

Muscat Bailey A grape stalk extract ameliorates high-fat diet-induced obesity by downregulating PPAR γ and C/EPB α in mice

BO-MI KIM¹, BYOUNG OK CHO^{2,3} and SEON IL JANG^{2,3}

¹Department of Chemical Engineering, Wonkwang University, Iksan, Jeonbuk 54538; ²Research Institute, Ato Q&A Co., Ltd., Jeonju, Jeonbuk 54840; ³Department of Health Management, Jeonju University, Jeonju, Jeonbuk 55069, Republic of Korea

Received April 20, 2018; Accepted September 13, 2018

DOI: 10.3892/ijmm.2018.3951

Abstract. Muscat Bailey A grape stalk is an organic waste produced in marked amounts during the vinification of grapes. Previous studies have indicated that grape stalk is rich in bioactive phenolic compounds, and exhibits antioxidant and UV-protective activities. However, its effects on obesity and obesity-associated disorders have not yet been investigated. The effects of grape stalk extract on improving metabolic features were examined using a high-fat diet (HFD)-induced obesity mouse model. Oral administration of 200 mg/kg/day grape stalk extract over 16 weeks markedly prevented HFD-induced obesity, hepatic steatosis, diabetic symptoms and the risk of developing cardiovascular disease. Furthermore, grape stalk extract prevented oxidative stress and inflammation caused by HFD in mice. The beneficial effect may be associated with CCAAT/enhancer-binding protein α and peroxisome-proliferator-activated receptor γ downregulation in liver tissue. Collectively, the results of the present study indicated that grape stalk extract may be a potent functional food ingredient for preventing obesity, hepatic steatosis and type 2 diabetes.

Introduction

Obesity, a metabolic disease caused by excessive nutrition primarily due to the consumption of high-fat diets (HFDs) affects millions of individuals in developed countries and its prevalence in developing countries is also gradually increasing. A study in developing countries indicated that overweight and obesity are increasing among women of reproductive age in urban Africa, with obesity among this age group more than

doubled or tripled in 12/24 countries surveyed (1), whereas in developed countries such as the USA, data from 2015 estimate that at least one-third (36.5%) of US adults are obese (2). This makes overweight and obesity global public health problems. Obesity is also viewed as the leading most preventable cause of mortality (2). There is evidence that the risks of coronary heart disease, ischemic stroke, hypertension, hyperlipidemia, liver disorders and Type 2 diabetes mellitus increase steadily with overweight and obesity (3-6). Oxidative stress serves a key function in obesity and its associated complications. Obesity is able to induce systemic oxidative stress through various biochemical mechanisms, such as superoxide generation, oxidative phosphorylation, glyceraldehyde auto-oxidation and protein kinase C activation (7). Other factors that contribute to oxidative stress in obesity include hyperleptinemia, low antioxidant defense, chronic inflammation and postprandial reactive oxygen species generation (7). Systemic oxidative stress and inflammation serve key functions in the pathogenesis of obesity-associated diseases, including atherosclerosis, insulin resistance, Type 2 diabetes and cancer (8).

The current rate at which the prevalence of obesity is increasing implies that diet control and exercise alone are insufficient to prevent or control obesity. Anti-obesity therapeutic agents such as orlistat, lorcaserin, liraglutide, phentermine/topiramate and naltrexone/bupropion are currently in use for treating obesity (9). Although these agents have proven beneficial in managing weight gain in obesity, they also led to marked side effects to patients such as stomach ache, paresthesia, vomiting, insomnia, constipation, headache and nausea. The majority of the drugs are contraindicated in patients with cardiovascular diseases and those with a high risk of cardiovascular diseases (9). For these reasons, there is a requirement to develop other anti-obesity agents with fewer or less marked side effects. Herbal medicines have been exploited and used for weight control in many countries. A good example is *Garcinia cambogia* with its main active compound, hydroxycitric acid, which is widely becoming a popular natural product ingredient in weight loss supplements and has no toxic effects. *G. cambogia* with its hydroxycitric acid has been revealed to be safe when taken orally as it did not lead to any abnormal changes in hepatic and testicular lipid

Correspondence to: Professor Seon Il Jang, Department of Health Management, Jeonju University, 303 Cheonjam, Wansan, Jeonju, Jeonbuk 55069, Republic of Korea
E-mail: sonjjang@jj.ac.kr

Key words: Muscat Bailey A grape stalk, obesity, high-fat diet, fatty liver, inflammation, oxidative stress

peroxidation, hematological, DNA fragmentation or histopathological changes, and was identified to be bioavailable in plasma following gas chromatography-mass spectrometry analysis (10-12). Furthermore, the main active compound of *G. cambogia* exhibits its anti-obesity properties in weight management in animals (12), including humans (13,14), by promoting fat oxidation, inhibiting ATP-citrate lyase, the building block for fat synthesis, and also lowering leptin levels in obese subjects (12). Furthermore, phytochemicals from plants as well as daily fermented foods have been identified to prevent weight gain and decrease the incidence of metabolic diseases by acting through several molecular mechanisms, such as cell signaling and modulation of gene expression, decreasing obesity-induced oxidative stress, production of inflammatory molecules and lipid accumulation (15,16).

Muscat Bailey A (MBA) grape (*Vitis labrusca* x *Vitis vinifera*) is one of the principal grape varieties grown in Korea. Its grape stalk is an organic waste produced in marked amounts during the vinification of grape. Previous studies have indicated that grape stalk is rich in bioactive phenolic compounds and exhibits antioxidant and UV-protective activities in *in vivo* studies in mice (17-19). Mattos *et al* (19) also suggested that the functional properties of grape wastes which are rich in phenolic compounds may be exploited to develop products ranging from medical to food applications. Although other parts of the grape plant such as the seed and fruits have been investigated for their anti-obesity effects for which they exhibit anti-obesity potencies (20,21), to the best of our knowledge, limited or no study has been performed to investigate the anti-obesity effects of the grape stalk. Therefore, in order to elucidate the effects of the grape stalk in obesity, a grape stalk extract was investigated for its effects on adipocyte differentiation in cell studies, and on HFD-induced obesity in mice *in vivo* studies. We hypothesize that grape stalk may also have anti-obesity potencies *in vitro* and *in vivo*. To test this hypothesis, the antioxidant capacity and phytochemical constituents of grape stalk harvested in different periods of the year were investigated. Furthermore, the effects of Muscat Bailey A grape stalk extracts (MGSE) were investigated on adipocyte differentiation and in HFD-induced mice *in vivo* studies, where a number of parameters associated with obesity were investigated. In the present study, *G. cambogia* extract (GCE) was used as a control, because of its main active compound, hydroxycitric acid, to determine the effects of MGSE.

Materials and methods

Grape stalk extracts preparation. Muscat Bailey A grape stalks were separately harvested in June, July, August and September 2016 from Jeongeup (Korea). Each harvest was washed and dried at 40°C for 72 h and then extracted (50 g) in 80% ethanol for 3 days. The extracts were filtered through a 0.45- μ m filter paper (Advantec; Toyo Kaisha, Ltd., Japan), concentrated at decreased pressure and freeze-dried to obtain the powder samples.

Total phenol and flavonoid contents, and *in vitro* antioxidant activity. The total phenolic and flavonoid contents of the various extracts harvested in the different time periods were determined using methods described previously by

Cho *et al* (22). The total phenolic and flavonoid contents were expressed as gallic acid and quercetin equivalents, respectively. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical-scavenging activities of the various extracts were determined as described previously (22). Butylated hydroxytoluene and Trolox were used as standards for the DPPH and ABTS scavenging activities, respectively. All extracts were investigated in a set of three separate experiments.

High-performance liquid chromatography (HPLC) analysis. HPLC was performed using an Agilent 1200 series instrument (Agilent Technologies, Inc., Santa Clara, CA, USA), equipped with a binary pump delivery system, a degasser (G1379A), an autosampler (G1313A) and a diode array detector (G1315B). Compound separation was performed on an AegisPak C₁₈ column (4.6x200 mm; 3 μ m pore size) through a gradient elution with 0.1% aqueous formic acid (A) and acetonitrile (B): 0 min, 20% B; 5 min, 20% B; 12 min, 30% B; 20 min, 60% B; 30 min, 80% B; 34 min, 80% B; 37 min, 60% B; 40 min, 20% B. The mixture was held for 10 min before returning to the initial conditions. The flow rate of the mobile phase was 0.5 ml/min and the column temperature was 35°C. The injection volume was 15 μ l and UV detection was monitored at 320 nm. All standards [(+)-catechin, (-)-epicatechin, rutin, *trans*-resveratrol and quercetin] were identified on the basis of retention times, and concentrations were calculated on the basis of comparison with sample peak areas obtained from various standards.

3T3-L1 cell culture, differentiation and Oil Red O staining. 3T3-L1 pre-adipocytes, from the American Type Culture Collection (Manassas, VA, USA), were grown in Dulbecco's medium Eagle's medium (DMEM; Hyclone; GE Healthcare; Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare), 1% penicillin (10,000 U/ml) and 1% streptomycin (10 g/ml). To induce adipocyte differentiation, the 3T3-L1 pre-adipocytes were seeded in 6-mm cell culture dishes at a density of 5x10⁴ cells/ml. After 2 days when the cells had reached 100% confluence, differentiation was induced by replacing the medium with DMEM containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μ M dexamethasone (DEX), 1 μ g/ml insulin and MGSE at 25, 50 and 100 μ g/ml. After 3 days, the medium was replaced with DMEM containing FBS, insulin and MGSE at 25, 50 and 100 μ g/ml. After 2 days, the medium was replaced with DMEM containing FBS, insulin and MGSE at 25, 50 and 100 μ g/ml. For Oil Red O staining, cells in wells were fixed with 10% formalin for 1 h and then washed with 60% propan-2-ol before being incubated in 5 ml Oil Red O working solution for 5 min at room temperature. Following incubation, the cells were immediately washed four times with distilled water and images were captured under a Leica light microscope. Then, Oil Red O was dissolved in 100% propan-2-ol and absorbance was determined using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at 540 nm.

Triacylglycerol (TG) content in 3T3-L1 adipocytes. Total TG content in 3T3-L1 adipocytes was determined using a commercial TG assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's protocol.

Table I. Total polyphenol and flavonoid contents of Muscat Bailey A grape stalk extracts.

Sample	Total polyphenol content (mg-gallic acid/g)	Total flavonoid content (mg-quercetin/g)
June	282.87±0.81	10.74±0.06
July	189.27±0.61	16.04±0.17
August	158.08±0.98	10.34±0.85
September	115.50±0.38	5.69±0.23

All extracts were investigated in a set of experiments performed three times.

Table II. Antioxidant activity of Muscat Bailey A grape stalk extracts.

Sample	DPPH IC ₅₀	ABTS IC ₅₀
June	29.10±0.67	101.47±0.99
July	45.26±0.71	107.84±0.39
August	63.29±0.85	148.90±0.49
September	88.49±2.39	238.74±1.46
BHT	75.96±1.27	-
Trolox	-	61.49±0.25

All extracts were investigated in a set of experiments performed three times. IC₅₀, half-maximal inhibitory concentration ($\mu\text{g/ml}$); DPPH, diphenyl-2-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); BHT, butylated hydroxytoluene.

Animals and diet. A total of 20 3-week-old C57BL/6N male mice weighing 19.5±1.0 g came from Orient Bio Inc. (Gwangju, Korea). The mice were housed under standard environmental conditions with a temperature of 22±2°C, humidity of 50-60% and a 12-h light/12-h dark cycle. Mice were fed on a commercial standard laboratory diet (AIN-76A; Research Diets, Inc., New Brunswick, NJ, USA) and water *ad libitum*. After 1 week of acclimatization, mice were divided into four groups (n=5) and fed for 16 weeks on either a non-fat diet or an HFD (Rodent Diet with 60 kcal% fat; Research Diets, Inc.) as follows: Group 1, mice were fed on non-fat diet (ND) and orally given 0.2 ml distilled water daily; group 2, mice were fed on HFD and orally given 0.2 ml distilled water daily; group 3, mice were fed on HFD and orally administered with 200 mg/kg MGSE daily; group 4, mice were fed on HFD and orally administered with 200 mg/kg GCE daily. The various extracts were dissolved in distilled water. The animal protocols were performed following approval from the Jeonju University Institutional Animal Care and Use Committee (#JJU-IACUC-2016-011).

Weight and histochemical analysis. The weight of the mice in each group was determined prior to and on the last day prior to sacrifice. Following sacrifice and collection of blood

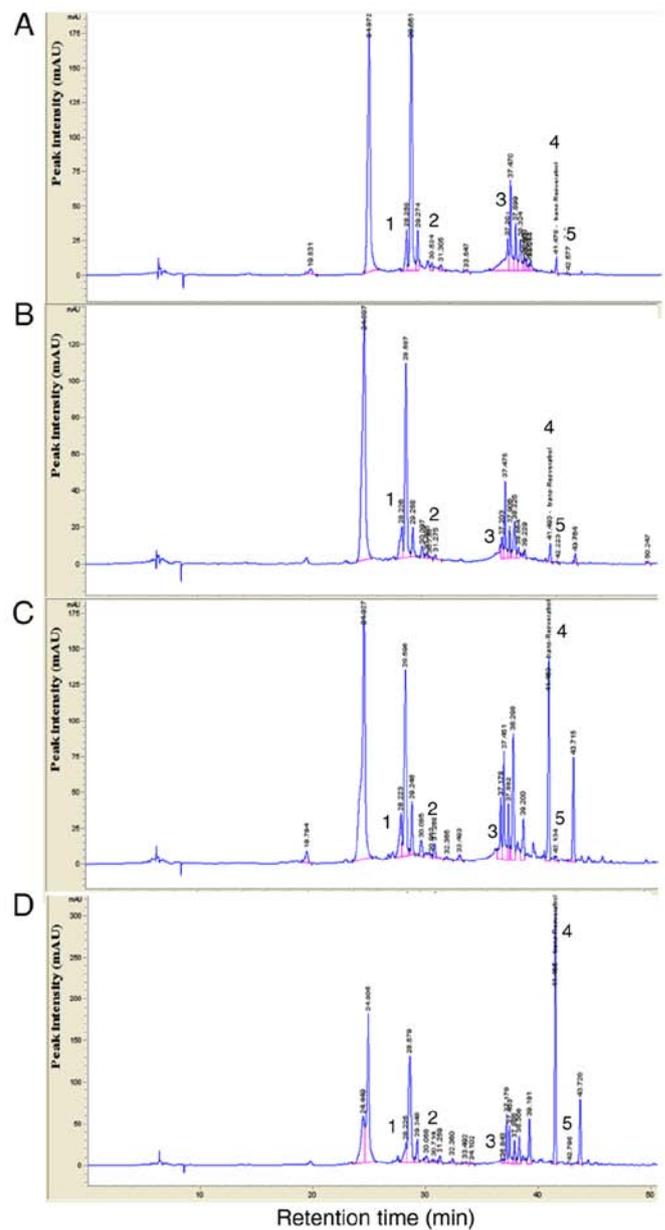


Figure 1. HPLC chromatograms of MGSE. Each MGSE collected in (A) June, (B) July, (C) August and (D) September 2016 was profiled using HPLC at 320 nm and compared with the conventional compounds using HPLC-diode array detector. Peak number represents the standard compound 1, (+)-catechin; 2, (-)-epicatechin; 3, rutin; 4, *trans*-resveratrol; 5, quercetin. HPLC, high-performance liquid chromatography; MGSE, Muscat Bailey A grape stalk extract; mAU, micro-absorbance units.

samples, adipose and liver tissues were removed and immediately weighed. For histological analysis, the adipose and liver tissues were fixed in 10% neutral formalin for 42 h, washed in several changes of PBS, dehydrated in a series of graded ethanol, cleared in two changes of xylene and embedded in three changes of paraffin wax. The tissues were blocked and sectioned (5 μm thick) using a microtome. The tissues sections were stained with hematoxylin and eosin stain.

Serum biochemical analysis. Blood samples were centrifuged at 2,000 \times g for 15 min at 4°C, and the serum was separated and stored at -70°C for subsequent experiments. Enzyme

Table III. Contents ($\mu\text{g/g}$) of flavonoids from Muscat Bailey A grape stalk extracts determined using high-performance liquid chromatography-diode array detector.

Sample collected	Final results, mg/g				
	1	2	3	4	5
June	39.98	19.07	15.71	0.11	0.17
July	22.64	9.49	6.17	0.09	0.05
August	17.51	9.56	3.21	0.46	0.09
September	17.94	0.59	0.74	0.91	0.08

Concentrations are expressed as free form. 1, (+)-catechin; 2, (-)-epicatechin; 3, rutin; 4, *trans*-resveratrol; 5, quercetin.

kits were employed to determine the serum concentrations of total cholesterol (TC; cat. no. AM202-K), high-density lipoprotein cholesterol (HDL-C; cat. no. AM203-K), TG (cat. no. AM157S-K), aspartate transaminase (AST; cat. no. AM101-K) and alanine transaminase (ALT; cat. no. AM101-K) (all Asan Pharmaceutical Co., Ltd., Gyeonggi, Korea), leptin (cat. no. MOB00; R&D Systems, Inc., Minneapolis, MN, USA) and insulin (cat. no. 80-INSMS-E01; Alpco Diagnostics, Windham, NH, USA). All experiments were performed according to the manufacturers' protocols and absorbances (TC, 492 nm; HDL-C, 492 nm; TG, 540 nm; AST, 492 nm; ALT, 492 nm; leptin, 450 nm; insulin, 450 nm) were determined using a Tecan spectrophotometer (Tecan Group, Ltd., Männedorf, Switzerland). Low-density lipoprotein cholesterol (LDL-C) was calculated using the following formula, $\text{LDL-C} = \text{TC} - \text{HDL-C} - (\text{TG}/5)$. Atherogenic index and cardiac risk factor were calculated using the following formulae: $\text{Atherogenic Index} = (\text{TC} - \text{HDL-C})/\text{HDL-C}$; $\text{Cardiac Risk Factor} = \text{TC}/\text{HDL-C}$.

Liver tissue analysis. Liver tissues (0.3 g) were homogenized in 0.5 ml PBS and centrifuged at $2,000 \times g$ for 15 min at 4°C to obtain the supernatant. The supernatants were stored at -70°C for subsequent experiments. The concentrations of catalase (cat. no. 707002), superoxide dismutase (SOD; cat. no. 706002, Cayman Chemical Company), malondialdehyde (MDA, cat. no. STA-330) and glutathione (GSH, cat. no. STA-312) (Cell Biolabs, Inc., San Diego, CA, USA) were determined using enzyme kits, following the manufacturers' protocols. Absorbances (catalase, 540 nm; SOD, 450 nm; MDA, 532 nm; GSH, 405 nm) were determined using a Tecan spectrophotometer.

Western blot analysis. Liver tissue samples (0.02-0.03 g) from each mice in each group ($n=5$) were mixed and protein was extracted using ice-cold lysis buffer (50 mM Tris/HCl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 2 mM Na_3VO_4 , 1 mM NaF, 20 mM $\text{Na}_4\text{P}_2\text{O}_7$, 0.02% NaN_3 and proprietary detergent; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Protease inhibitor cocktail (cat. no. P2714; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and phosphatase inhibitor cocktail (cat. no. P0044; Sigma-Aldrich; Merck KGaA) was added at 100-fold dilution. To ensure equal loading of proteins, the protein concentration in various

samples were determined using Quick Start™ Bradford Protein Quantification reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (80 μg) were loaded and separated by SDS-PAGE (4-20% gel) and transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked at room temperature with 5% (w/v) skimmed milk (EMD Millipore, Billerica, MA, USA) in TBS-T [10 mM Tris/HCl (pH 7.5), 150 mM NaCl and 0.1% (v/v) Tween-20]. The membranes were then incubated on a shaker overnight at 4°C with the following antibodies: Anti-peroxisome-proliferator-activated receptor γ (PPAR γ ; 1:500 dilution; cat. no. MAB3632, EMD Millipore), anti-CCAAT/enhancer-binding protein α (C/EBP α ; 1:1,000 dilution; cat. no. 2295S, Cell Signaling Technology, Danvers, MA, USA), anti-adiponectin (1:1,000 dilution; cat. no. sc-136131; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-leptin (1:1,000 dilution; cat. no. AB3521; EMD Millipore), anti-inducible nitric oxide synthase (iNOS; 1:2,000 dilution; cat. no. 610329; BD Biosciences, San Jose, CA, USA) and anti- β -actin (1:5,000 dilution; cat. no. 612657; BD Biosciences). Stripping buffer was used to strip primary antibodies. The secondary antibodies (1:5,000 dilution) used were horseradish peroxidase-conjugated anti-goat (cat. no. sc-2768; Santa Cruz Biotechnology, Inc.), anti-chicken (cat. no. SA1-72004; Invitrogen; Thermo Fisher Scientific, Inc.), anti-mouse (cat. no. 10004302, Cayman Chemical Company) or anti-rabbit (cat. no. sc-2357; Santa Cruz Biotechnology, Inc.) antibodies. Proteins were detected using the SuperSignal West Dura Stable Peroxide solution (Thermo Fisher Scientific, Inc.) and chemiluminescence detection systems (Alliance version 15.11; UVITEC, Cambridge, UK).

Statistical analysis. Results are expressed as the mean \pm standard deviation. Statistical analyses were performed using the SPSS statistics program (version 22; IBM Corp., Armonk, NY, USA). Differences between the variables were determined using one-way analysis of variance with Duncan's multiple range test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results and Discussion

Obesity has been associated with several pathologies such as the metabolic syndrome, hypertension, atherosclerosis, liver

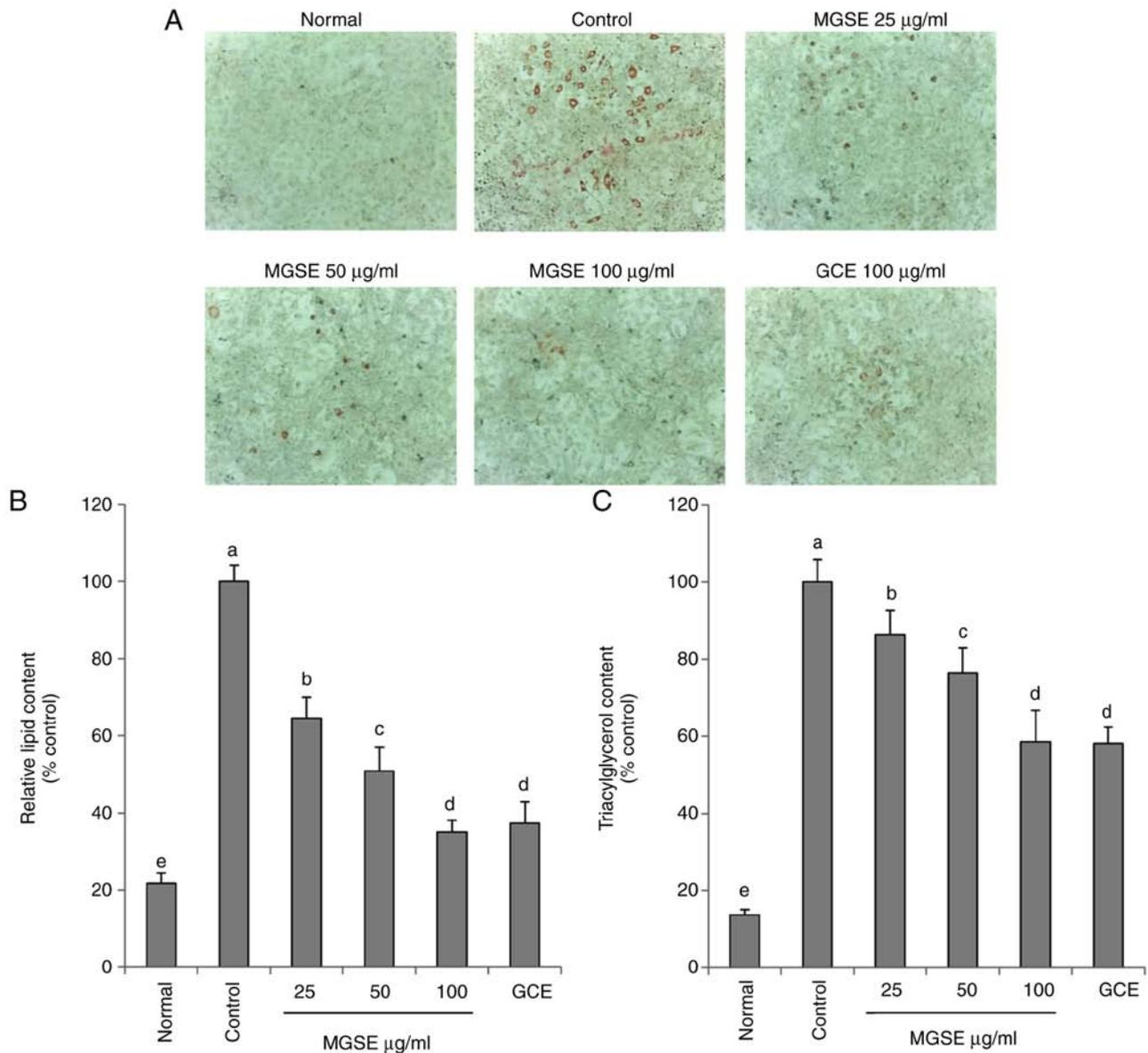


Figure 2. Microscopic (magnification, x100) images of lipid accumulation in control and experimental adipocytes treated with or without MGSE at various concentrations and GCE at 100 µg/ml (A). (B) Oil Red O stain was dissolved using propan-2-ol and lipid contents were determined using a microplate reader. (C) Adipocyte cell lysates were used to quantify triacylglycerol levels. Results are presented as the mean \pm standard deviation. Results were significantly different ($P < 0.05$) from all other results, unless labelled with the same letter. MGSE, Muscat Bailey A grape stalk extract; GCE, *Garcinia cambogia* extract.

diseases, oxidative stress and inflammation. In addition, the majority of available therapies designated for the prevention and/or treatment of obesity, and its associated abnormalities are either insufficient or renders patients with severe side effects. Therefore, a number of studies have focused on alternative therapies from agricultural products because of the recently uncovered biological actions of plant extracts and phytochemicals, to be used for preventing and treating obesity, and a number of previous studies have revealed the anti-obesity, antioxidative and anti-inflammatory potentials of plant extracts and dietary phytochemicals (17,20,21,23). In the present study, the anti-obesity effects of MGSE (200 mg/kg) on HFD-induced obese C57BL/6N mice were investigated for 16 weeks.

Initially, the time of harvest of the grape stalk for which the polyphenol and flavonoid yield are highest, and also

exhibiting the greatest *in vitro* antioxidant activities, was determined. The harvest time markedly affected the total polyphenol and flavonoid contents of MGSE, with the June harvest yielding the highest polyphenol content and the July harvest yielding the highest flavonoid content (Table I). Furthermore, the time of harvest affected the antioxidant activities of MGSE as revealed using a DPPH and ABTS assay with the highest antioxidant activity determined in the June sample (Table II). HPLC analysis indicated that (+)-catechin, (-)-epicatechin, rutin and quercetin gradually decreased from June to September whereas *trans*-resveratrol exhibited an increasing tendency from June to September (Table III; Fig. 1). The results clearly indicated that the harvest period affects the polyphenol and therefore the antioxidant activity of Muscat Bailey A grape. A similar study, but on rabbiteye blueberry leaves concluded that blueberry

Table IV. Effect of MGSE on body weight, weight gain, liver and epididymal adipose weight in HFD-induced obese mice.

Parameter	Normal	HFD control	MGSE	GCE
Initial body weight, g	21.18±0.74 ^a	21.72±0.63 ^a	21.56±0.62 ^a	21.70±0.87 ^a
Final body weight, g	30.12±2.20 ^a	47.98±1.61 ^b	45.28±0.88 ^c	44.94±1.50 ^c
Weight gain, g	8.94±2.29 ^a	26.27±1.72 ^b	23.72±0.62 ^c	23.24±1.68 ^c
Liver weight, g	1.10±0.10 ^a	1.96±0.24 ^b	1.57±0.24 ^c	1.55±0.30 ^c
Epididymal adipose weight, g	1.19±0.18 ^a	2.33±0.24 ^b	1.80±0.23 ^c	2.01±0.21 ^{b,c}

Results are presented as the mean ± standard (n=5). Results were significantly different ($P < 0.05$) from all other results, unless labelled with the same letter Normal, mice fed on the control diet (negative control); HFD, mice fed on the HFD (positive control); MGSE, mice fed on the HFD and administered with 200 mg/kg/day MGSE; GCE, mice fed on the HFD and administered with 200 mg/kg/day GCE. MGSE, Muscat Bailey A grape stalk extract; GCE, *Garcinia cambogia* extract; HFD, high-fat diet.

leaves from different seasons exhibited different bioactive secondary metabolite content and different antioxidant activities (24). Therefore, on the basis of these results, grape stalk harvested in June was selected for the investigation of its potential anti-obesity effects.

The 3T3-L1 cell line is the most widely used cell line for evaluating anti-obesity activities of a number of agents including plant extracts. This cell line can be stimulated by metabolic-regulating hormones such as IBMX, DEX and insulin to accumulate intracellular lipid droplets (25). A dose-dependent study was performed to investigate the effects of MGSE on fat and TG accumulation in the 3T3-L1 adipocyte cells. The results indicated that MGSE dose-dependently decreased lipid accumulation and TG content in 3T3-L1 adipocyte cells (Fig. 2). Similar previous studies but on other parts of the grape plant such as the skin and the seeds also revealed the anti-adipogenic effects of these parts of grape in 3T3-L1 adipocytes at the induction of differentiation and, primarily, on the PPAR γ signaling pathway (26,27).

Following the anti-adipogenic effects of MGSE on lipid accumulation in 3T3-L1 adipocytes, the focus was turned to elucidating the anti-obesity, anti-diabetic, antioxidative, anti-inflammatory and liver protective effects of MGSE in HFD-induced obesity in *in vivo* mouse studies. Obesity was induced in mice by feeding them on an HFD for 16 weeks. When the mice fed on the HFD were orally administered daily with 200 mg/kg MGSE or GCE, a decrease in body, liver and epididymal fat weights was observed (Table IV), compared with the mice fed on only HFD. The efficacy of grape stalk in the prevention of lipid accumulation in 3T3-L1 adipocytes was confirmed in the *in vivo* studies as MGSE treatment decreased epididymal fat and body weight of the mice. Blood serum analysis further revealed that total cholesterol, TG and LDL concentrations were significantly decreased in the mice fed on the HFD and administered with MGSE or GCE compared with the HFD-fed group. On the other hand, the high-density lipoprotein concentrations increased in the HFD-fed mice that were administered with MGSE or GCE (Fig. 3A-D). However, the differences were not statistically significant. Abdominal fat distribution is associated with increased serum TG, cholesterol and LDL-C (28). In addition, increased concentrations of TG lead to accumulation of TC, and LDL-C can build up on artery walls, thereby increasing the risk of heart disease (29).

However, increased HDL-C levels attenuate the accumulation of LDL-C and protect against heart disease by transporting LDL-C, TC and TG from the arteries (30). The results of the present study indicated that MGSE and GCE have the potential to decrease TG, TC and LDL-C in obese mice, thereby decreasing or even preventing the risk of heart disease. The atherogenic index and cardiac risk factors were significantly decreased when obese mice were administered with MGSE or GCE (Fig. 3E and F).

Leptin, a key hormone, predominantly produced by adipose cells, regulates energy balance, appetite and adiposity, and its secretion levels are positively associated with TG stores in adipose tissue (31). Diabetes in obesity has been associated with changes in insulin secretion. Several risk factors in subjects with hypertension are associated with insulin resistance, including low HDL-C, high TG levels and glucose intolerance (32). Adipocytes contribute to insulin resistance via adipocytokine and hormone secretion, where retinol-binding protein 4, adiponectin, leptin, interleukin (IL)-6, IL-1 β and tumor necrosis factor α (TNF- α) have been observed (33). Noteworthy, the results from the MGSE-treated group indicated significantly decreased insulin (Fig. 3G), leptin (Fig. 3H and I), IL-6, IL-1 β , TNF- α and iNOS levels (Fig. 4) compared with those of the HFD-fed group. However, the adiponectin level was increased in the MGSE-treated group (Fig. 3I). This increase in adiponectin level in the liver can be beneficial as a decrease in circulating adiponectin in Type 2 diabetes contributes to an abnormal increase in glucose production. In addition, low adiponectin signaling and high insulin levels indicate an insulin resistance state (34). These results suggested that MGSE might have a beneficial effect in Type 2 diabetes by ameliorating insulin resistance in HFD-induced obesity that leads to diabetes. This test, to the best of our knowledge, was performed on MGSE-treated HFD mice for the first time and also confirms the anti-inflammatory effects of MGSE in obesity. A similar effect was observed in the GCE-treated HFD mice. The beneficial anti-inflammatory effects of MGSE may be associated with the high levels of phenolic compounds, which are known for their anti-inflammatory potential.

AST and ALT serve important functions in the formation of amino acids in the liver, and their activity increases with HFD-induced liver damage. Fatty liver disease is a disease in

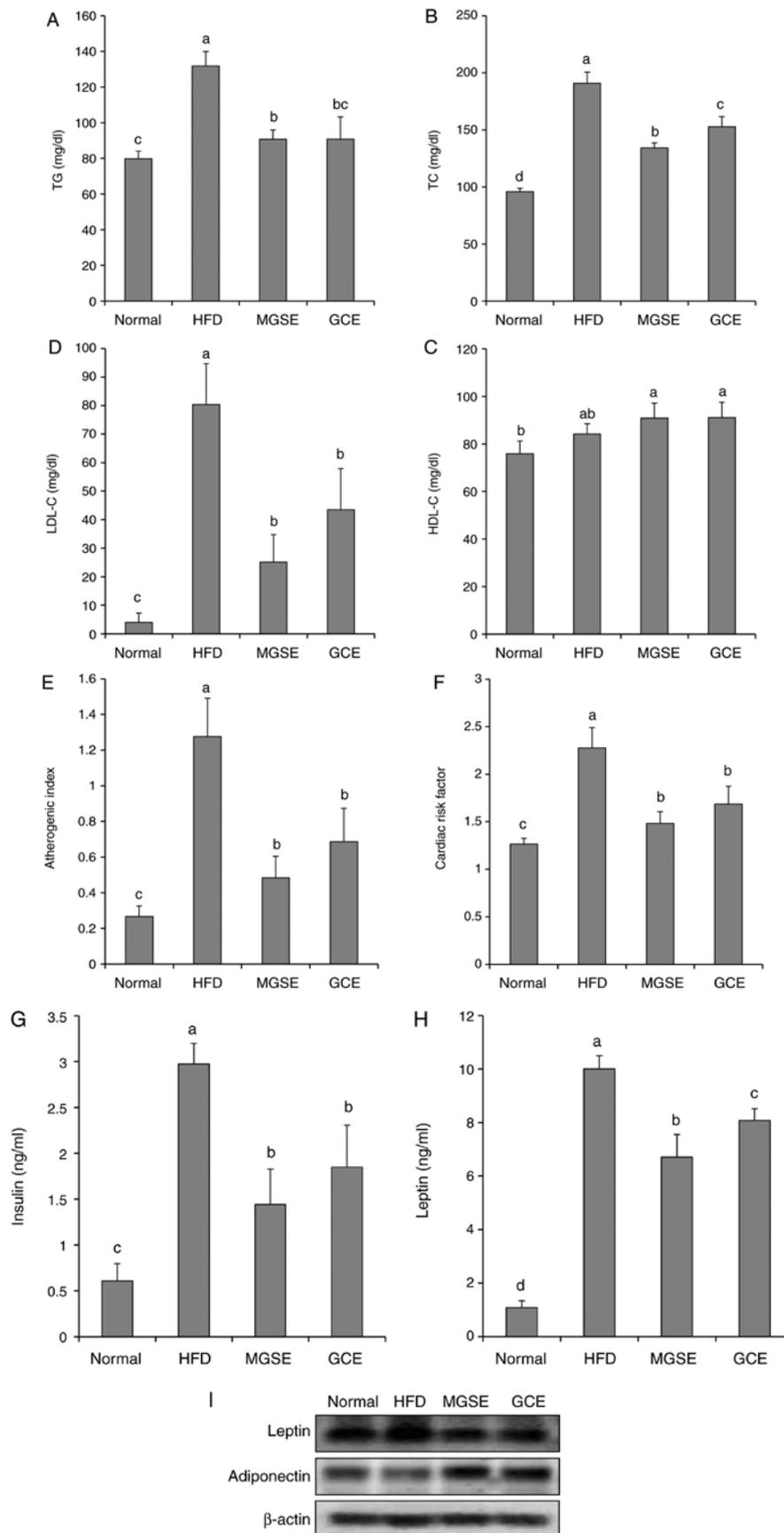


Figure 3. Effects of MGSE on serum lipid profiles of (A) TG, (B) TC, (C) LDL-C and (D) HDL-C, (E) atherogenic index, (F) cardiac risk factor, (G) insulin and (H) leptin, and (I) the expression of leptin and adiponectin in HFD-induced obesity in mice. Results are presented as the mean \pm standard deviation. Results were significantly different ($P < 0.05$) from all other results, unless labelled with the same letter. Normal, mice fed on the control diet (negative control); HFD, mice fed on the HFD (positive control); MGSE, mice fed on the HFD and administered with 200 mg/kg/day MGSE; GCE, mice fed on the HFD and administered with 200 mg/kg/day GCE. MGSE, Muscat Bailey A grape stalk extract; GCE, *Garcinia cambogia* extract; TG, triacylglycerol; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HFD, high-fat diet.

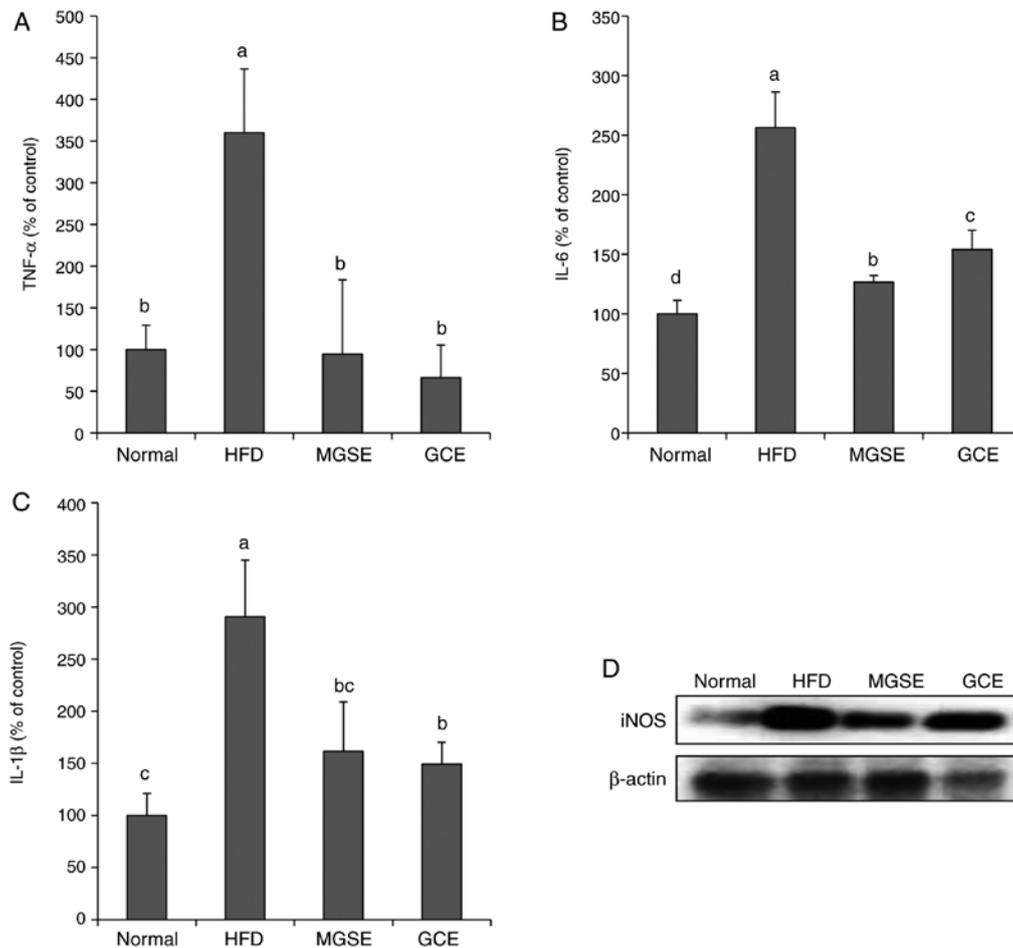


Figure 4. Effects of MGSE on the inflammatory response induced by HFD in obese mice. Levels of the pro-inflammatory cytokines (A) TNF- α , (B) IL-6 and (C) IL-1 β were determined in serum samples of mice. Results are presented as the mean \pm standard deviation. Results were significantly different ($P < 0.05$) from all other results, unless labelled with the same letter. (D) Effects of MGSE on the expression of iNOS. Liver tissue samples (0.02–0.03 g) from each mice in each group ($n=5$) were mixed and the expression levels of the indicated proteins was determined by western blotting. Normal, mice fed on the control diet (negative control); HFD, mice fed on the HFD (positive control); MGSE, mice fed on the HFD and administered with 200 mg/kg/day MGSE; GCE, mice fed on the HFD and administered with 200 mg/kg/day GCE. MGSE, Muscat Bailey A grape stalk extract; GCE, *Garcinia cambogia* extract; HFD, high-fat diet; TNF- α , tumor necrosis factor α ; IL, interleukin; iNOS, inducible nitric oxide synthase.

which abnormally large numbers of lipid droplets, primarily composed of TG, accumulate in liver cells. Most importantly, the non-alcoholic fatty liver disease caused by excessive caloric consumption induces chronic inflammation (hepatic steatosis) in the presence of oxidative stress that may result in cancer (35). Therefore, decreasing serum levels of AST, ALT and pro-inflammatory cytokines as well as upregulating the antioxidants defense system will definitely prevent fatty liver diseases in obesity. The results of the present study indicated that MGSE decreased serum levels of the liver enzymes AST and ALT (Fig. 5); decreased serum IL-6, IL-1 β and TNF- α , and iNOS expression in the liver (Fig. 4); prevented lipid peroxidation in the liver; and also upregulated GSH, SOD and catalase in liver tissues (Fig. 6). Increases in GSH, SOD and catalase levels explain the decrease in lipid peroxidation exhibited by MDA levels, and further explain the significantly decreased serum levels of AST and ALT observed in MGSE-treated mice. Furthermore, photomicrographs of liver samples stained with H&E revealed that the livers of mice fed on a normal diet exhibited no pathological abnormalities (Fig. 7). However, in livers of mice fed on the HFD, the hepa-

cytes exhibited a number of large vacuoles within a number of lipid droplets (Fig. 7). Treatment with MGSE attenuated the hepatic steatosis. The GCE-treated group exhibited a similar effect. These results therefore indicated that MGSE treatment exerts hepatoprotective functions in obesity and liver damage via regulation of chronic inflammation and oxidative stress.

To determine the molecular mode-of-action of MGSE in obesity, the protein expression levels of C/EBP α and PPAR γ in the mice liver tissue were determined. PPAR γ and C/EBP α co-regulate the transcriptional pathway of adipogenesis. In the early phase of adipogenesis, C/EBP β and C/EBP δ are expressed, but PPAR γ and C/EBP α are induced. C/EBP α binds to the PPAR γ promoter, leading to the expression of PPAR γ . PPAR γ serves a dominant function in the differentiation and maturation of adipocytes (36,37). Previous studies have identified that an HFD induces the expression of adipogenic genes such as those encoding PPAR γ and C/EBP α (38–40). This was also true in the present study as mice fed on an HFD exhibited an increase in the expression of phosphorylated PPAR γ and C/EBP α protein. However, when the mice fed on the HFD were administered with MGSE, the expression of phosphorylated PPAR γ and

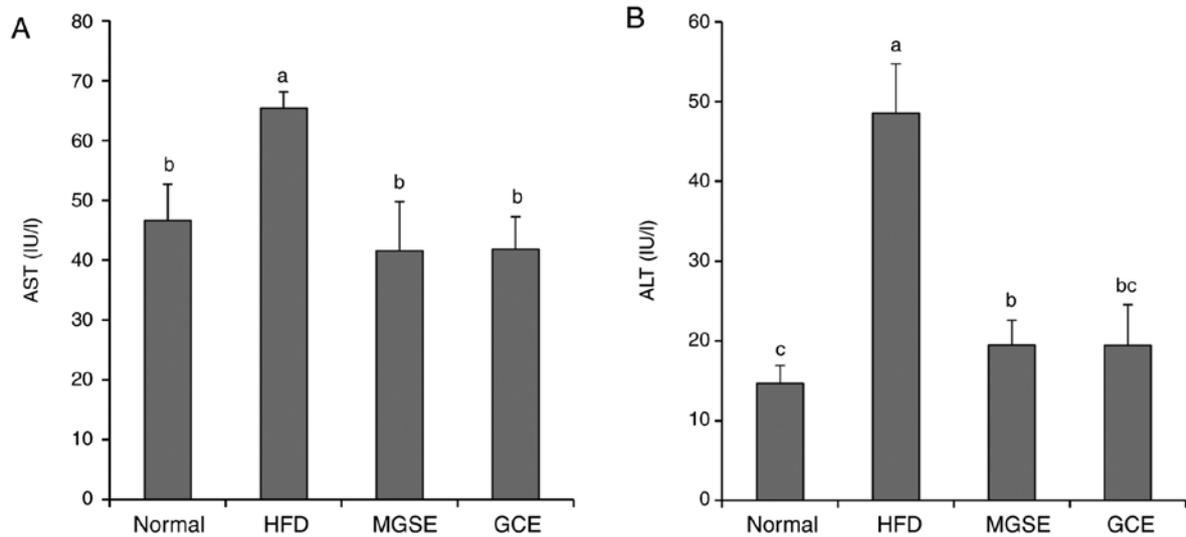


Figure 5. Effects of MGSE on (A) AST and (B) ALT levels in HFD-induced obesity in mice. Results are presented as the mean \pm standard deviation. Results were significantly different ($P < 0.05$) from all other results, unless labelled with the same letter. Normal, mice fed on the control diet (negative control); HFD, mice fed on the HFD (positive control); MGSE, mice fed on the HFD and administered with 200 mg/kg/day MGSE; GCE, mice fed on the HFD and administered with 200 mg/kg/day GCE. MGSE, Muscat Bailey A grape stalk extract; GCE, *Garcinia cambogia* extract; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HFD, high-fat diet.

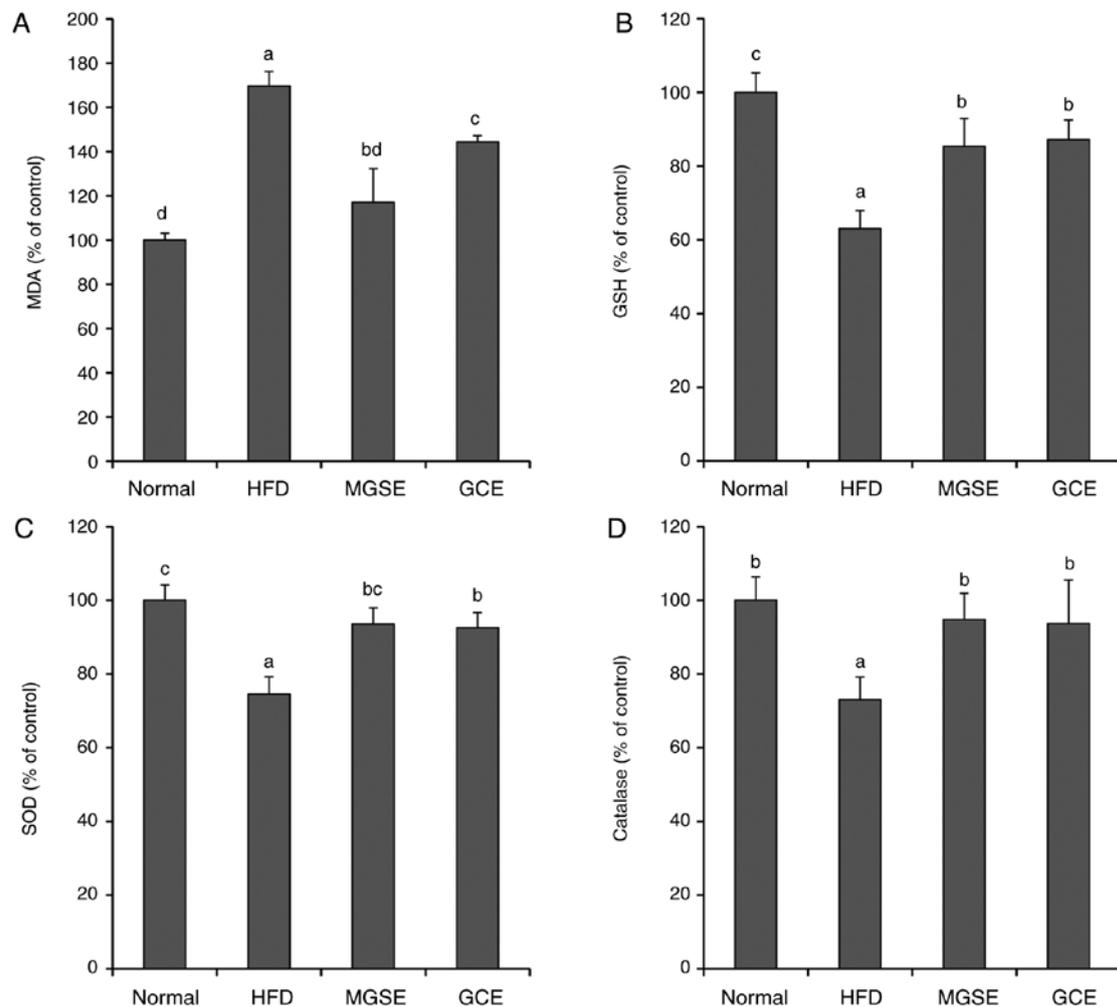


Figure 6. Effects of MGSE on (A) lipid peroxidation, (B) GSH concentration, (C) SOD and (D) catalase activity in liver tissues of mice. Lipid peroxidation was quantified as a level of MDA. Results are presented as the mean \pm standard deviation. Results were significantly different ($P < 0.05$) from all other results, unless labelled with the same letter. Normal, mice fed on the control diet (negative control); HFD, mice fed on the HFD (positive control); MGSE, mice fed on the HFD and administered with 200 mg/kg/day MGSE; GCE, mice fed on HFD and administered with 200 mg/kg/day GCE. MGSE, Muscat Bailey A grape stalk extract; GCE, *Garcinia cambogia* extract; HFD, high-fat diet; MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase.

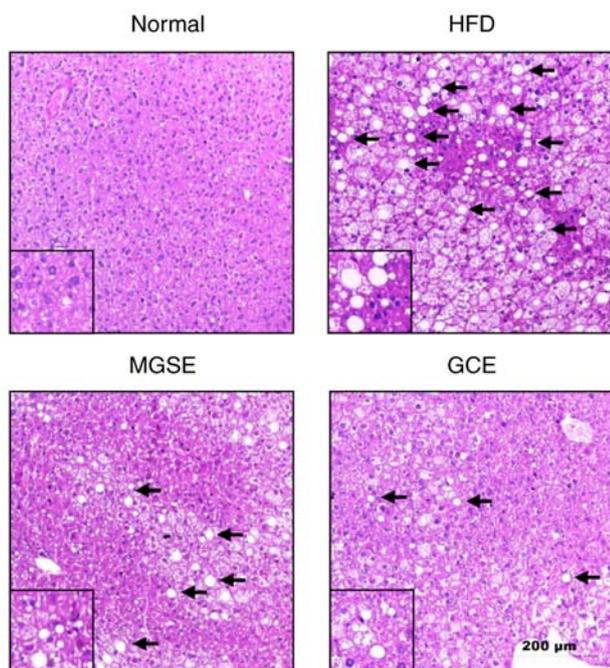


Figure 7. Effect of MGSE on liver morphology (magnification, x100) in HFD-induced obese mice exhibiting hepatic steatosis. Normal, mice fed on the control diet (negative control); HFD, mice fed on the HFD (positive control); MGSE, mice fed on the HFD and administered with 200 mg/kg/day MGSE; GCE, mice fed on the HFD and administered with 200 mg/kg/day GCE. Arrows indicate lipid droplets. MGSE, Muscat Bailey A grape stalk extract; GCE, *Garcinia cambogia* extract; HFD, high-fat diet.

C/EBP α were downregulated (Fig. 8). The GCE-treated group exhibited a similar effect. These results may provide a clue to the understanding of the mechanism of action of MGSE on obesity in mouse models. However, in-depth molecular studies are required.

A number of studies have indicated that polyphenol compounds such as resveratrol, rutin and quercetin attenuate HFD-induced obesity in rodents (41-43). It was previously identified that catechin and epicatechin-rich grape seed extract suppressed HFD-induced obesity in mice (44). Rahman *et al* (45) demonstrated that *Cosmos caudatus* Kunth leaf extract including catechin, epicatechin, quercetin and rutin exhibited an anti-obesity effect in HFD-fed rats. Rodriguez Lanzi *et al* (46) also identified that grape pomace extract contained various compounds including resveratrol, catechin, epicatechin, quercetin and rutin, inhibited differentiation of 3T3-L1 cells and decreased white adipose fat weight in rats. In addition, it has been identified that resveratrol suppresses differentiation of 3T3-L1 adipocytes (47). The results of the present study indicated that MGSE decreased the lipid and TG content of 3T3-L1 cells, and also that catechin, epicatechin, rutin and resveratrol attenuated lipid content in 3T3-L1 cells (data not shown). Therefore, the results of the present study suggest that the anti-obesity effect of MGSE may be associated with polyphenol compounds such as catechin, epicatechin, rutin and resveratrol.

In conclusion, the results of the present study indicated that the harvest period affects secondary metabolite content and therefore the antioxidant abilities of the grape stalk. Furthermore, it has been identified that MBA grape stalk

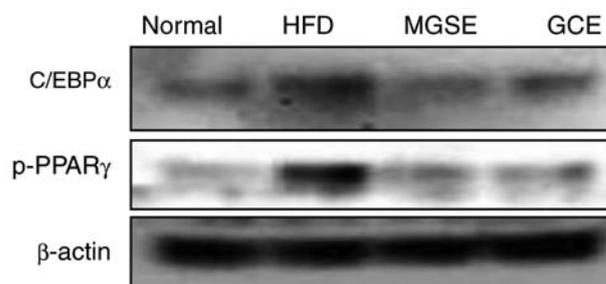


Figure 8. Effects of MGSE on the expression of C/EBP α and phosphorylated PPAR γ . Liver tissue samples (0.02-0.03 g) from each mice in each group (n=5) were mixed and the expression levels of the indicated proteins was determined by western blotting. Normal, mice fed on the control diet (negative control); HFD, mice fed on the HFD (positive control); MGSE, mice fed on the HFD and administered with 200 mg/kg/day MGSE; GCE, mice fed on the HFD and administered with 200 mg/kg/day GCE. MGSE, Muscat Bailey A grape stalk extract; GCE, *Garcinia cambogia* extract; HFD, high-fat diet; C/EBP α , CCAAT/enhancer-binding protein α ; PPAR γ , peroxisome-proliferator-activated receptor γ ; p-, phospho-.

harvested in June has beneficial effects in HFD-induced obesity that can lead to Type 2 diabetes, hepatic steatosis, cardiovascular disorders, inflammation and oxidative stress. MGSE led to no evident hepatotoxicity while it improved liver test results. Detailed mechanisms of action of MGSE remain to be investigated further. However, these results suggested that MBA grape stalk may be a potential nutraceutical for the prevention/treatment of obesity and obesity-associated disorders.

Acknowledgements

The authors thank Mr. Denis Nchang Che (Department of Food Science and Technology, Chonbuk National University, Jeonju, Republic of Korea), Mr. Jae Young Shin (Department of Health Management, Jeonju University, Jeonju, Republic of Korea) and Miss Hyun Ju Kang (Ato Q&A Co. Ltd., Jeonju, Republic of Korea) for animal care, and Mr. Sang Jun Kim (Jeonju AgroBio-Materials Institute, Jeonju, Republic of Korea) for HPLC analysis.

Funding

The present study was supported financially by the Ministry of Trade, Industry, and Energy, Korea, under the Regional Specialized Industry Development Program (grant no. R0006168) supervised by the Korea Institute for Advancement of Technology.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BMK, BOC and SIJ designed the research. BMK and BOC performed the research. BMK, BOC and SIJ analyzed the data and wrote the manuscript. SIJ supervised the research project. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal protocols were performed following approval from Jeonju University Institutional Animal Care and Use Committee (#JJU-IACUC-2016-011).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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