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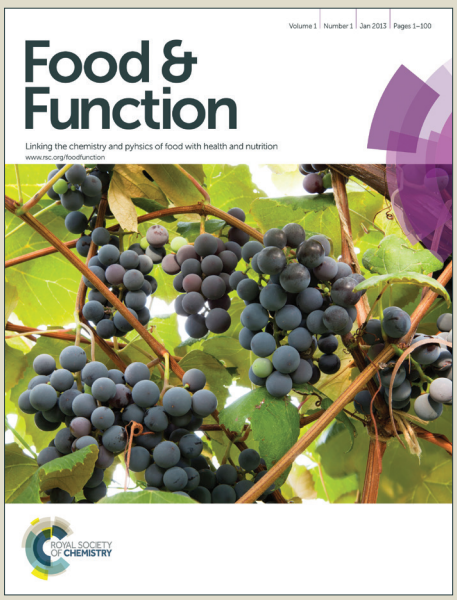

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Polyphenolic extract from *Hibiscus sabdariffa* reduces body fat by inhibiting hepatic lipogenesis and preadipocyte adipogenesis

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Abstract

Diets high in fat lead to excess lipid accumulation in adipose tissue, which is a crucial factor in the development of obesity, hepatitis, and hyperlipidemia. In this study, we investigated the anti-obesity effect of *Hibiscus sabdariffa* extract (HSE) in vivo. Hamsters fed a high-fat diet (HFD) develop symptoms of obesity, which were determined based on body weight changes and changes in plasma and serum triglycerides, free fatty acid concentrations, total cholesterol levels, LDL-C levels, HDL-C levels, and adipocyte tissue weight. HFD-fed hamsters were used to investigate the effect of HSE on symptoms of obesity such as adipogenesis and fatty liver, loss of blood glucose regulation, and serum ion imbalance. Interestingly, HSE treatment effectively reduced the effect of the HFD in hamsters in a dose-dependent manner. Further, after inducing the maturation of preadipocyte, *Hibiscus sabdariffa* polyphenolic extract (HPE) was shown to suppress the adipogenesis of adipocytes. However, HPE does not affect the viability of preadipocytes. Therefore, both HSE and HPE are effective and viable treatment strategies for preventing the development and treating the symptoms of obesity.

KEYWORDS: *Hibiscus sabdariffa* extract; polyphenol; obesity; adipocyte tissue weight; hepatoprotection; adipogenesis.

1. Introduction

Obesity is one of the major public health problems in developed countries. Obesity is a metabolic disease characterized by an increase in fat mass and body weight.[1] The metabolic disorders associated with obesity show symptoms of insulin resistance, nonalcoholic fatty liver disease (NAFLD), atherosclerosis, degenerative disorders (e.g., dementia), immune-mediated disorders, and cancers.[1-2] Extensive adipose tissue remodeling occurs during the development of obesity in the form of adipocyte hypertrophy, adipocyte hyperplasia, and angiogenesis.[3]

During adulthood, changes in adipocyte number result from a complex interplay between the proliferation of preadipocytes and the differentiation of preadipocytes to adipocytes.[4] Under normal conditions, adipocyte tissue development begins during gestation and proceeds until adolescence through increased proliferation of preadipocytes and their subsequent differentiation into adipocytes.[5] Progenitor preadipocytes are committed to differentiate into white adipocytes of the adipose and also comprise a significant portion of fat tissue (15–50%).[4, 6] Differentiated adipocytes store fatty acids in the cytoplasm in the form of triglycerides (TGs) with the involvement of various enzymes such as fatty acid synthase (FASN) acetyl-CoA carboxylase (ACC). Sterol regulatory element binding protein 1(SREBP-1) is a master transcriptional regulator that plays a critical role in regulating fatty acid synthesis and has two types of patterns: a precursor segment and a mature segment. ALK 7 plays an important role in maintaining the balance between energy expenditure and fat accumulation by suppressing adipocyte mitochondrial biogenesis, fatty acid oxidation, and β-AR mediated-lipolysis.[7-8]

Several studies have demonstrated the efficiency of various natural compounds to suppress adipogenesis and thereby prevent obesity in animal models.[9-12]

Polyphenols are a large and ubiquitous group of bio-active phytochemicals,[13] which are well known antioxidant agents that have effective anti-inflammatory, anti-allergy, hepatoprotective, antithrombotic, antiviral, and anti-carcinogenic activities.[14]

Serum total cholesterol (TC), TG, free fatty acids (FFA), high density lipoproteins (HDL), low density lipoproteins (LDL), and blood glucose levels of high-fat diet (HFD)-fed rats can be regulated by treating with polyphenols obtained from various sources.[15-17] In addition, various polyphenols have shown promising hepatoprotective effects against drug-induced hepatic necrosis and cholestatic liver injury in rodents.[18]

Hibiscus Sabdariffa, a tropical herb that has been used worldwide in cold and hot beverages, is known for its potential protective effects against many diseases such as hypertension, inflammation, and liver disease.[19-20] In our previous work, HSE inhibited LDL oxidation in vitro and decreased serum cholesterol levels in cholesterol-fed rats and rabbits. Further, polyphenol-rich HSE has demonstrated chemopreventive properties by acting through the Mitogen-activated protein kinases (MAPK) signaling pathway.[21-23] HSE capsules are also known to reduce serum cholesterol in human subjects.[19] Our previous study also showed that HSE can act as an adjuvant for preventing obesity in human.[24]

In this study, HSE and the HSE polyphenol fraction (HPE) were tested for anti-obesity activity in the HFD-induced obesity animal model. The inhibitory effect of HSE on 3T3-L1 adipocytes was examined to determine the influence of HSE on 3T3-L1 preadipocyte differentiation. We also monitored improvements in metabolic syndrome and symptoms of liver damage following HSE anti-obesity treatment.

2. Material and methods

2.1. Preparation of HSE and HPE

HSE was prepared from H. *sabdariffa* (Malvaceae) and analyzed according to our previous report.[21] Briefly, H. *sabdariffa* L. (150 g) was macerated with hot water (95°C, 6000 mL) for 2 h, and the aqueous extract was evaporated under a vacuum at -85°C. The extracted solution was filtered, lyophilized to obtain 75 g of HSE, and stored at 4°C before use. To prepare the HPE, 100 g of H. *sabdariffa* L. was extracted three times with 300 mL of methanol at 50°C for 3 h. The extracts were filtered and the solvent was removed using a vacuum rotary evaporator. The residue was dissolved in 500 mL of water (50°C) and extracted with 200 mL of hexane to remove some of the pigments. The aqueous phase was extracted three times with 180 mL of ethyl acetate, which was then evaporated under reduced pressure. The residue was redissolved in 250 mL of water, lyophilized to yield approximately 2 g of HPE, and stored at -20°C before use. HPLC analysis was performed on a Hitachi system series L-6200A (Toyko, Japan) equipped with a Hitachi D-7000 chromatography data station software, a Hitachi L-4250 UV–visible detector. a column. Wavelength monitoring was performed at 260 nm. Separation of polyphenols was carried out on a Mightysil RP-18 (250 × 4.6 mm) column from Kanto (Kanto Chemical, Toyko, Japan). The elution solvents consisted of acetic acid/water (2:98, v/v, solvent A) and 0.5% in water/acetonitrile (50:50, v/v, solvent B). All solvents were filtered with a 0.22 μm membrane filter. The flow rate was 1.0 mL min^{-1} and the injection volume was 20 μL. Quantification of polyphenols was done by the external standard method. Triplicate tests were conducted for each sample. The HPE extract was analyzed by HPLC as protocatechuic acid (24%), catechin (3%), gallocatechins (2%), caffeic acid (20%), and gallocatechin gallates (28%) and with 74% polyphenol content, and HSE also

analyzed by HPLC as protocatechuic acid (9%), catechin (10%), caffeic acid (18%), gallocatechin gallates (20%) and with 2% polyphenol content. The biochemical constituents and characteristics of HPE and HSE were published in our previous study.[25]

2.2. Animals and experimental design

All animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University in Taichung, Taiwan (No.513). Male Syrian hamsters, aged 7 weeks and weighing 120 g, were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and housed under standard laboratory conditions (18-23°C, 55-60% humidity, and a 12 h light/dark cycle) for 1 week before the experiments were performed to allow the animals to adapt to their environment. All Syrian hamsters were randomly divided into six groups (n=8/group) as follows: control (normal meals); HFD (normal meals containing 10% coconut oil and 0.5% cholesterol); and HFD supplemented with 0.5%, 1.0%, or 2.0% HSE. After 10 weeks of feeding on the aforementioned diets, the Syrian hamsters were fasted for 12-14 h and then sacrificed. Whole blood and livers were then collected from the Syrian hamsters.

2.3. Blood sample analysis

Blood samples from the Syrian hamsters were collected in EDTA tubes and immediately centrifuged at 1500 x g for 10 min. Then, the serum was decanted, and the samples were stored at 4°C. Biochemical examinations were performed within 1 h of specimen collection. Serum levels of TG, TC, HCL-C, LDL-C, alanine transaminase (ALT), and aspartate aminotransferase (AST) were measured using clinical chemistry reagent kits (Randox Laboratories, Antrim, UK). Free fatty acids were assayed using a free fatty acid quantification kit (BioVision, Mountain View, CA)

according to the manufacturer's protocol. Briefly, the free fatty acids in the plasma of Syrian hamsters were converted to their CoA derivatives, which were subsequently oxidized with the concomitant generation of color. FFA was then easily quantified using a colorimetric (spectrophotometrically at λ=570 nm) method.

2.4. Determination of TC and TG in the liver

Portions of the fresh livers from the Syrian hamsters were collected for liver lipid extraction. Liver tissues (1.25 g) were 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 x g for 10 min), the lower clear organic phase solution was transferred into a new tube and lyophilized. The lyophilized powder was dissolved in chloroform:methanol (1:2) to make the liver lipid extract and stored at -20℃ for less than 3 days. The liver TGs and liver cholesterol in the lipid extracts were measured by enzymatic colorimetric methods using commercial kits (HUMAN, Wiesbaden, Germany).

2.5. Cell culture

Mouse embryo derived 3T3-L1 cells (BCRC 60159) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). The culture medium included DMEM, 10% calf serum, 1.5 g/L sodium bicarbonate, and 100 U/mL penicillin-streptomycin. The cells were maintained at 37°C in humidified 5% CO_2 incubator.

2.6. MTT assay

The 3T3-L1 pre-adipocytes were plated into 24-well plates at a density of 1 x 10^4 cells/well. After 24 h, the culture medium was replaced with the indicated concentration of MLE or MLPE, and the cells were incubated for 2-14 days. Culture solutions were removed and replaced with new culture medium. The sterile filtered

142 MTT solution (5 mg/mL) in phosphate buffered saline (PBS, 0.85% NaCl, 2.68 mM
143 KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2 PO_4 were dissolved in distilled water, pH
144 7.4) was added to each well at a final concentration of 0.5 mg MTT/mL. Unreacted
145 dye was removed after 4 h. The insoluble formazan crystals were dissolved in 1000
146 mL/well of isopropanol and measured spectrophotometrically using a Hitachi U2900
147 spectrophotometer (Hitachi, Ltd., Tokyo, Japan) at 563 nm. Inhibition (%) was
148 expressed as the percentage of cell growth compared to non-treated control cells.

149 **2.7. Adipocyte maturation assay**

150 Mature adipocyte were seeded in a 6-well plate (3 x 10^6 cells) and treated with the
151 indicated concentration of HSE for 14 days. After being washed twice with PBS, the
152 cells were fixed with 4% formaldehyde for 30 min and then stained with 0.05 g/mL
153 Oil Red O or 1 μg/mL Nile red for 30 min at room temperature. The red lipid droplets
154 in the Oil Red O were visualized using a light microscope. Lipid-bounded Nile red
155 fluorescence was detected, and the lipid content in the cells was immediately analyzed
156 and quantified by flow cytometer (Becton Dickinson, Mountain View, CA, USA).

157 **2.7. Annexin V-FITC/PI double staining analysis**

158 Annexin V-FITC/PI double staining of the cells was determined using an Annexin
159 V-FITC kit (Vybrant™ Apoptosis Assay, V-13242, Molecular Probes, Eugene, OR,
160 USA) to detect early apoptosis, late apoptosis, and necrosis induced by HSE or HPE.
161 Mature 3T3-L1 adipocytes (1 x 10^6 cells/dish) were added to each well of a 6-cm dish
162 at 37°C in culture medium containing the indicated concentration of HSE or HPE for
163 72 h. Approximately 1 x 10^5 cells were then stained for 10 min at room temperature
164 with Annexin V-FITC and PI in a Ca^{2+}-enriched binding buffer (Annexin V-FITC kit)
165 and analyzed by FACScan flow cytometry. Annexin V-FITC and PI emissions were
166 detected in the FL1 and FL2 channels of a FACScan flow cytometer using the

emission filters of 525 and 575 nm, respectively. Approximately 1 x 10^4 counts were made for each sample. The percentages of normal (Annexin V-FITC$^-$/PI$^-$), early apoptotic (Annexin V-FITC$^+$/PI$^-$), late apoptotic (Annexin V-FITC$^+$/PI$^+$), and necrotic cells (Annexin V-FITC$^-$/PI$^+$) were calculated using CELL Quest software.

2.8. Western blot analysis

Equal amounts of protein samples (50 μg) were subjected to SDS − polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat milk powder containing 0.05% Tween 20 in PBS and then incubated with the primary antibody at 4°C overnight. Next, the membranes were washed three times with 0.05% Tween 20 in PBS and incubated with an anti-mouse secondary antibody conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The bands were detected by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed on ECL hyperfilm using a FUJFILM Las-3000 (Tokyo, Japan). Protein quantitation was determined by densitometry using the FUJFILM MultiGauge V2.2 software.

2.9. Statistical analysis

The data were analyzed using an unpaired t test and represented as the means (standard deviation (SD). Significant differences were evaluated using an unpaired *t* test and considered significant at the level $p < 0.05$. All data collected were analyzed using an unpaired t test after one-way ANOVA analysis showed a significant difference among all the groups ($p < 0.05$).

3. Results

3.1. The effect of HSE on body weight and food efficiency

To verify the effect of HSE in vivo, male Syrian hamsters were fed a high-fat diet feed containing 10% coconut oil and 0.2% cholesterol for 10 weeks. Consumption of the HFD for 10 weeks resulted in a significant increase in the body weight gain and feeding efficiency of the hamster. The body weight gain of the HFD group was significantly increased compared with that of the control group. HSE supplementation did not cause any change in the food intake of the HFD-fed hamsters. However, supplementation of HSE in the HFD group resulted in a significant decrease in body weight gain compared with the HFD group (Table 1). HPE showed similar effects to HSE.

3.2. Analysis of plasma and liver lipid levels

The results from the analysis of plasma lipid levels are shown in Figure 1. Hamsters fed a normal diet were used as a control. The following groups were analyzed: HSE-0%, hamsters fed a high-fat diet without HSE; HSE 0.5%, hamsters fed a high-fat diet with 0.5% HSE; HSE 1%, hamsters fed a high-fat diet with 1% HSE; and HSE 2%, hamsters fed a high-fat diet with 2% HSE. Triglycerides (Figure 1A), fatty acid concentrations (Figure 1B), total cholesterol content (Figure 1C), LDL-C (Figure 1D), HDL-C (Figure 1E), and LDL-C/HDL-C (Figure 1F) were decreased in the serum from hamsters treated with HSE. The results from the analysis of liver lipid levels are shown in Figure 2. Hamsters fed a normal diet were used as a control. The following groups were analyzed: HSE-0%, hamsters fed a high-fat diet without HSE; HSE 0.5%, hamsters fed a high-fat diet with 0.5% HSE; HSE 1%, hamsters fed a high-fat diet with 1% HSE; and HSE 2%, hamsters fed a high-fat diet with 2% HSE. Liver cholesterol content (Figure 2A) and liver triglyceride levels (Figure 2B) were

decreased in a dose-dependent manner following HSE treatment.

3.3. Effect of HSE on the liver function and blood biochemical parameters

Plasma ALT and AST levels were analyzed in hamsters fed HFD or HFD containing 0.5% HSE, 1.0% HSE, or 2.0% HSE. ALT (Figure 2C) and AST (Figure 2D) values were decreased in a dose-dependent manner following HSE treatment for 10 weeks (n=8/group). BUN and CRE levels were not significantly changed compared with the control (Table 2). Glucose and ketone body values were not significantly changed compared with the control (Table 2). After 10 weeks of being fed a HFD or a HFD containing 0.5% HSE, 1.0% HSE, or 2.0% HSE, serum ion levels were analyzed in the male hamsters (n=8/group). Potassium and sodium levels were not significantly changed compared with the control (Table 2).

3.4. The effect of HSE and HPE on premature adipocyte cell viability

The MTT assay performed on 3T3-L1 cells at different time points (2, 4, 6, 8, 10, 12, or 14 days) showed that HSE (1 or 2 mg/mL) and HPE (0.25 or 0.5 mg/mL) did not alter cell viability (Figure 3). Therefore, a concentration of 2 mg/mL of HSE was considered to be a non-lethal dose for HSE treatment. Similarly, concentrations of HPE up to 0.5 mg/mL did not affect cell viability (Figure 3).

3.5. The effect of HSE and HPE on the adipogenesis of preadipocytes

Oil Red O was used to stain 3T3-L1 cells. Then, after differentiation of 3T3-L1 cells, a strong reduction in fatty molecules was observed at 1 mg/mL and 2 mg/mL of HSE when compared with the completely differentiated adipocytes (Figure 4). The loss of fat molecules was due to the suppression of preadipocyte differentiation by HSE treatment (Figure 4). HPE was more efficient in suppressing adipogenesis to that of HSE (Figure 4). The markers of adipocyte differentiation, such as SREBP 1 and ALK7, and the markers of free fatty acid synthesis, such as FASN, were found to be

decreased in a concentration-dependent manner following treatment with HSE. The phosphorylation of ACC and AMPK was also increased as treating with HSE. The results show a decrease in adipocyte differentiation and fatty acid synthesis, which reflects an ameliorating effect of HSE on obesity. A similar trend was observed in the 3T3-L1 pre-adipocytes when treated with HPE. HPE was more efficient in regulating the markers of adipocyte differentiation and free fatty acid synthesis (Figure 5).

3.6. The effect of HSE and HPE on mature adipocytes

Flow cytometry was used to determine the number of apoptotic cells following HSE and HPE treatment. HSE and HPE were found to be effective in reducing the number of mature adipocytes. The percentage of apoptotic cells among the total mature adipocytes increased following treatment with HSE or HPE in a dose dependent manner (Figure 6). HPE was more efficient in stimulating cell apoptosis of adipocyte. Similar concentrations of HSE and HPE did not affect preadipocyte cell viability.

4. Discussion

An accumulation of excess adipose tissue causes obesity and is a major risk factor for type 2 diabetes and cardiovascular disease.[26] Adipocytes are the primary sites of energy storage, and an accumulation of triglycerides occurs during nutritional excess. Adipocyte dysfunction is a critical phenomenon that plays an important role in the development and progression of obesity. It is therefore considered to be a risk factor for the development of various diseases, including coronary heart disease, hypertension, type 2 diabetes mellitus, cancer, respiratory complications, and osteoarthritis.[1] Recent reports have outlined that administration of an efficient anti-obesity drug may decrease energy/food intake and increase energy expenditure, as well as decrease preadipocyte differentiation and proliferation, decrease lipogenesis and increase lipolysis, and decrease fat oxidation.[27]

The prevalence of both obesity and type-2 diabetes has increased sharply in Western countries. Therefore, therapeutic strategies for treating these diseases are being actively pursued at various levels. Our results highlight the promising anti-obesity potential of HSE. Specifically, our results show that HSE and its polyphenolic fraction HPE can inhibit the lipid accumulation that results from fat feeding. HSE is already known to be rich in antioxidants such as polyphenolic and flavonoids, which can decrease oxysterols in bile acid metabolism and block lipid accumulation in the liver that results from the bile salt system.[28] Therefore, the effect of the HSE could be due to the presence of polyphenols in the HSE, and HSE treatment can thereby effectively decrease the cholesterol in plasma and organs. Daily take of HSE decreased body weight, BMI and body fat, and reduced abdominal fat distribution in human.[24] The anti-obesity effects of HSE and HPE were also reflected by the effective adipolytic activity demonstrated against post-confluent 3T3-L1 mature adipocytes. Interestingly,

neither HSE nor HPE showed any cytotoxic effects against 3T3-L1 preadipocyte cells. However, the anti-obesity effects of HSE are more desirable. Analysis of the molecular markers of fatty acid synthesis and accumulation also reveal that HSE and HPE have similar effects in regulating fatty acid accumulation. Polyphenols are known to activate the AMPK pathway, which mediates FASN and ACC activities[29] and regulates fatty acid accumulation. HSE exhibit activities against atherosclerosis, liver disease, cancer, diabetes and metabolic syndromes.[30] Comparative analysis of the effects of HSE and HPE further proves that the active metabolites of HSE are also present in HPE. Previous study showed that *H. sabdariffa* polyphenol-rich extracts (HPE) more effective decrease plasma cholesterol and LDL-C than crude HSEs.[31] Active polyphenols such as protocatechuic acid, GCG, and caffeic acid, which are present in HPE, are known to potentially reduce total cholesterol levels in the plasma in various animal models.[25]

Additionally, the anti-obesity effects of HSE and its polyphenol content were determined in animal models by analyzing body weight, fat mass, feed intake, plasma lipid profiles, and biochemical examination of liver and renal function in HFD-fed hamsters. Our results showed that supplementation with either HSE or HPE significantly reduced body weight gain, fat mass, and plasma TG, TC, and LDL cholesterol concentrations. High levels of cholesterol in the plasma are commonly observed in obese patients. In our HFD-fed animal model, not only were the levels of cholesterol elevated, the levels of TG and LDL were also increased in the plasma.[32]

Obesity also causes chronic inflammation in the liver, which can result in liver damage.[33] Our results showed that a HFD increases ALT and AST levels in the plasma, which indicates a damaged liver in the HFD-fed hamsters.[34] To better understand the effects of a HFD on liver function, the enzyme activities of ALT and AST were

306 measured.[35] ALT and AST measurements in the HFD-fed hamsters collectively
307 reflected the severity of liver damage caused by the HFD. Both ALT and AST are
308 leakage enzymes, and their elevation in the circulation indicates significant
309 hepatocellular damage. Toxicity, inflammation, hypoxia, and tissue trauma may be the
310 underlying reason for their elevation.[36] Liver abnormalities are often characterized by
311 an increase in liver weight, an increase in ALT and AST activities, and the occurrence
312 of inflammatory infiltrates in rats. Meanwhile, renal abnormalities are characterized
313 by an increase in kidney weight and pararenal fat accumulation when a HFD is
314 consumed.[35] However, AST is also found in the heart, kidneys, brain, and skeletal
315 muscle and has been used as a nonspecific marker for other organ damage.[36-37] Our
316 results reveal that treatment with HSE significantly reduced ALT and AST levels in
317 the circulation. Therefore, HSE ameliorated the potential liver damage caused by the
318 HFD. The reduction of liver enzymes following HSE treatment indicates a decrease in
319 necrosis and fat deposition in liver cells.[38]
320 Further analysis shows that the HSE treatment did not cause any marked change in
321 renal functional markers such as BUN and CRE, indicating that HSE administration
322 does not affect the renal function.[39] Insulin resistance is usually accompanied by
323 unregulated blood glucose levels, ion levels, and ketone bodies in the plasma, which
324 increase the risk for diabetic ketoacidosis.[40] HSE supplementation in HFD-fed
325 hamsters did not change blood glucose levels, ion levels, or ketone body levels in the
326 plasma. Therefore, HSE does not affect insulin function. The polyphenol and
327 flavonoid composition of HSE contributes to its hepatic protective activities and
328 protective effects against hypolipidemia.[25, 41] Additionally, our previous work showed
329 that HSE effectively reduces serum cholesterol levels in 42 men and women
330 volunteers after 4 weeks of administration.[42] A HFD has been known to cause an

increase in liver mitochondrial ROS production.[43] It is widely recognized that ROS can cause cell damage via the mechanisms involved in lipid peroxidation, which results in tissue injury, particularly in the liver.[44] Dietary polyphenols contain a number of phenolic hydroxyl groups and have been shown to have various beneficial effects, which are primarily due to their ROS scavenging activities.[45] Polyphenols are widely distributed in vegetables, fruits, and beverages and are present as an integral part of the human diet.[46-47] Various polyphenol-rich sources, such as tea, pomegranates, grape juice, apples, and pecan nuts, have demonstrated hepatic protection against many insults.[45, 47-50] Various herbal extracts, such as *Chrysanthemum morifolium* extract, *Morinda citrifolia* L extract, and *Coix lachryma-jobi* L. extract, that are abundant in polyphenols have also shown to provide protection against obesity-related liver damage.[51-54]

In conclusion, HSE treatment improved HFD-induced obesity and lipid accumulation-induced damage in the liver in an animal model of obesity and inhibits adipogenesis in preadipocytes. The major active components responsible for the anti-obesity effects of HSE are polyphenols; therefore, polyphenolic-rich HPE treatment is a safe therapy for treating HFD-induced obesity and liver disease.

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Acknowledgements

This work was financially supported by Ministry of Science and Technology, of Taiwan (MOST 99-2632-B-040-001-MY3) and Chienkuo Technology University, Taiwan (CTU-101-RP-BS-002-048).

Conflict of interest

The authors declare no competing financial interest.

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549 **Figure Caption**

550 **Figure 1. The effect of HSE supplementation on plasma lipid levels in HFD-fed**
551 **hamsters.** Male Syrian (7 weeks old) hamsters were individually housed and
552 maintained at 25℃ with a 12 h light/dark cycle. The induced group of Syrian
553 hamsters was maintained on a HFD containing 10% coconut oil and 0.2% cholesterol
554 for 10 weeks. The levels of triglycerides (A), fatty acids (B), total cholesterol (C),
555 LDL-C (D), HDL-C (E), and LDL-C/HDL-C (F) were measured in HFD-fed hamsters
556 that had been supplemented with or without various concentrations of HSE.
557 Corresponding levels in hamsters fed a normal diet (Control, C) were used as the
558 control. The data are shown as the mean ± SD: *, $p < 0.05$ as compared to the HFD
559 group. #, $p < 0.05$ as compared to the normal group.
560
561 **Figure 2. The effect of HSE supplementation on liver lipid and serum levels in**
562 **HFD-fed hamsters.** Male Syrian (7 weeks old) hamsters were individually housed
563 and maintained at 25℃ with a 12 h light/dark cycle. The induced group of Syrian
564 hamsters was maintained on a HFD containing 10% coconut oil and 0.2% cholesterol
565 for 10 weeks. The liver cholesterol content (A), the levels of liver triglycerides (B),
566 serum ALT (C) and serum AST (D) activities were measured in HFD-fed hamsters
567 that had been supplemented with or without various concentrations of HSE.
568 Corresponding levels in hamsters fed a normal diet (Control, C) were used as the
569 control. The data are shown as the mean ± SD: *, $p < 0.05$ as compared to the HFD
570 group. #, $p < 0.05$ as compared to the normal group.
571
572 **Figure 3. HSE is not cytotoxic to 3T3-L1 preadipocyte cells.** 3T3-L1 preadipocyte
573 cells were incubated with various concentrations of HSE (1 or 2 mg/mL) or HPE (0.5

or 1 mg/mL) at 37°C for the indicated times. 3T3-L1 preadipocyte cells treated without any extract served as the negative control. Cell viability was measured using an MTT assay. The data were shown as the means ± SD from three replicates per treatment.

Figure 4. HSE and HPE inhibited adipogenesis of mature adipocyte cells. Post-confluent 3T3-L1 pre-adipocytes were treated with HSE or HPE at the indicated concentrations for 14 days. Then, the cells were stained with Oil Red O (A), and flow cytometry was performed (B). The fluorescence was quantified by flow cytometry (C). The data were shown from three replicates per treatment. *, $p < 0.05$, compared to the Mature group. In the Pre group, 3T3-L1 preadipocytes treated without any extract served as the negative control. In the Mature group, 3T3-L1 preadipocytes were induced to become mature adipocytes and were treated without any extract.

Figure 5. HSE and HPE inhibited adipocyte differentiation. HSE and HPE reduced the expression of the adipocyte differentiation markers SREBP1 and ALK7. Post-confluent 3T3-L1 pre-adipocytes were treated with HSE or HPE at the indicated concentrations for 14 days. Proteins isolated from the cells were analyzed by Western blot using the appropriate antibody. All data are expressed as the fold change relative to the untreated control cells. The data were shown as the means ± SD from three replicates per treatment. In the Mature group, 3T3-L1 preadipocytes were induced to become mature adipocytes and were treated without any extract.

Figure 6. HSE and HPE induced apoptosis of mature adipocytes. Mature adipocytes were treated with various concentrations of HSE or HPE for 72 h. Then,

599 the cells were stained with Annexin V/PI and analyzed by flow cytometry (A). The
600 lower-right quadrant shows the early apoptotic cells, and the upper-right quadrant
601 shows the late apoptotic cells. The fluorescence was quantified by flow cytometry.
602 The percentage of apoptotic cells is the sum of the early and late apoptotic cells (B).
603 The data shown were from three replicates per treatment. *, $p < 0.05$, compared with
604 the Mature group. **, $p < 0.01$, compared with the Mature group. In the Pre group,
605 3T3-L1 preadipocytes were treated without any extract and served as the negative
606 control. In the Mature group, 3T3-L1 preadipocytes were induced to become mature
607 adipocytes and were treated without any extract.
608
609

610 **Table 1. A comparison of body weight and adipose tissue content in HFD-fed hamsters**

	Control	HFD	HFD+0.5% HSE	HFD+1.0% HSE	HFD+2.0% HSE
Body weight change	24.26±4.66	32.48±10.96#	23.72±6.31*	20.17±13.01*	20.85±9.52*
Feeding efficiency	0.67±1.69	0.48±1.24	-0.04±1.13	-0.82±3.15	-1.08±2.87
Gonadal fat tissue /body weight	0.17±0.01	0.22±0.03#	0.19±0.02*	0.18±0.03*	0.17±0.02*
Pararenal fat tissue /body weight	0.12±0.01	0.17±0.03#	0.14±0.02	0.14±0.03	0.12±0.04*
Total fat tissue weight /body weight	0.30±0.02	0.39±0.07#	0.34±0.04*	0.33±0.07*	0.31±0.07*

611 Each value is expressed as the mean ± SD (n=8/group). Statistical significance was analyzed with ANOVA. #, $p < 0.05$ as compared to the

612 control group. *, $p < 0.05$ as compared to the HFD group

613

614 **Table 2. Effects of HSE on the Serum Biochemical Parameters in HFD-fed hamsters**

	ND	HFD	HFD + HSE 0.5%	HFD + HSE 1.0%	HFD + HSE 2.0%
BUN (mg/dL)	13.32 ± 1.81	15.09 ± 2.58	14.56 ± 1.72	13.42 ± 4.47	13.36 ± 1.86
UA (mg/dL)	1.00 ± 0.16	1.01 ± 0.25	1.02 ± 0.33	1.00 ± 0.18	1.00 ± 0.13
Creatinine (mg/dL)	0.77 ± 0.05	0.82 ± 0.06	0.82 ± 0.04	0.79 ± 0.03	0.78 ± 0.05
Ketone body (mmol/L)	0.19 ± 0.07	0.19 ± 0.07	0.19 ± 0.05	0.19 ± 0.06	0.19 ± 0.07
Sodium (mmol/L)	146.92 ± 1.56	149.33 ± 3.60	148.92 ± 1.44	147.83 ± 1.47	147.17 ± 1.47
Potassium (mmol/L)	6.61 ± 0.48	6.98 ± 0.42	6.90 ± 0.52	6.73 ± 0.47	6.72 ± 0.46
Glucose (mg/dL)	16.42 ± 9.07	28.58 ± 8.34^{b}	23.75 ± 5.34	23.08 ± 7.59	21.50 ± 6.02^{c}

615 Each value is expressed as the mean ± SD (n=8/group). Statistical significance was analyzed with ANOVA.

616

617

Figure 1

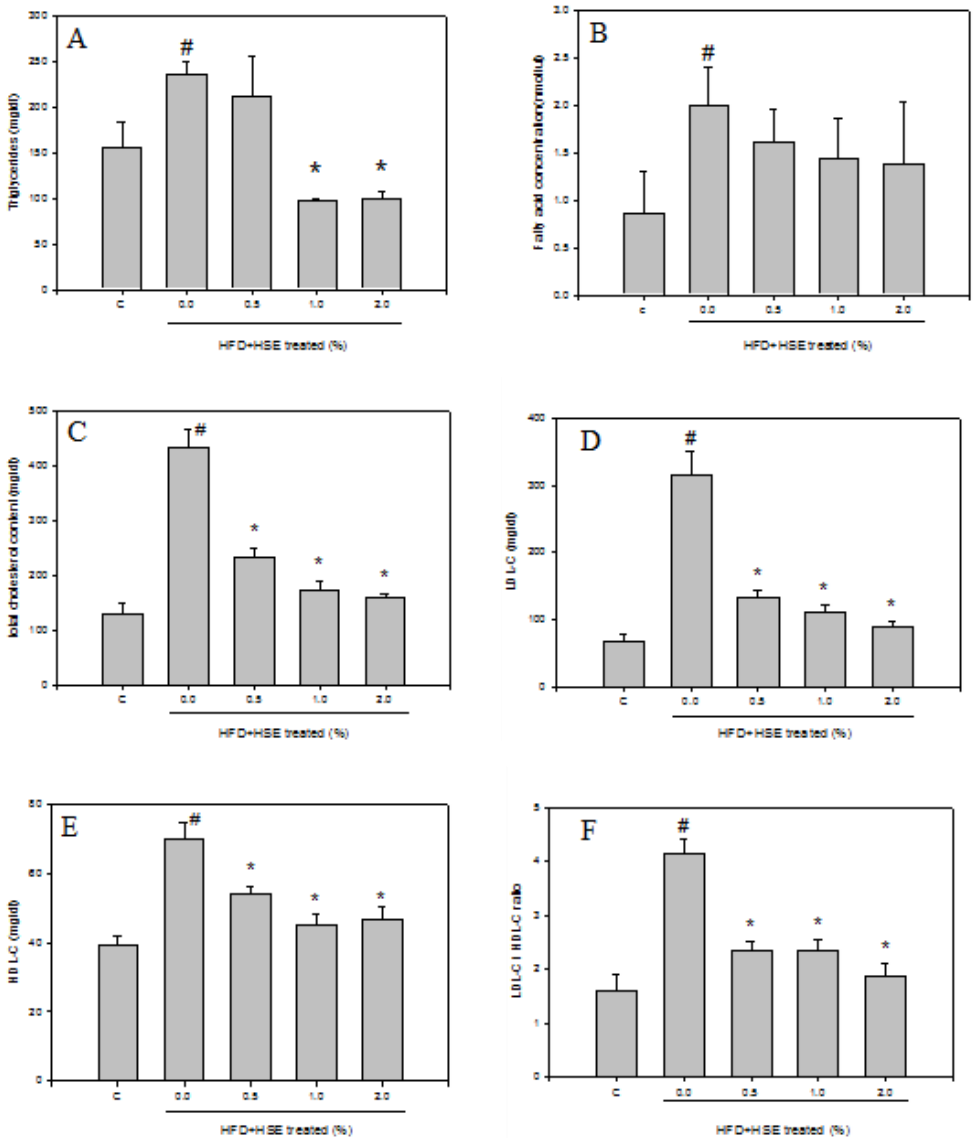


Figure 2

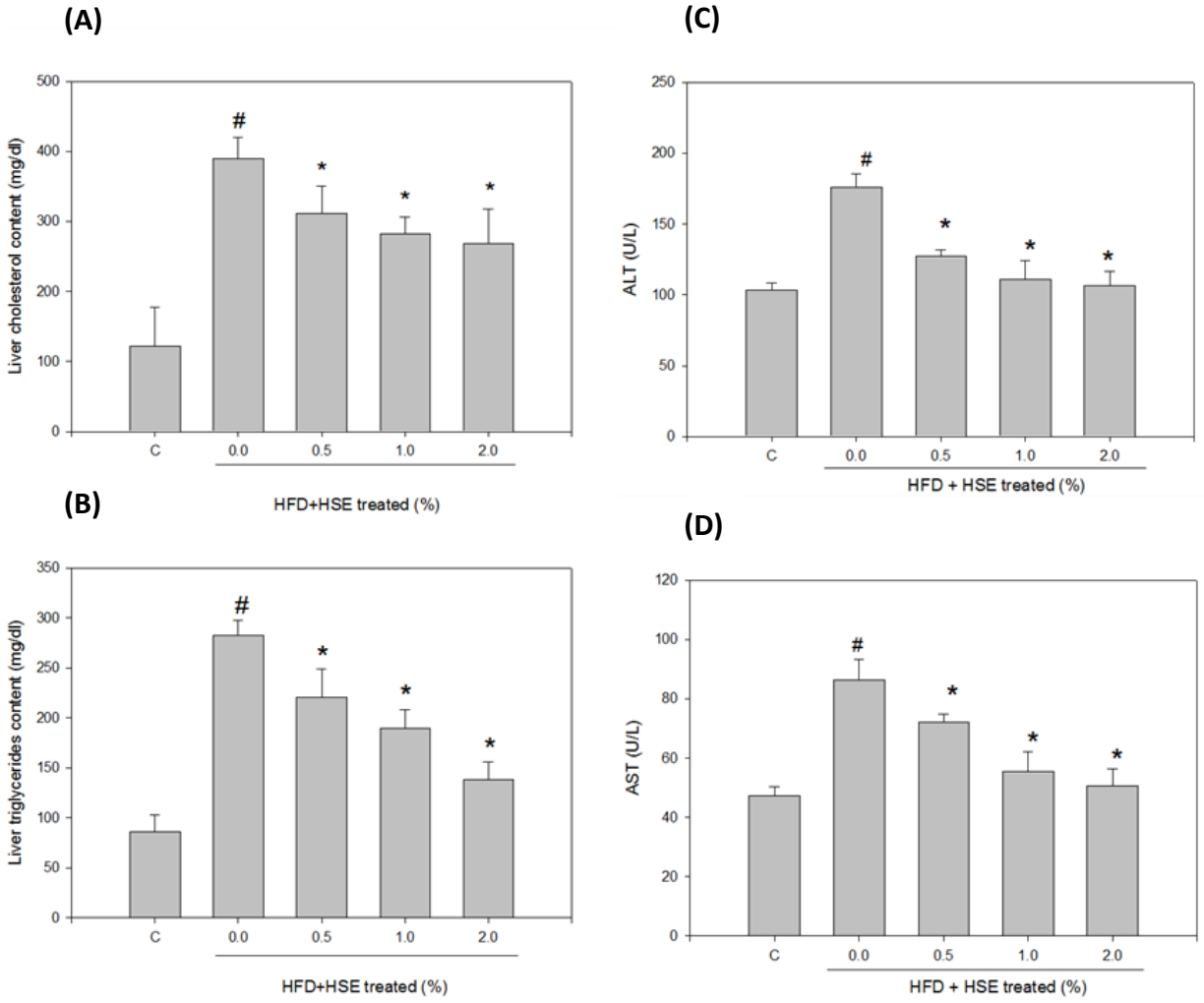


Figure 3

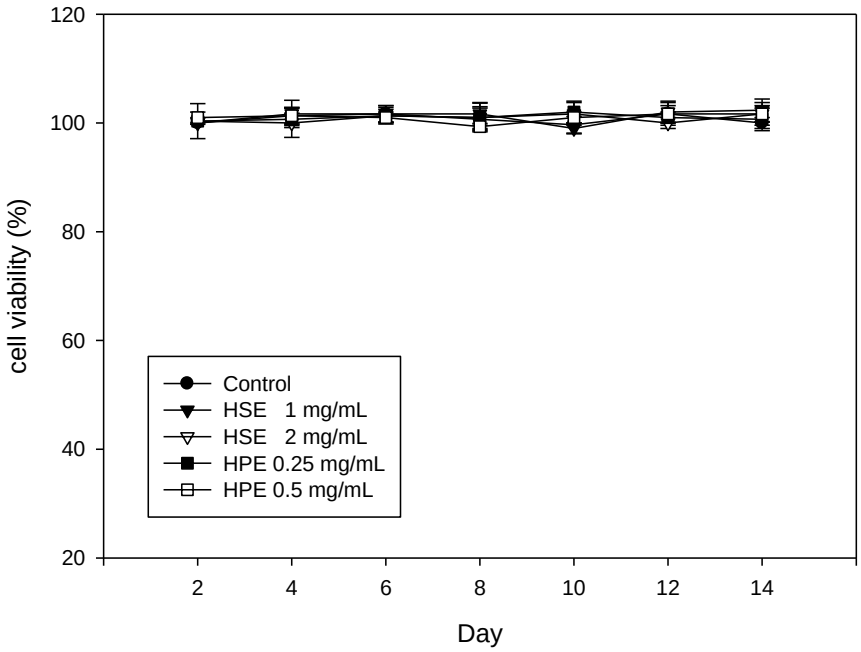


Figure 4

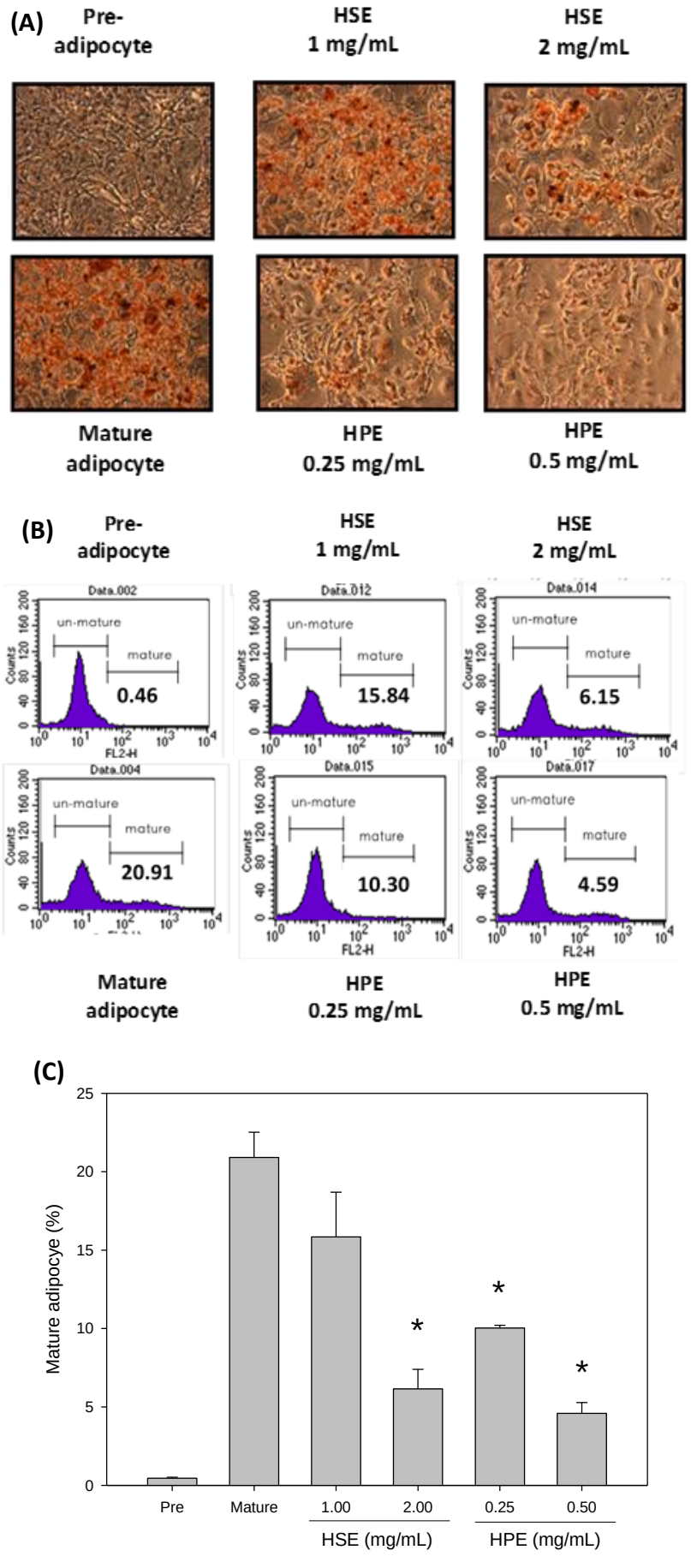


Figure 5

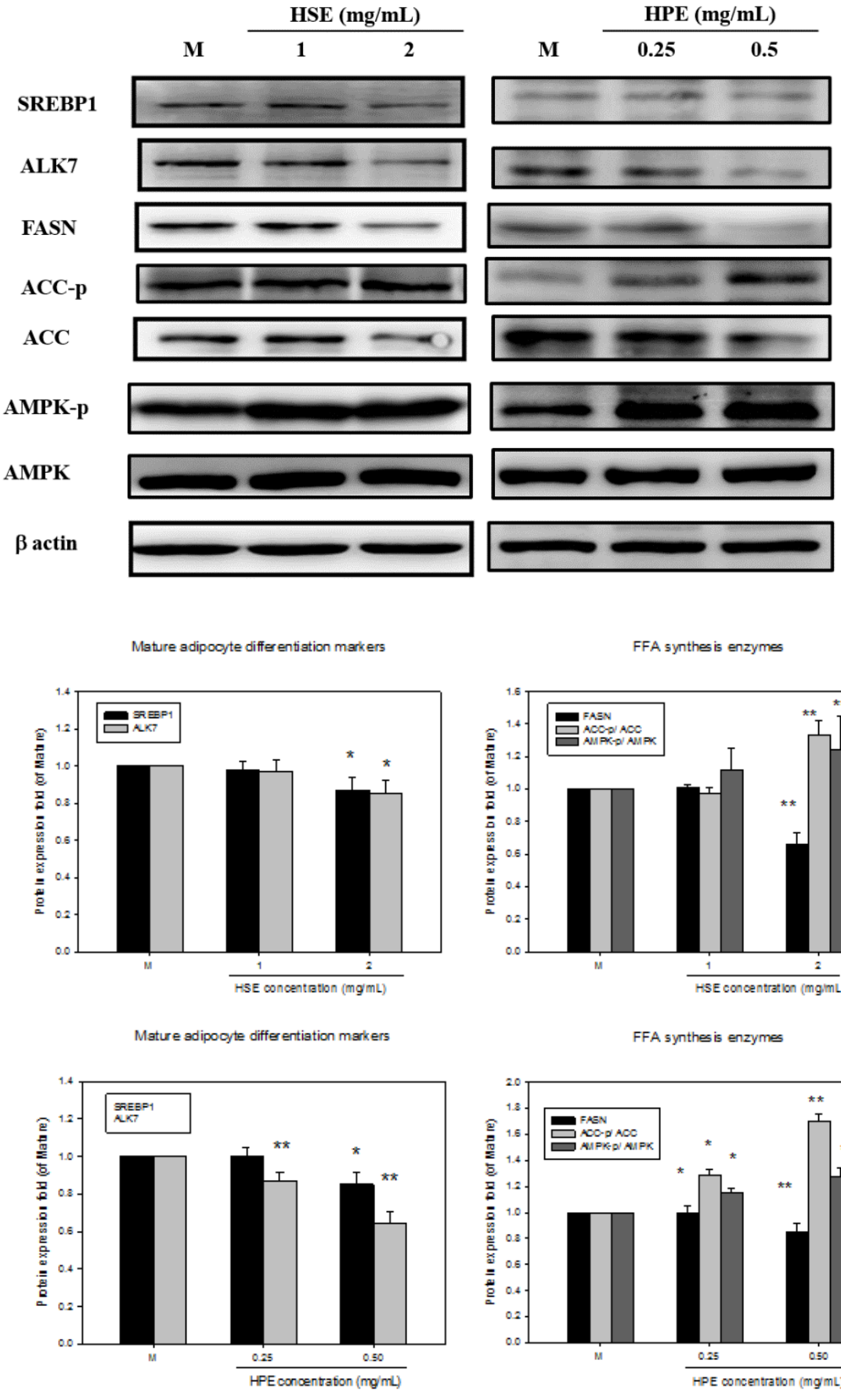
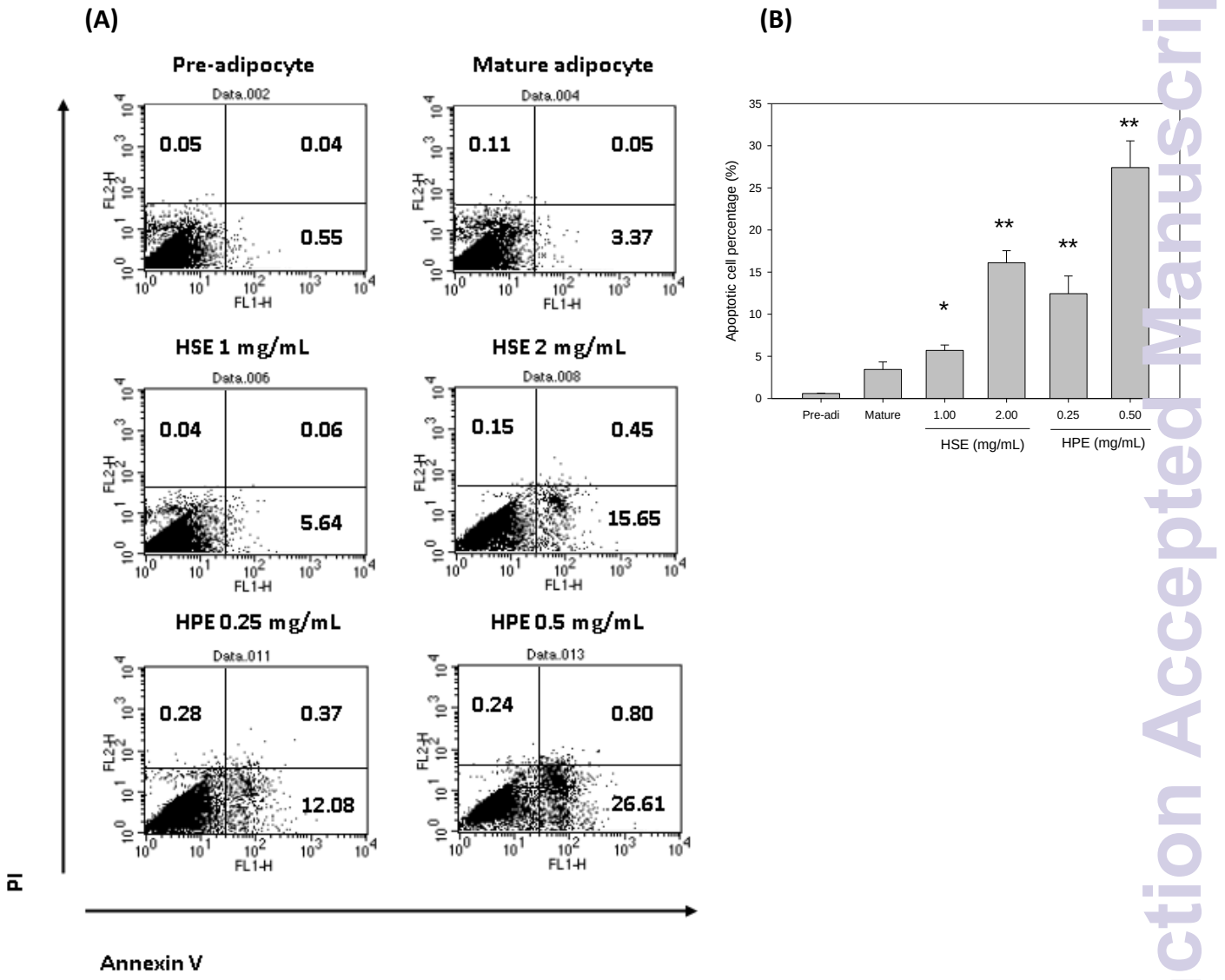


Figure 6



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