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RESEARCH PAPER

Anti-hyperlipidemic and Fat Pad Lowering Effect of Standardized Tea Seed Cake Extract in Mice Fed High-fat and High-carbohydrate Diet

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Abstract *Camellia sinensis* L. O. Kuntze has traditionally been used in Asian countries as a stimulant, a diuretic, and to prevent obesity. Studies in green tea are well-documented in literature, though the potential of fruit from the plant has never been studied in details. In the present study, the anti-adipogenic effect of aqueous extract of green tea seed cake extract (GTE) were analysed *in-vitro* and the anti-obesity effects were determined *in-vivo* in murine model fed high-fat and high-carbohydrate diets. We found that GTE inhibited the lipid accumulation in 3T3-L1 adipocyte and significantly lowered body weight, body fat-pad weights, blood lipid, glucose, insulin, and leptin levels in mice fed high-fat and high-carbohydrate diets. GTE suppressed the expression of important adipogenic genes (PPARγ, C/EBPα, SREBP, LPL, aP2 and leptin) and lipid metabolism genes (FAS). Furthermore, GTE induced the levels of adiponectin in serum and adipose tissue, as well as AMP-activated protein kinase (AMPK) in liver tissue. These results suggested that GTE ameliorates its anti-obesity effect through multiple cells signaling pathways in diets induced obesity in mice.

Keywords: green tea seed cake, high-fat diet, high-carbohydrate diet, anti-obesity, adipose tissue, AMPK

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1. Introduction

Obesity is a complex, multi-factorial metabolic disorder that requires long term management. It is commonly associated with type II diabetes mellitus, hypertension, coronary heart disease, and cancer [1,2]. Obesity results from the excessive growth and expansion of adipose tissue due to an imbalance between energy intake and expenditure. Excess energy is stored in fat cells that enlarge or increase in number [3,4]. Adenosine monophosphate (AMP)-activated protein kinase (AMPK) plays an important role in regulating glucose metabolism, including glucose transport, lipogenesis, gluconeogenesis, and lipolysis [5]. AMPK is a serine-threonine kinase that is activated in response to increase in the intracellular AMP/ATP ratio within the cell and the activation of AMPK has been characterized on liver metabolism [6]. Binding of AMP with AMPK allosterically phosphorylates and activates AMPK, which in turn inactivates anabolic pathways involved in fatty acid and cholesterol synthesis and activates catabolic pathway regulating the enzymes of

energy metabolism [7]. AMPK inhibits de novo synthesis by inactivating acetyl-CoA carboxylase (ACC-1), and fatty acid synthetase (FAS) which catalyse the key regulatory steps in fatty acid and sterol synthesis and activates fatty acid oxidation [8]. Carnitine palmitoyltransferase-1 (CPT-1) transfers cytosolic long-chain fatty acyl CoA into the mitochondria for oxidation. It is allosterically inactivated by malonyl CoA and catalyzed by the enzyme malonyl-CoA decarboxylase (MLYCD). Glycerol-3-phosphate acyl transferase-1 (GPAT-1) is the enzyme that esterifies fatty acids to glycerol to form triglyceride for storage. AMPK also regulates the ligand-activated transcriptional factors such as peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα), and sterol regulatory element binding proteins (SREBP), which are the central regulators of adipogenesis and lipogenesis [9,10]. Thus, the AMPK pathway is regarded as a potential therapeutic target for metabolic disorders.

Green tea (*Camellia sinensis*) contains many bioactive compounds such as catechins, caffeine, saponins, theanine, vitamins, oil mineral and trace elements [11-13]. The components of green tea have many biological and biochemical effects such as anti-mutation [14], anti-carcinogenesis [15-18], antioxidation [19,20], apoptosis-inducing [21] and anti-angiogenesis [22]. The major bioactive compounds present in green tea seed are saponin glycosides [23], kaempferol [24], and naringenin [25]. The present study was conducted to evaluate the effect of standardized green tea seed cake extract (GTE) supplementation on adipocyte differentiation, and on body weight gain in mice fed high-fat and high-carbohydrate diets using biochemical markers to elucidate the molecular mechanism for such an effect.

2. Materials and Methods

2.1. Extract preparation and analysis

Green tea [*Camellia sinensis* (L.) O. Kuntze] seeds were collected from Myungin Shin GwangSu tea garden, Suncheon, Korea. Dried tea seeds were pressed to remove oil and the pomace powder (360 g) was extracted with distilled water (1.8 L) at 90°C for 1.5 h. The resulting suspension was filtered and lyophilized. The yield of the lyophilized residue was 64.5 g. Ten grams of extract were dissolved in 80% methanol and the saponin glycosides were quantified by preparative high-performance liquid chromatography (HPLC) by the method described previously [26]. Teasaponin (96%, $C_{57}H_{90}O_{26}$, molecular weight 1200, Aladdin Chemistry Co. Ltd., Shanghai, China) was used as standard. For the analysis of naringenin as an index material for GTE, one hundred milligrams of GTE was added in 1 mL of sodium acetate buffer (0.02 M, pH 5.0) containing 1 mL of Novozym 33095 (Novozymes, Neumatt, Dittingen, Switzerland), 1 mL of Pectinex ultra SP-L (Novozymes) and 1 mL of Viscozyme (Novozymes) and incubated at 37°C for 24 h with agitation (150 rpm). This suspension (200 μL) was mixed with 800 μL of ethanol and centrifuged (at 10,000 rpm, at 4°C for 10 min). The supernatant was analyzed by HPLC at 280 nm with a 1 mL/min flow rate and the injection volume was 20 μL. The mobile phase consisted of 10% acetonitrile in 0.1% trifluroacetic acid (A)/ 50% acetonitrile in 0.1% trifluroacetic acid (B). The gradient system used was initially A (80%)/B (20%) at 0 min to A (20%)/B (80%) at 60 min. Naringenin (95%, Sigma, St. Lous, MO, USA) was used as standard.

Saponins and naringenin were characterized by LC/MS analysis (Fig. 1). For saponin glycosides, LC/TOF-MS (1050-1300 *m/z*) was performed on a LTQ Orbitrap XL instrument (Thermo Scientific, Bremen, Germany) as described [13]. High-resolution mass analysis for naringenin was conducted by using hybrid high-performance liquid chromatography-mass spectrometer (Shimadzu, LCMS-IT-TOF). ESI-MS was performed in the positive mode under the following operating parameters: probe voltage, 4.5 kV; nitrogen gas flow, 1.5 L/min; MS range, 150 ~ 1,500 *m/z*; Curved desolvation line (CDL) temperature, 200°C; event time, 100 ms; ion accumulation time, 20 ms.

2.2. Cell culture

The 3T3-L1 cell line was obtained from the Korean Cell Line Bank, Seoul, Korea. 3T3-L1 cells were cultured in DMEM medium (Gibco, Grand Island, NY, USA) supplemented with $NaHCO_3$ (3.7 g/L), 100,000 IU/L penicillin, 100 mg/L streptomycin, and 10% (v/v) fetal bovine serum (FBS).

2.3. Cell viability assay

Cell viability was determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. 3T3-L1 cells (1×10^4 cells/well) were seeded in a 96-well plate for 24 h and treated with different concentration of GTE for 24 h, at 37°C in a humidified 5% CO_2 incubator. 0.5% MTT solution (Sigma) was added in the medium and incubated for 4 h at 37°C. MTT medium was aspirated and dimethyl sufoxide (DMSO) (Sigma) was added to dissolve the formazan crystals for 15 min. Absorbance was measured at 540 nm using a microplate reader (Biochrom Ltd., Cambridge, UK). Cell viability was calculated as relative percentage compared to control.

2.4. Oil red O staining

3T3-L1 cells (3×10^3 cells/cm^2) were seeded into six-well

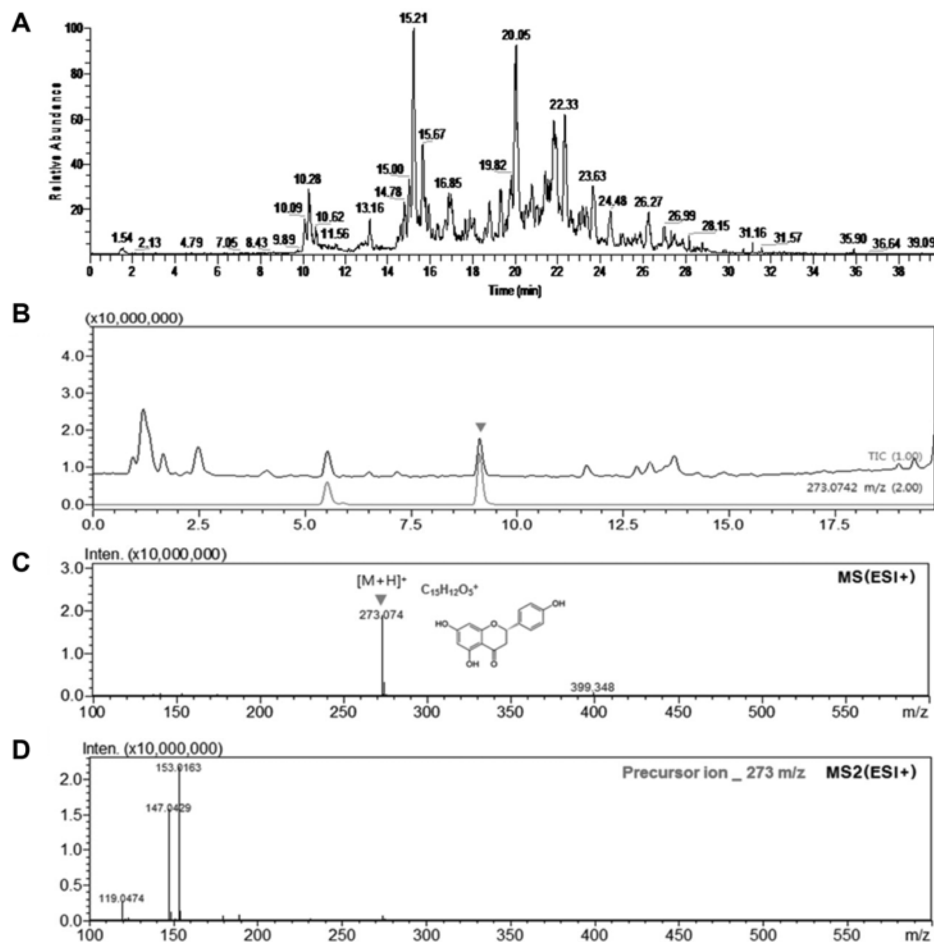

Fig. 1. LC-MS and LC-MS/MS analysis of saponin glycosides and naringenin in green tea seed extract. (A) Base peak intensity chromatogram of saponin-rich fraction of green tea seed cake aqueous extract, *m/z* = 1050-1300. (B) LC/MS chromatogram of GTE after enzymatic hydrolysis, *m/z* = 273.0742. (C) LC/MS/MS chromatogram of naringenin of the GTE. (D) Product-ion mass spectra of naringenin.

plates. Two days after reaching confluence, cells were kept for another 24 h in this state to arrest cell division. At this point (day 0), culture medium was changed to differentiation-induction medium (1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 10 μg/mL insulin). After 2 days, cells were maintained in maintenance medium (10 μg/mL insulin in culture medium). Maintenance medium was changed every 2 days until day 8. To the differentiation-induction medium and maintenance medium, different concentrations of GTE were added. Epigallocatechin gallate (EGCG, 50 μg/mL) (Sigma) was taken as a positive control. Cells were washed with PBS and fixed with 10% formalin for 1 h. Again, the cells were washed with 60% 2-propanol to dryness and then incubated with Oil red O working solution for 3 h. Once again, cells were washed four times with distilled water and completely dried cells were washed with 100% 2-propanol to extract staining dye of the cells. The absorbance of extracted Oil red O solution was measured at 520 nm using a 96 well plate.

2.5. Animals

All animals in the study were treated according to the National Institutes of Health Guide for the care and use of Laboratory Animals and the experimental protocol was approved by the Chonnam National University Ethical community for Animal studies (CNU IACUC–YS–2013-3). Fifty four 8-week-old female ICR mice (Taconic, Chungbuk, Korea) were randomly assigned to 9 groups of 6 mice each after one week of acclimation. All animals were housed under standard conditions (12 h light/dark cycles, at 22°C and relative humidity 50 ± 5%) and given diets and water ad libitum. The control groups were fed with normal diet (normal diet control (NC); NIH #31 M Rodent Diet, Taconic), high-fat diet (high-fat diet control (HFC); 45% fat, D12451 Research Diets, New Brunswick, NJ, USA) and high-carbohydrate diet (high-carbohydrate diet control (HCC); 70% carbohydrate, TD.98090 Harlan Teklad, Seoul, Korea). One group served as positive control group fed with high-fat diet plus 50 mg/kg/day Orlistat® (HF-O). The mice

in other four group received high-fat diet plus 100 mg/kg/day GTE (HF-100), high-fat diet plus 300 mg/kg/day GTE (HF-300), high-carbohydrate diet plus 100 mg/kg/d GTE (HC-100) and high-carbohydrate diet plus 300 mg/kg/day GTE (HC-300). One another group was fed with high-fat diet for 4 week to induce obesity pre-treatment and then fed with 300 mg/kg/day GTE (PHF-300). All the drugs were administered by oral gavage once a day and control groups were administered distilled water without drugs at the same time. Body weight and food intake were measured twice per week on a group basis. After 10 week of study, mice were anesthetized with ether after an overnight fasting and blood was collected from abdominal vena cava of each mice. Serum samples were stored at –80°C until analysis. Perirenal white adipose (WAT), parametrial WAT, mesenteric WAT and liver tissues were excised and weighed. The mesenteric adipose and liver tissues were removed under aseptic conditions and snap frozen in liquid nitrogen and stored at −80°C for RNA and protein isolation.

2.6. RNA isolation and RT-PCR analysis

The totals RNA from liver tissues were isolated by using TRI reagent (Sigma, USA) according to the manufacturer's instructions. The isolated RNA was subjected to DNase treatment and 500 ng of total RNA was used for cDNA synthesis using a RevertAid Premium First Strand cDNA Synthesis Kit (Thermo scientific, EU). Reverse transcription polymerase chain reaction (RT-PCR) was used to analyze the mRNA expression level of genes using gene specific primers (Table 1). PCR reaction was performed by using Takara Ex Taq Hot Start Version (Takara, Japan). PCR reactions consisted of an initial denaturing cycle at 94°C for 3 min, followed by 35 amplification cycles: 94°C for 30 sec, 60°C for 45 sec, and at 72°C for 1 min. One additional cycle of 72°C for 7 min was run to allow trimming of incomplete polymerizations. Amplified products were separated by electrophoresis on 1.5% agarose gel and visualized by UV transillumination.

2.7. Western blot analysis

The liver tissues were homogenized at 4°C using PRO-PREP Protein Extraction Solution (iNTRON Biotechnology Inc., Gyeongi-do, Korea) according to the manufacturer's instructions and protein concentration was measured by BCA protein Assay Reagent (Pierce, Rockford, IL, USA). Protein samples (30 μg) were resolved in loading buffer, separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were then blocked in Tris-buffered saline (TBS)-Tween 20 solution containing 3% bovine serum albumin and incubated overnight at 4°C with phospho-AMPKα (Cell Signaling Technology, Danvers, MA) and β-actin (Santa Cruz, CA) primary antibodies. Protein bands were visualized using corresponding secondary antibody conjugated to horseradish peroxidase and enhanced chemiluminescence (Amersham Bioscience, UK).

2.8. Biochemical analyses

The level of total cholesterol (TC), triacylglycerol (TG), HDL cholesterol, LDL cholesterol, free fatty acid (FFA), glucose, alanine transaminase (ALT) and aspartate transaminase (AST) in serum were measured according to the kit manufacturer's instruction (Wako Chemicals, Osaka, Japan). Serum insulin level was measured by immunoassay using a mouse insulin ELISA kit (Shibayagi, Shibukawa, Japan). Serum concentration of leptin, adiponectin, IL-6,

Table 1. Gene-specific primers along with their product size and gene bank accession number used for PCR

Gene	Primer sequence	Gene bank accession no.	Size (bp)
PPARγ	F 5'-CTG GCC TCC CTG ATG AAT AA-3' R 5'-GGG TGA AGG CTC ATG TCT GT-3'	NM_001127330.1	393
SREBP	F 5' -TTG CAC CAG AGA GCA TTT TG-3' R 5'-GAA AAT GAG AGG CTG GTT GC-3'	NM_033218.1	593
C/EBPα	F-5'-TTA CAA CAG GCC AGG TTT CC-3' R 5'-CCA CAG GGG TGT GTG TAT GA-3'	NM_007678.3	629
FAS	F-5'-AAA GGA CCT GCC CAA TCT CT-3' R-5'-TGA TCA AAC TCA GGC TGC AC-3'	NM_007988.3	245
aP2	F-5'-CAG CCT TTC TCA CCT GGA AG-3' R-5'-TCG ACT TTC CAT CCC ACT TC-3'	NM_024406.2	352
Leptin	F-5'-CTC ATG CCA GCA CTC AAA AA-3' R-5'-AGG TGA CCA AGG TGG CAT AG-3'	NM_008493.3	465
LPL	F-5'-AAG CCC CAC AAG TGT AGT CG-3' R-5'-CGG ACA CAA AGT TAG CAC CA-3'	NM_008509.2	402
Actin	F-5'-GTT GGT TGG AGC AAA CAT CC-3' R-5'-GAG GGT GAG GGA CTT CCT GT-3'	NM_007393.3	151

IL-10 and TNF-α level and mesenteric adipose tissue concentration of leptin, adiponectin and IL-6 were measured by using mouse ELISA kit (Abcam, Cambridge, MA, USA). The total proteins of mesenteric adipose tissue were extracted using TRI reagent and resuspended in 1% SDS solution according to the manufacturer's instructions and protein concentration was measured as mentioned above.

2.9. Fecal lipid concentration

Fecal lipid was extracted as described [27]. Briefly, for the analysis of fecal lipids, feces were collected for 3 weeks at the end of the experiment. Fecal samples were cleaned and dried at 70°C for 1 h, 100 mg aliquots of feces were incubated with 2 mL of chloroform-methanol (2:1) at 60°C for 30 min with constant agitation and then centrifuged. One milliliter of water was added to the supernatant, vortexed and centrifuged (2,000 rpm for 10 min). The lower phase was collected in a new tube and the sample was evaporated to dryness, resuspended in 0.5 mL of Triton X-100/methanol (2:1). The quantities of total cholesterol and triacylglycerol in the fecal lipid extracts were measured according to the kit manufacturer's instruction (Wako Chemicals).

2.10. Statistical analysis

Data are expressed as mean±SEM. Differences in hemodynamic parameters between treatments groups were analyzed by Two-Way ANOVA. Differences in cell culture system data were tested by One-Way ANOVA followed by Tukey-Kramer Multiple Comparisons Test (SPSS 21). Values of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Inhibition of lipid accumulation

To investigate the effect of GTE on adipocyte differentiation, pre-adipocyte 3T3-L1 cells were differentiated into mature adipocytes in the presence of various concentration of GTE. EGCG (50 µg/mL) was used as positive control. Treatment of cells with GTE during differentiation suppressed the accumulation of lipid in a dose-dependent manner as measured by Oil Red O staining (Fig. 2A). The amount of triglycerides was significantly decreased at 50, 75, and 100 µg/mL in compare to control (Fig. 2B). It is important to note that inhibitory effect of GTE (100 µg/mL) was equal to the effect of positive control used. We also examined the effect of GTE on 3T3-L1 cell viability. GTE showed no significant effect on 3T3-L1 viability (Fig. 2C). The result suggests that GTE prevents the differentiation of pre-adipocyte 3T3-L1 cells whereas cell viability was not affected at the experimental concentration.

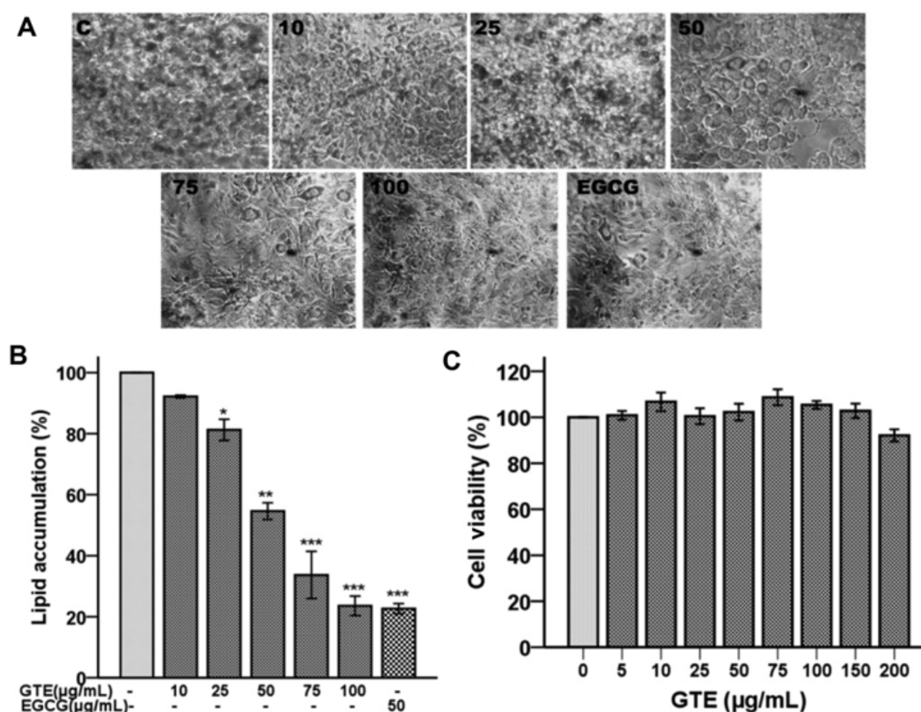

Fig. 2. Aqueous extract of green tea seed cake inhibits adipocyte differentiation. (A) Representative photograph of Oil-Red O stained 3T3-L_1 cells. Stained 3T3-L_1 was observed under an inverted phase-contrast microscope and photographed at x100 magnification. (B) Quantitative analysis of adipocyte differentiation measured by Oil red O staining. (C) The effect of GTE on cytotoxicity of 3T3-L_1 cells.. Data are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$ *vs*. control.

Table 2. Effect of aqueous extract of green tea seed cake on food intake and serum biochemical parameters in high-fat and high-carbohydrate diet induced obesity in mice

Parameters	NC	HFC	HCC	HFO	HF-100	HF-300	PHF-300	HC-100	HC-300
Food intake (kcal/day)	28.07 ± 2.43	27.79 ± 1.56	24.77 ± 0.44	29.59 ± 0.28	26.59 ± 2.1	24.59 ± 2.64	28.58 ± 1.31	24.57 ± 1.588	23.88 ± 1.96
Serum									
TG (mg/dL)	186.93 ± 25.73*#	287.15 ± 26.20	239.56 ± 29.39	214.09 ± 21.53*	226.94 ± 25.82*	200.94 ± 28.31*	213.82 ± 23.02*	217.85 ± 37.03#	175.32 ± 24.90#
TC (mg/dL)	69.27 ± 2.41*#	145.83 ± 18.59	106.48 ± 7.63	85. 24 ± 5.23*	129.28 ± 23.04	82.81 ± 3.89*	93.28 ± 2.50*	68.92 ± 4.05#	66.26 ± 1.83#
FFA (μEq/L)	179.35 ± 26.54*#	247.98 ± 28.52	216.07 ± 38.66	154.02 ± 12.66*	142.63 ± 9.29*	133.76 ± 21.90*	173.52 ± 26.08*	145.81 ± 21.10#	121.03 ± 11.08#
HDL (mg/dL)	49.14 ± 2.68*#	36.93 ± 4.12	39.38 ± 3.40	48.60 ± 5.62*	43.89 ± 7.87*	48.97 ± 3.95*	47.85 ± 1.59*	45.51 ± 7.32#	50.87 ± 3.94#
LDL (mg/dL)	20.35 ± 1.37*#	54.29 ± 3.83	47.54 ± 2.47	27.92 ± 1.49*	43.24 ± 5.27	24.29 ± 3.47*	21.72 ± 1.46*	23. 84 ± 1.36#	20.48 ± 2.91#
Glucose (mg/dL)	108.75 ± 11.66*#	136.85 ± 13.68	130.45 ± 20.59	120.34 ± 9.95*	126.36 ± 17.15*	103.68 ± 11.26*	106.61 ± 3.74*	118.11 ± 19.89#	107.60 ± 6.41#
ALT (IU/L)	36.86 ± 3.34*#	87.73 ± 8.65	57.86 ± 9.56	40.80 ± 3.95*	56.31 ± 10.84*	49.09 ± 5.63*	46.25 ± 3.08*	42.67 ± 3.08#	36.99 ± 00.84#
AST (IU/L)	48.34 ± 0.95*#	109.63 ± 4.36	78.06 ± 8.13	74.56 ± 1.59*	59.23 ± 1.05*	55.35 ± 2.37*	56.38 ± 5.75*	40.95 ± 6.98#	31.46 ± 0.60#
Feces									
TG (mg/g)	2.08 ± 0.14	1.57 ± 0.95	2.26 ± 0.90	2.93 ± 0.19*	1.81 ± 0.13	3.42 ± 0.21*	2.87 ± 0.18*	3.21 ± 0.70#	3.60 ± 0.11#
TC (mg/g)	3.48 ± 0.41#	2.72 ± 0.14	2.26 ± 0.38	3.89 ± 0.26	3.07 ± 0.84*	4.97 ± 0.45*	4.86 ± 0.44*	3.38 ± 0.83#	4.95 ± 0.58#

Data are expressed as mean ± SEM (n = 6). *P < 0.05, significantly different from HFC. #P < 0.05, significantly different from HCC.

3.2. Effect of GTE on body mass gain and food intake

To investigate whether GTE could modulate obesity in mice model, eight week old female ICR mice were randomly assigned to 9 groups (n = 6). The first group was fed with normal diet, five groups were fed with high-fat diet and the other three groups were fed high-carbohydrate diet. GTE was orally administered daily to the treatment groups. Food intake was measured twice per week. The food intake among high-fat and high-carbohydrate diet fed groups were not statistically significant. However, the food intake in the first group fed normal diet was slightly higher than the other groups (Table 2).

After 10 weeks, the total body weight of mice in HFC group and HCC group were significantly higher than mice in NC group (Fig. 3). The body weight of mice in HF-100 and HF-300 groups were significantly decreased compared with the HFC group (Figs. 3A and 3B). The PHF-300 group was fed with high-fat diet for 4 weeks to induce obesity before treatment. After treatment of 300 mg/kg GTE to PHF-300 group, body weight gain was significantly reduced during 7 ~ 10 weeks. The positive control HF-O group showed decrease in body weight gain, however, gastro-intestinal side effects such as oily and loose stools were observed. Next, the treatment of GTE suppressed the body weight gains in high-carbohydrate diet fed groups from the early week of experiment. The significant effect on body weight gain was observed during 3 ~ 10 weeks and 2 ~ 10 weeks in HC-100 and HC-300 groups, respectively. Overall, GTE treatment in mice fed high-fat diet suppressed further gains in body weight throughout the study whereas slimming effect was observed in GTE treatment groups fed high-carbohydrate diet.

3.3. Effect of GTE on liver and adipose tissue mass gain

As shown in Fig. 3E, the weight of WAT in the high-fat diet plus treatment groups were significantly decreased in compared to HFC group (P < 0.05). Also, the weights of WAT in the HC-100 and HC-300 groups were significantly decreased than HCC group (Fig. 3F). Liver weight in mice fed high-fat and high-carbohydrate diet control groups were higher compared to the respective GTE treatment groups (Figs. 3G and 3H) but not statistically significant.

3.4. Serum and fecal biochemical parameters

The results of serum biochemical parameters are shown in Table 2. The serum levels of TG, TC, FFA, LDL cholesterol,

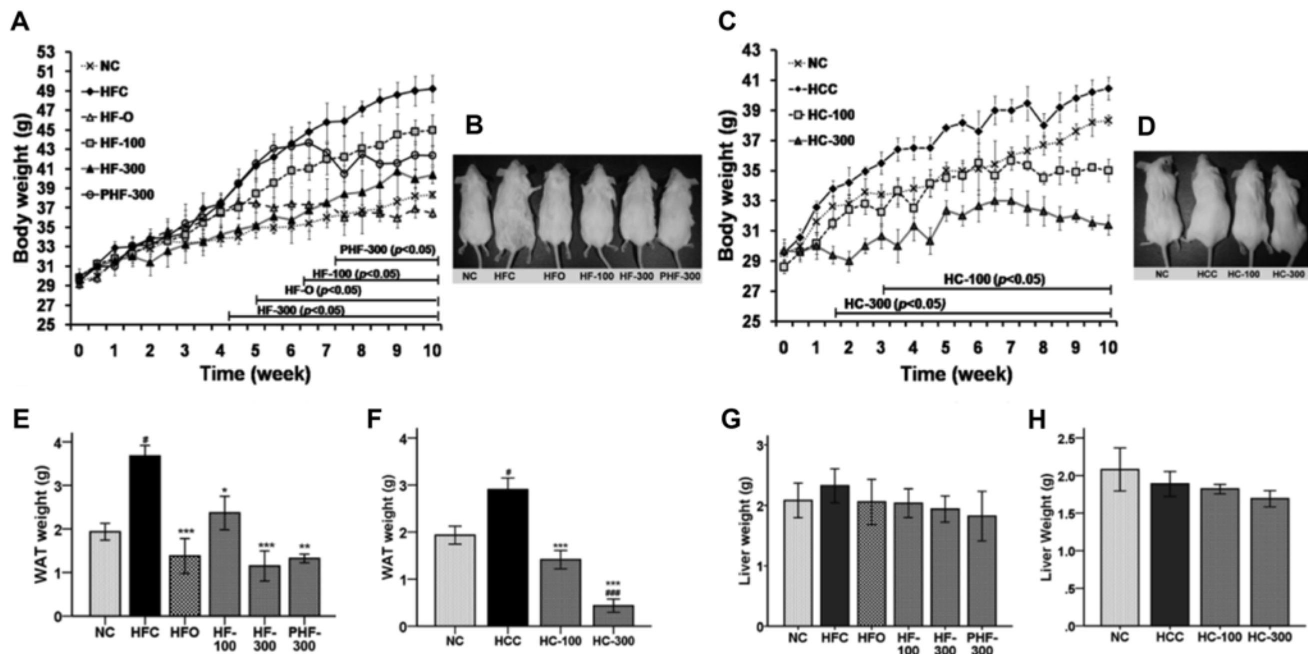

Fig. 3. Effect of GTE on body weight gain, adipose and liver tissue weight. (A) The anti-obesity effect of GTE in mice fed high-fat diet. (B) Representative picture of mice fed high-fat diet. (C) Effect of GTE on body weight gain in mice fed high-carbohydrate diet. (D) Representative picture of mice fed high-carbohydrate diet. (E, F) Effect of GTE on fat-pad weights in mice fed high-fat and high-carbohydrate diet. (G, H) Effect of GTE on liver weights in mice fed high-fat and high-carbohydrate diet. Data are expressed as mean ± SEM (n = 6). $^{*}P < 0.05$, $^{**}P < 0.005$ and $^{***}P < 0.001$ *vs*. control and $^{\#}P < 0.05$ *vs*. NC.

glucose, ALT and AST were significantly ($P < 0.05$) higher in the HFC and HCC groups than those in NC and treatment groups. Significant decreases in HDL cholesterol were observed in the HFC and HCC groups than those in the treatment groups. High-fat diet plus GTE administration caused a significant ($P < 0.05$) decrease in TG, TC, FFA, LDL cholesterol, glucose, ALT and AST levels by 1.4-, 1.7-, 1.8-, 2.2-, 1.3-, 1.7- and 1.9-fold, respectively, in HF-300 group. The high-fat diet induced elevation in the serum TG, TC, FFA, LDL cholesterol, glucose, ALT and AST levels were significantly reversed when the mice were fed with GTE by 1.3-, 1.5-, 1.4-, 2.5-, 1.2-, 1.8- and 1.9-fold, respectively in PHF-300 group. High-carbohydrate diet plus GTE administration also caused a significant ($P < 0.05$) decrease in TG, TC, FFA, LDL cholesterol, glucose, ALT and AST levels by 1.1-, 1.5-, 1.4-, 2-, 1.1-, 1.3- and 1.8-fold, respectively in HC-100 group and by 1.3-, 1.6-, 1.7-, 2.3-, 1.2-, 1.5- and 2.5-fold, respectively in HC-300 group. However, the level of HDL cholesterol was increased in HF-100 group (by 1.1-fold), HF-300 group (by 1.3-fold), PHF-300 group (by 1.3-fold), HC-100 group (by 1.1-fold) and HC-300 group (by 1.2-fold) in compare to their respective control groups.

The fecal level of TG and TC were analyzed from the feces collected for 3 weeks at beginning and end of the experiment (Table 3). A significantly higher level of fecal TG and TC in the treatment groups were observed during the final 3 week of the experiment than those in HFC and HCC groups.

3.5. Effect of GTE on serum insulin, leptin, adiponectin, IL-10, IL-6, and TNF-α level

The effect of GTE on serum insulin, leptin, adiponectin, IL-10, IL-6 and TNF-α levels are shown in Table 3. Serum insulin, leptin, IL-10, IL-6 and TNF-α level in the HFC and HCC groups were significantly increased in compare to those in the NC and GTE treatment groups. However, serum adiponectin levels were significantly increased by the treatment of GTE in compare to HFC and HCC groups.

3.6. Effect of GTE on adipose tissue leptin, IL-6 and adiponectin levels

The levels of leptin, IL-6 and adiponectin in mesenteric adipose tissue are shown in Fig. 4. High-fat and high-carbohydrate diet fed mice exhibited significant ($P < 0.01$) increase in adipose tissue leptin and IL-6 concentrations than ND fed mice, whereas, treatment with GTE significantly decreased the leptin and IL-6 levels in adipose tissue. The concentrations of adiponectin in mesenteric WAT were significantly higher by 1.3-, 2.4- and 1.9-fold in HF-100, HF-300 and PHF-300 groups, respectively against HFC group (Fig. 4E) and by 1.4- and 2.4-fold in HC-100 and HC-300 groups, respectively against HCC group (Fig. 4F).

Effect of GTE on expression levels of biomarkers involved

Table 3. Effect of aqueous extract of green tea seed cake on serum adipokines levels in high-fat and high-carbohydrate diet induced obesity in mice

Parameters	NC	HFC	HCC	HFO	HF-100	HF-300	PHF-300	HC-100	HC-300
Insulin (pg/mL)	1240.19 ± 64.97*#	2586.08 ± 222.76	2586.08 ± 102.10	1699.65 ± 40.3.76*	1862.08 ± 37.12*	998.86 ± 204.20*	938.52 ± 71.07*	785.37 ± 92.82#	655.42 ± 21.48#
Leptin (pg/mL)	183.80 ± 7.45*#	428.20 ± 2.73	323.75 ± 44.19	218.17 ± 74.15*	284.91 ± 49.55*	236.13 ± 15.73*	200.75 ± 39.94*	222.64 ± 6.01#	124.88 ± 4.37#
Adiponectin (pg/mL)	1007.96 ± 103.51*#	856.56 ± 270.00	910.61 ± 174.70	1522.16 ± 130.28*	1340.90 ± 69.88*	1835 ± 99.35*	1784.68 ± 134.74*	1434 ± 174.70#	2048 ± 49.35#
IL-10 (pg/mL)	105.03 ± 42.63*	336.51 ± 35.99	135.58 ± 47.124	125.15 ± 0.73*	115.07 ± 44.82*	59.00 ± 2.19*	47.87 ± 4.89*	58.349 ± 5.66#	66.85 ± 15.05#
IL-6 (pg/mL)	35.19 ± 2.83*#	162.37 ± 10.28	93.47 ± 8.63	40.38 ± 4.29*	78.93 ± 7.48*	34.68 ± 6.48*	59.01 ± 10.38*	44.82 ± 3.98#	26.48 ± 2.94#
TNF-α (pg/mL)	0.56 ± 0.07*#	1.68 ± 0.13	1.05 ± 0.11	0.86 ± 0.03*	1.02 ± 0.12	0.75 ± 0.18*	0.86 ± 0.03*	0.77 ± 0.05#	0.42 ± 0.02#

Data are expressed as mean ± SEM (n = 6). *$P < 0.05$, significantly different from HFC. #$P < 0.05$, significantly different from HCC.

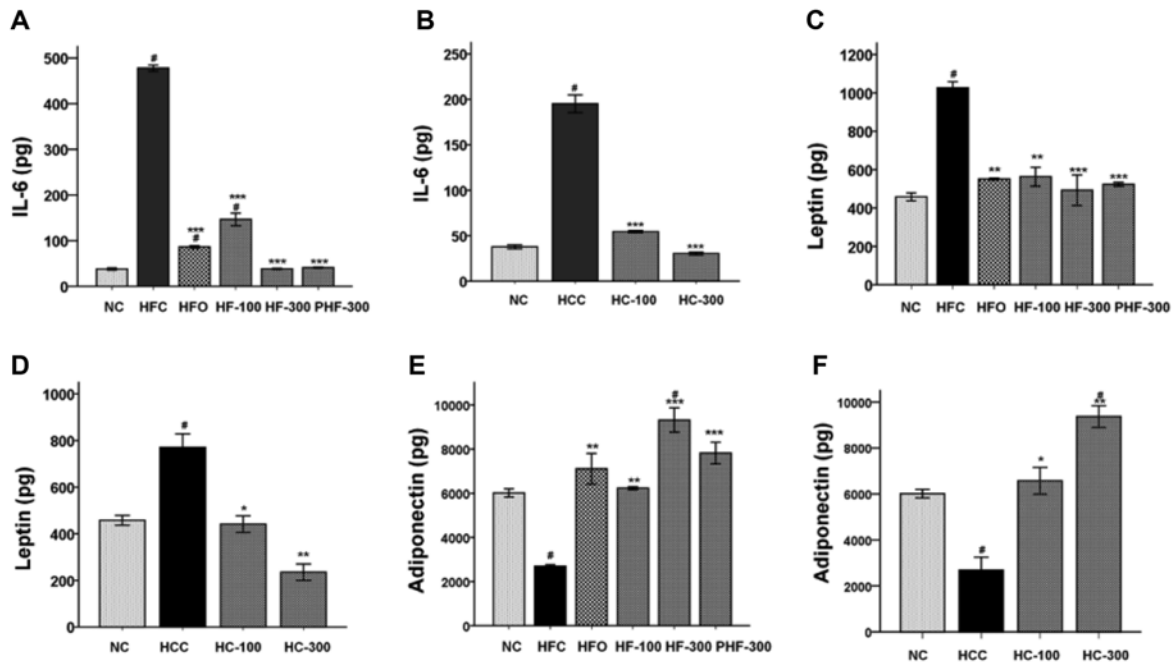

Fig. 4. Effect of GTE on adipose tissue adipokine levels in high-fat and high-carbohydrate diet induced obese mice. The levels of adipokine are presented against 50 μg of total protein in the extraction sample. Data are expressed as mean ± SEM (n = 6). *$P < 0.05$, **$P < 0.005$ and ***$P < 0.001$ *vs*. control and #$P < 0.05$ *vs.* NC.

in lipogenesis and adipogenesis.

We used liver tissue to examine the expression levels of biomarkers, which are key transcriptional factors for lipogenesis and adipogenesis, to investigate if the anti-obesity effect of GTE was due to molecular signaling involved in lipogenesis and adipogenesis. The mRNA expression levels of PPARγ, C/EBPα, and SREBP, which are key transcription factors for the regulation of adipogenesis were down-regulated in the GTE treatment groups fed high-fat diet than that observed in HFC (Fig. 5A). In addition, the target genes lipoprotein lipase (LPL), FAS, adipocyte protein 2 (aP2), and leptin of lipogenesis and adipogenesis transcription factors tended to be decreased by GTE treatment (Fig. 5B). GTE treatment also suppressed the expression levels of these genes in mice fed high-carbohydrate diets (Figs. 5C and 5D). We further assessed the activities of AMPKα in liver tissue by measuring phosphorylated- AMPKα levels. p-AMPKα protein levels in GTE treatment groups were increased in compare to both high-fat and high-carbohydrate fed control groups

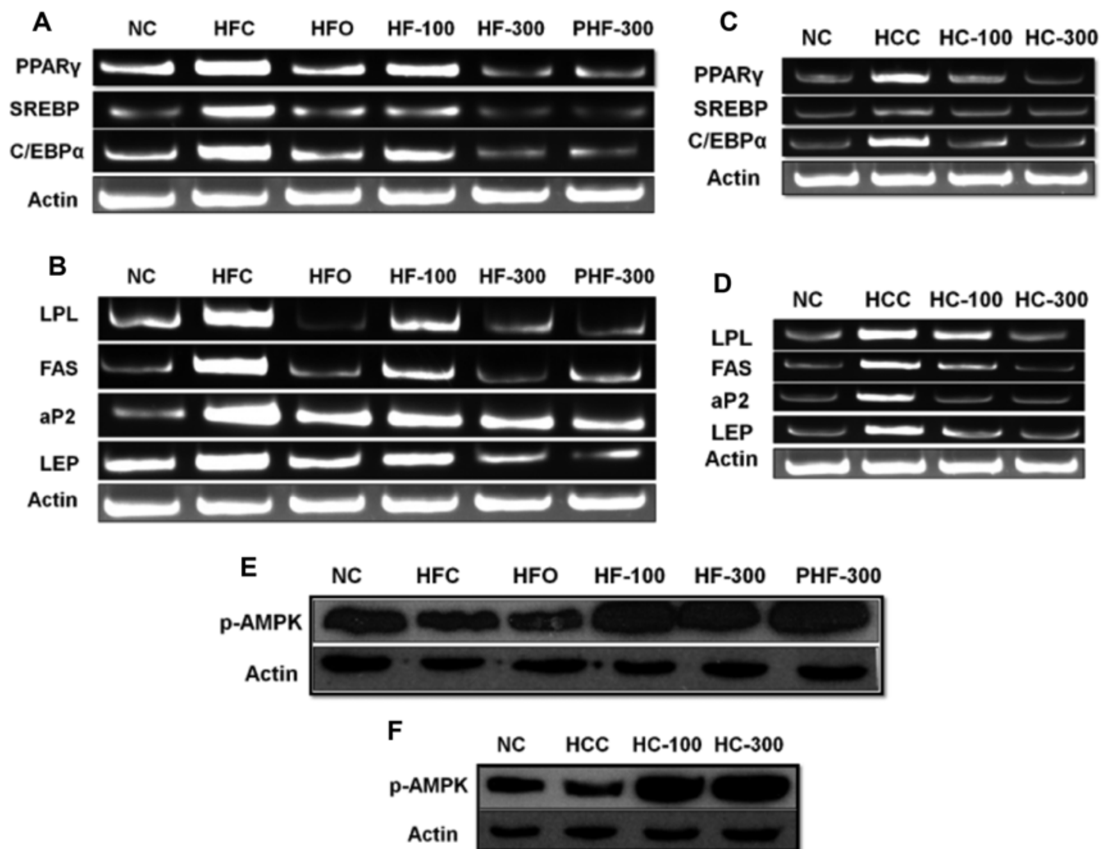

Fig. 5. Effect of GTE on expression levels of adipogenesis and lipogenesis-related biomarkers in the liver. mRNA expression of genes related to adipogenesis and lipogenesis in liver in high-fat (A, B) and high-carbohydrate (C, D) diet fed mice, analyzed by RT-PCR. Representative western blots are shown for actin and phosphorylated AMPK (p-AMPK) in liver in high-fat (E) and high-carbohydrate (F) diet fed mice.

mice (Figs. 5E and 5F). Thus, our results demonstrated the AMPK activating effect of GTE.

4. Discussion

Obesity has become a major public health problem due to its associated risk factors and diets are prevalence to induce obesity whereas natural product shows anti-obesity effect involving diverse mechanisms, such as regulation of appetite, food intake, fatty acid oxidation, lipogenesis and adipogenesis [28-30].

In the present study, anti-obesity effect of GTE was investigated using high-fat and high-carbohydrate diet induced obese mice model. In order to attain the characterization of bioactive compounds content of GTE, thorough LC/MS analysis was carried out using reference standard. GTE consisted of saponins (11.7%) and naringenin (0.3%) as bioactive components of green tea and proteins (~15%), polysaccharides (~65%) and ashes as non-bioactive components, whereas, other bioactive components such as EGCG, ECG, kaempferol, etc were absent in aqueous extract of green tea seed cake (data not shown). Molecular mass of all the reported tea saponins falls between *m/z* 1050 to *m/z* 1300 [23]. Hence, we screened the molecular mass of saponin rich fraction using *m/z* 1050 to *m/z* 1300 (Fig. 1A). The identification and quantification of naringenin was performed by enzymatic hydrolysis of GTE and hybrid LCMS-IT-TOF analysis. The results obtained are summarized in Fig. 1.

The results from Oil red O staining assay demonstrated that GTE prevents preadipocyte differentiation and lipid accumulation in a dose-dependent manner. Excessive growth of adipose tissue mass due to both increased fat cell number and size results in obesity [31]. Adipose tissue plays critical role in energy balance and changes in body mass based on metabolic requirements of the organism. In *in-vivo* experiment, we observed that GTE effectively protected high-fat and high-carbohydrate diet induced body weight gain. Reduction in body weight gain in GTE treatment group mice were accompanied by reduction in body fat mass, since treatment with GTE significantly

suppressed the weight of adipose tissue. However, no significant differences in the daily food intake was observed in GTE treatment groups in compared to respective high-fat and high-carbohydrate diet fed control groups. Thus, reduction in body weights in GTE treatment groups was independent of the food intake. In our study, GTE significantly lowered the serum lipid and glucose levels when compared with diet fed control groups. The treatment of GTE also lowered serum and mesenteric adipose tissue levels of leptin and IL-6 whereas adiponectin levels were significantly increased. Moreover, the levels of HDL were also improved in GTE treatment groups. GTE significantly increased the fecal excretion of TG and total cholesterol. Thus, GTE ameliorates the serum biochemical parameters in diet induced obesity.

We confirmed that GTE has anti-adipogenic effect via inhibition of PPARγ, C/EBPα, and SREBP, which are the key transcription factors in regulation of adipogenesis and induces the expressions of the aP2, LPL, FAS and other genes involved in lipid metabolism [32]. GTE also suppressed the target genes (LPL, aP2, FAS and leptin) of these transcription factors [33,34]. Activation of AMPK attenuates the synthesis of glycerol lipids and augments fatty acid oxidation in metabolic tissues and has been described as a master regulator of lipid metabolism [35] and to observe the effects of GTE on AMPK, protein levels of p-AMPKα in mice's liver were measured using Western blot. We observed that supplementation of GTE increased the protein levels of p-AMPKα in a dose-dependent manner. Activation of AMPK eventually changes the diverse metabolic pathways by suppressing the expression of genes involved in adipogenesis.

Our studies in mice revealed that GTE does not affect food intake, rather it may suppresses anabolic pathways and stimulates catabolic pathways. Traditional medicine usually uses natural products to enhance the efficacy and at the same time reduce the side effects. Our results provide a molecular basis for understanding the effect of GTE on anti-hyperlipidemic and fat pad lowering effect. The molecular mechanisms underlying the anti-obesity effect of GTE appear to involve the expression of adiponectin and activation of AMPK, together with the suppression of key transcription factors of adipogenesis and their target genes. The treatment of GTE seems to play an important role in regulating the adipogenesis and lipid metabolism in mice fed high-fat and high-carbohydrate diet. Our results clearly demonstrated that GTE displays remarkable bioactivity for prevention of adipogenesis and obesity related metabolic disorder by inhibiting adipocyte differentiation, body weight gain and by reducing body fat pad and serum lipid levels. Therefore, GTE could be incorporated into diets to intervene obesity metabolism.

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