NLFE, 1.5% NLFE, 1 mg/kg simvastatin (Z) or 100 mg/kg silymarin (S) for 6 weeks ($n = 10$). Data show values from male mice ($n = 10$) on the indicated treatments for 6 weeks as the mean ± SD. All data are [(week X − week 0)/week 0]×100%. [b] $p < 0.05$, with respect to high-fat diet-treated group. [c] $p < 0.01$, with respect to high-fat diet-treated group.

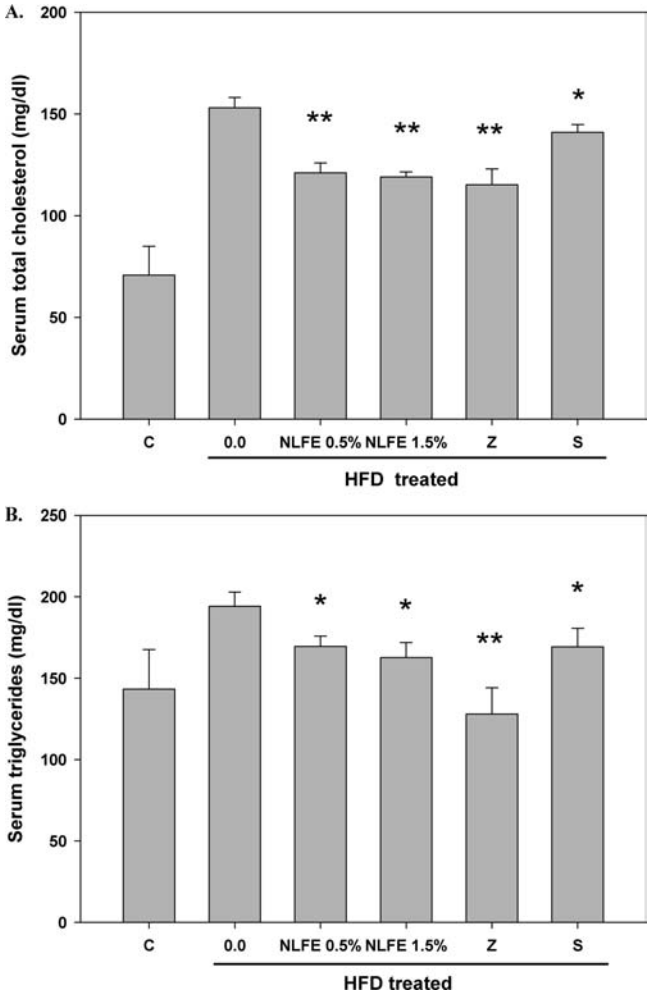


Figure 3. Effects of a NLFE supplement on plasma cholesterol and triglycerides in C57BL/6 mice fed HFD: (**A**) plasma cholesterol and (**B**) triglycerides in C57BL/6 mice fed HFD and HFD containing 0.5% NLFE, 1.5% NLFE, simvastatin (Z), and silymarin (S). Corresponding levels in C57BL/6 mice fed a normal diet (C) were used as a control. Data are shown as the mean ± SD: (*) $p < 0.05$ compared with HFD group; (**) $p < 0.01$ compared with HFD group.

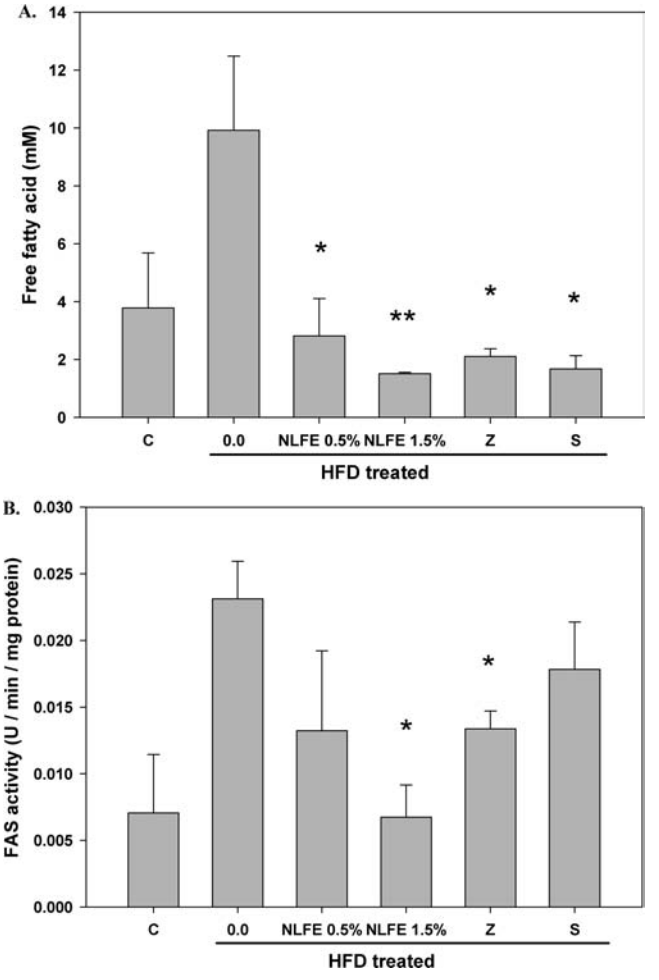


Figure 4. Effects of a NLFE supplement on plasma free fatty acid and the activities of fatty acid synthase in C57BL/6 mice fed HFD: (**A**) plasma free fatty acid and (**B**) the activities of fatty acid synthase in C57BL/6 mice fed HFD, and HFD containing 0.5% NLFE, 1.5% NLFE, simvastatin (Z), and silymarin (S). Corresponding levels in C57BL/6 mice fed a normal diet (C) were used as a control. Data are shown as the mean ± SD: (*) $p < 0.05$ compared with HFD group; (**) $p < 0.01$ compared with HFD group.

shown in **Figures 2**−**4**, NLFE reduced the levels of GOT, GPT, FFA, and FAS activity to a greater extent than simvastatin and silymarin in order to inhibit lipid accumulation.

Effect of NLFE, Simvastatin, and Silymarin on Liver Weight and Hepatic Lipids. We examined the fat content of the liver in mice fed with HFD, 0.5% NLFE, 1.5% NLFE, 1 mg/kg simvastatin, and 100 mg/kg silymarin. We compared the liver weight of different groups. **Table 3** shows that the liver weight of mice fed with a normal diet, HFD alone, 0.5% NLFE, 1.5% NLFE, simvastatin, and silymarin was 4.58 ± 1.65 g, 5.28 ± 0.26 g, 5.21 ± 0.33 g, 4.81 ± 0.13 g ($p < 0.01$), 5.16 ± 0.40 g ($p < 0.05$), and 4.67 ± 0.13 g ($p < 0.01$), respectively. The TG content in mice fed with a normal diet, HFD, 0.5% NLFE, 1.5% NLFE, simvastatin, and silymarin was 227.12 ± 101.72 mg/dL, 624.90 ± 27.03 mg/dL, 389.88 ± 52.09 mg/dL ($p < 0.01$), 325.68 ± 86.56 mg/dL ($p < 0.01$), 473.06 ± 142.81 mg/dL ($p < 0.05$), and 565.16 ± 94.97 mg/dL ($p < 0.05$), respectively. The cholesterol content in mice fed with a normal diet, HFD, 0.5% NLFE, 1.5% NLFE, simvastatin, and silymarin was 221.23 ± 27.18 mg/dL, 908.62 ± 269.22 mg/dL, 424.23 ± 18.90 mg/dL ($p < 0.01$), 374.76 ± 2.99 mg/dL ($p < 0.01$), 374.7 ± 78.08 mg/dL ($p < 0.01$), and 425.34 ± 67.47 mg/dL ($p < 0.01$), respectively. As shown in **Table 3**, we suggest that NLFE is better than simvastatin and silymarin in reducing TG content.

Effect of NLFE, Simvastatin, and Silymarin on Body Fat in HFD-Fed Mice. The body fat index is a ratio of the total adipose tissue (retroperitoneal, epididymal, and mesenteric) and body weight. **Figure 5A** shows the epididymal fat tissue from each group dissected at the end of the experiments. Quantification of the body fat index showed that there was an increase in the body fat index in the HFD group in contrast to decreased values for rats from the groups of mice fed with NLFE ($p < 0.01$), simvastatin ($p < 0.05$), and silymarin ($p < 0.05$; **Figure 5B**).

Effect of NLFE, Simvastatin, and Silymarin on Hepatic Lipid Metabolism in HFD-Fed Mice. Previous in vitro experiments showed that activation of AMPK-p could limit lipid accumulation and the activation of FAS and ACC. In animal experiments, we examined the protein change of liver lipid metabolism in mice fed with HFD, 0.5% NLFE, 1.5% NLFE, simvastatin, and silymarin. By using Western blot, we analyzed the protein expression of AMPK-p, FAS, ACC, and HMGCR (**Figure 6**). All groups of mice were similar in reducing FAS as simvastatin and silymarin. The group of mice fed 1.5% NLFE had enhanced activity of AMPK-p compared to simvastatin and silymarin. When the expression of ACC and HMGCR are compared,

Table 3. Comparison of Liver Weight, Triglycerides, and Cholesterol Content in Simvastatin, Silymarin, and NLFE Treated Mice[a]

		HFD treated				
		NLFE (%)				
	C	0	0.5	1.5	Z	S
liver relative weight (g/100 g body weight)	4.58 ± 1.65	5.28 ± 0.26	5.21 ± 0.33	4.81 ± 0.13[c]	5.16 ± 0.40[b]	4.67 ± 0.13[c]
liver triglycerides content (mg/dL)	227.12 ± 101.72	624.90 ± 27.03	389.88 ± 52.09[c]	325.68 ± 86.56[c]	473.06 ± 142.81[b]	565.16 ± 94.97[b]
liver cholesterol content (mg/dL)	221.23 ± 27.18	908.62 ± 269.22	424.23 ± 18.90[c]	374.76 ± 62.99[c]	374.70 ± 78.08[c]	425.34 ± 67.47[c]

[a] Animals were fed a normal diet (C), a high fat diet (HFD alone), or HFD pair feeding with a 0.5% NLFE, 1.5% NLFE, 1 mg/kg simvastatin (Z) or 100 mg/kg silymarin (S) for 6 weeks ($n = 10$). Data show values from male mice ($n = 10$) on the indicated treatments for 6 weeks as the mean ± SD. [b] $p < 0.05$, with respect to high-fat diet-treated group. [c] $p < 0.01$, with respect to high-fat diet-treated group.

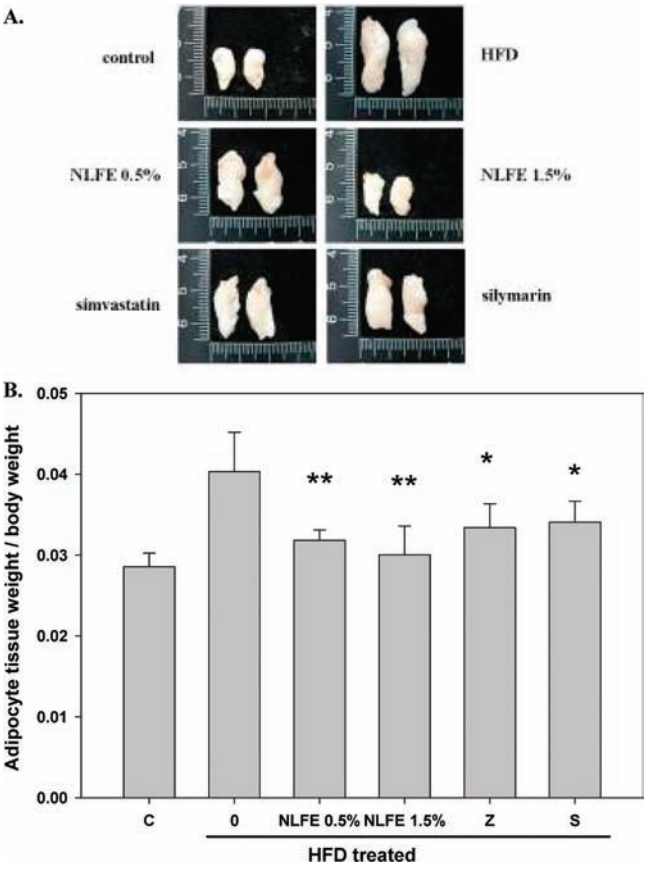


Figure 5. NLFE treatments reduced body fat accumulation. (**A**) Representative gonadal fat tissue from each group was dissected at the end of experiment. Animals were fed a normal diet (C) or HFD, or HFD pair feeding with 0.5% NLFE, 1.5% NLFE, simvastatin (Z), and silymarin (S) for 6 weeks ($n = 10$). (**B**) Body fat index (total adipose tissue/body weight) of each group. Data are shown as the mean ± SD: (∗) $p < 0.05$ compared with HFD group.

NLFE, simvastatin, and silymarin reduced the expression of ACC and HMGCR. Therefore, in vivo experiments revealed that through lipid metabolism-related enzymes, NLFE lowered lipid accumulation, and it was much more effective than simvastatin and silymarin.

DISCUSSION

Excess energy is conserved in the form of fat in adipose tissues, leading to obesity and obesity-associated fatty liver disease (FLD) (*28*). Obesity is closely associated with lifestyle-related diseases, such as hyperlipidemia, hypertension, arteriosclerosis, type 2 diabetes mellitus, and cancer (*29*). Some recent studies have focused on the search for functional food ingredients or herbal extracts that can suppress the accumulation of body fat. Polyphenols have a variety of biological functions, including antioxidant, anti-inflammatory, and anticancer effects (*30*). In an in vitro study, rutin has been shown to be the major low-density lipoprotein (LDL) antioxidant compound of mulberry (*31*).

We first examined the effects of NLFE on plasma cholesterol and TG concentrations in HFD-fed mice. We used clinical drugs, simvastatin, silymarin (a potent antioxidant, liver protector, and anticancer agent), and NLFE to treat HFD-fed mice. The results showed that with respect to weight reduction (**Table 1**), serum GOT, GPT, cholesterol, TGs, free fatty acids, and FAS reduction (**Figure 2**−**4**), feeding with NLFE was better than feeding with simvastatin and silymarin. Our observations are in agreement with other reports that have shown that flavonoids can significantly reduce the levels of total cholesterol and also markedly decrease weight in animals with a high-cholesterol diet (*31*).

We further compared the effects of NLFE, simvastatin, and silymarin on lipid accumulation, TGs, and cholesterol content in the liver. The results were consistent with the findings that hepatic triacylglycerol and cholesterol levels were significantly decreased in the HFD plus flavonoid groups compared to those in the HFD group (*32*). The NLFE may effectively reduce both the TGs and total cholesterol in plasma and liver in HFD-fed hamsters (*24*). When the TG content in the liver is compared, NLFE reduced TG much better than simvastatin and silymarin. Therefore, NLFE can indeed reduce the lipid accumulation and it showed better results than simvastatin and silymarin.

As shown in our data, NLFE decreases in the levels of serum and hepatic lipids, such as total cholesterol, triglycerides, and free fatty acid in mice compared to those for mice fed with HFD only, which could be attributed to the inhibition of lipid absorption in the gastrointestinal tract. Dietary lipids are absorbed into the bloodstream as chylomicron; triglycerides in these chylomicrons are then digested as fatty acids and glycerol by lipoprotein lipase and are eventually transported and stored in the liver and adipose tissues in the form of triglycerides. The remnants of the chylomicrons are taken up mainly by the liver and are then transformed into lipoproteins, such as VLDL, which transport triglycerides synthesized in the liver to adipose tissues, and LDL, which transports cholesterol to peripheral tissues (*33*).

In this study, we found that NLFE suppressed lipid accumulation in vivo, mainly by the activation of the AMPK/ACC pathway (**Figure 6**). In fact, our previous in vitro report and other research indicated that polyphenol extracts from many plants can activate AMPK (*34*). We also found that NLFE can limit the expression of FAS in vivo. FAS is the main enzyme in lipogenesis, which is a curative index in obesity treatment. On the other hand, AMPK has been highly valued in the research of lipometabolism; AMPK can adjust the synthesis of fatty acids (*35*).

Through the activation of AMPK, NLFE can limit the expression of ACC and FAS to decrease lipid accumulation in the liver. We suggest that AMPK is pivotal in shutting down anabolic pathways and promoting catabolism in response to an

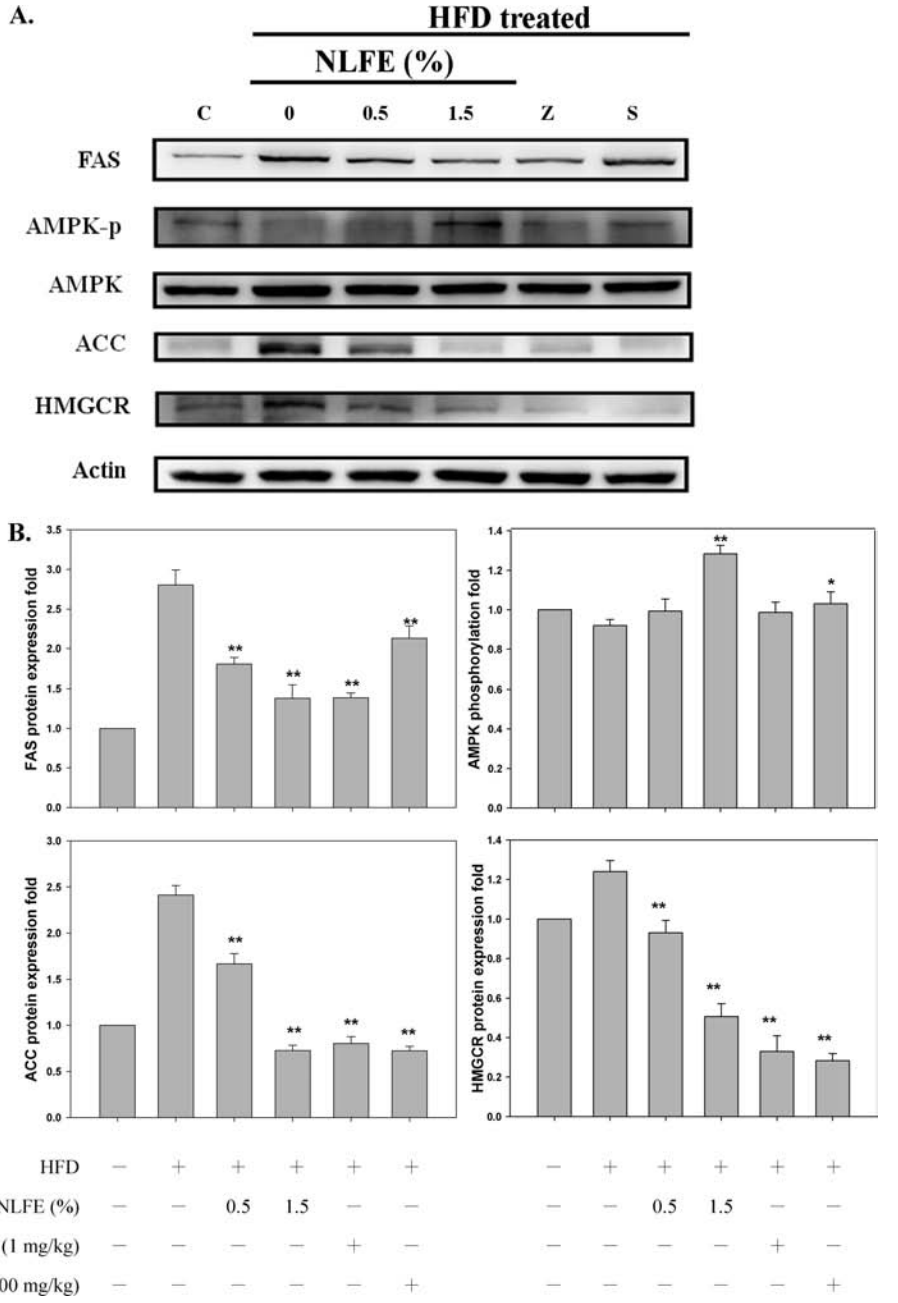


Figure 6. (**A**) Comparison of NLFE, simvastatin (Z), and silymarin (S) regulating the hepatic lipid metabolism enzyme expression in HFD-fed mice. NLFE treatment not only increased the phosphorylation of AMPK but also decreased ACC, FAS, SREBP-1, and HMGCR protein expression in mice fed with the HFD. Animals were fed a normal diet (C) or a HFD or HFD pair feeding with 0.5% NLFE or 1.5% NLFE diet, 1 mg/kg simvastatin (Z) and 100 mg/kg silymarin (S) for 6 weeks ad libitum. (n = 10). The results from three repeated and separate experiments were similar. (**B**) Represents the average of three independent experiments. The results are expressed as the mean ± SD: (∗) $p < 0.05$ compared with HFD group; (∗∗) $p < 0.01$ compared with HFD group.

increase in the AMP/ATP ratio by down-regulating the activity of key enzymes of intermediary metabolism.

Gallic acid and rutin are two of the major polyphenols in NLFE. Gallic acid reduced oxidative stress in the hepatic tissue of rats with HFD-induced obesity and suppressed HFD-induced dyslipidaemia, hepatosteatosis, and oxidative stress in rats (*36*). Gallic acid induced apoptosis and inhibited proliferation in 3T3-L1 preadipocytes (*37*). However, inhibitory effects of phenolic acids on 3T3-L1 preadipocytes may provide a proposed mechanism for antiobesity and have further implication in in vivo antiobesity effects. Rutin is found in many plants and is also an important dietary constituent of food and plant-based beverages (*31*). Rutin was identified as the major LDL antioxidant compound of mulberry in an in vitro study (*38*). Recent research has suggested that the reduced glucolipotoxic effects of rutin are via activation of AMPK signaling to inhibit the activities of lipogenic enzymes, such as FAS and ACC (*39*). In an in vivo study, uptake of rutin has been demonstated to significantly decrease body weight, liver, and adipose tissue weights and also to decrease hepatic TGs and cholesterol levels in rats fed a HFD (*32*).

In conclusion, by use of a HFD-fed animal model, NLFE significantly decreased blood and liver lipid accumulation by activation of the AMPK pathway. The effects of activating AMPK and inhibiting FAS, ACC, and HMGCR by NLFE were similar to the effects of rutin in a previous study (*39*). Taken together, our data suggest that the prevention of lipid accumulation may be attributable to rutin and gallic acid components in NLFE.

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