

Anti-obesity effects of pectinase and cellulase enzyme-treated *Ecklonia cava* extract in high-fat diet-fed C57BL/6N mice

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Abstract. The present study investigated the anti-obesity effects of enzyme-treated *Ecklonia cava* extract (EEc) in C57BL/6N mice with high-fat diet (HFD)-induced obesity. The EEc was separated and purified with the digestive enzymes pectinase (Rapidase X-Press L) and cellulase (Rohament CL) and its effects on the progression of HFD-induced obesity were examined over 10 weeks. The mice were divided into 6 groups (n=10/group) as follows: Normal diet group, HFD group, mice fed a HFD with 25 mg/kg/day *Garcinia cambogia* extract and mice fed a HFD with 5, 25 or 150 mg/kg/day EEc (EHD groups). Changes in body weight, fat, serum lipid levels and lipogenic enzyme levels were determined. The body weight and liver weight were increased in the HFD group compared with those in the ND group, which was significantly reduced by EEc supplementation. In addition, significant reductions in epididymal, perirenal and mesenteric white adipose tissues were present in the EHD groups and all three EHD groups exhibited decreases in insulin, leptin and glutamate pyruvate transaminase levels compared with those in the HFD group. In addition, EEc treatment significantly decreased the serum and hepatic triglyceride levels compared with those in the HFD group. However, the levels of high-density lipoprotein cholesterol/total cholesterol ration increased significantly in EHD-25 and -150 groups compared with those in the HFD group. Changes in adipogenic and lipogenic protein expression in the liver was assessed by western blot analysis. The EHD-25 and -150 groups exhibited reduced levels of CCAAT/enhancer-binding protein α and peroxisome proliferator activated receptor γ . However, the phosphorylation ratios of AMP-activated protein kinase and acetyl-CoA carboxylase

were significantly increased in the liver tissue obtained from the EHD (5, -25 and -150 mg/kg/day) groups compared with those in the HFD group. EEc supplementation reduced levels of sterol regulatory element-binding protein-1c, adipose fatty acid-binding protein, fatty acid synthase and leptin, while it significantly increased glucose transporter type 4 and adiponectin protein levels in the liver tissues of all three EHD groups compared with those in the HFD group. Taken together, these results suggest that EEc exerts anti-obesity effects by reducing body weight and the serum and hepatic levels of obesity-associated factors. Thus, EEc supplementation reduces HFD-induced obesity in C57BL/6N mice and has the potential to prevent obesity and subsequent metabolic disorders.

Introduction

Obesity is the most common metabolic disease worldwide and represents a serious human health issue (1-3). It is a state of energy imbalance caused by excessive energy storage and insufficient energy expenditure (4,5), and is closely associated with a high-calorie diet, high blood pressure, cardiovascular disease, atherosclerosis, osteoarthritis, nonalcoholic fatty liver disease (NAFLD) and metabolic syndrome (6-8). Besides increased morbidity rates, obesity is also associated with a variety of metabolic syndromes, including type 2 diabetes, insulin resistance, hyperlipidemia, hypertension, stroke, cardiac injury, heart disease and cancer (9,10).

Exercise and dietary control are effective therapeutic strategies for obesity but pharmacotherapy is another important option for direct treatment. A variety of drugs that provide appetite inhibition, heat production, satiety enhancement and inhibition of fat absorption have been developed to treat obesity (11,12). Currently available therapeutic agents include orlistat, phentermine and phendimetrazine. However, these drugs have side effects, including abdominal distension, increased bowel movements, diarrhea, fever, anorexia, nasal congestion (orlistat), insomnia, cardiovascular disease, extreme fatigue (phendimetrazine) and depression (phentermine and phendimetrazine) (13,14). In fact, due to the adverse effects of these types of pharmaceutical approaches, certain anti-obesity medicinal products have been withdrawn from the market following their approval (15). Thus, the development of safe and effective anti-obesity drugs is of utmost importance.

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In addition, dietary supplements and natural herbal components are increasingly being recognized as viable alternative therapies. However, there remains a requirement for functional agents derived from safe and natural sources that are effective for obesity control with minimal side effects relative to those of artificially synthesized drugs.

Several studies have attempted to derive anti-obesity agents from seaweeds that contain a variety of vitamins and dietary fibre with low energy content. The development of functional foods from seaweed has led to additional research demonstrating improvements in hyperlipidemia, suppression of cholesterol accumulation, improvement of bowel activity and release of heavy metals (16-18).

Brown algae have potential therapeutic value due to the abundance of bioactive substances, including sulfated polysaccharides, proteins, dietary fibres, carotenoids, alginates, fucoidans and phlorotannins, contained in them (19-25). Furthermore, polysaccharides, including alginate and fucoidan, reduce serum cholesterol and triglycerides (TG) (26-29). Therefore, components of seaweed are likely to serve as safe anti-obesity agents that lack adverse side effects, and which may be ingested for long periods of time. *Ecklonia cava* is an edible species of brown algae found in the ocean off the coasts of Japan and Korea (30). Phlorotannins, which are polyphenol components of *E. cava*, have been isolated and demonstrated to include fucodiphloretol G, eckol, 8-8'-bieckol, dieckol, eckstolonol, phlorofucofuroeckol A, phloroglucinol and dioxinodehydroeckol (31-33). Studies on *E. cava* have demonstrated its anti-inflammatory (24,34,35), anti-oxidative (22-25,32,36-39), anti-bacterial (40,41), anti-cancer (42-44) and hair growth (45,46) effects as well as its actions against Alzheimer's disease (47,48).

Studies on the anti-obesity effects of *E. cava* have been performed in zebrafish (49,50), mice (51-53) and cell cultures (54-57), with most of these studies focusing on identifying a method to effectively extract phlorotannins (e.g., eckol, dieckol and phlorofucofuroeckol-A). For this purpose, a previous study by our group investigated a variety of methods, including hot-water extraction, ethanol extraction and enzyme extraction, and assessed the yield efficiency and economic efficiency (58). Hot water treatments (60°C and 90°C), ethanol treatments (60 and 80%) and enzymatic treatments (Protex 6L, an endo-type protease; Rapidase press L, a pectinase cellulase/hemicellulase enzyme complex; Rohament CL, a cellulase β glucanase/hemicellulase enzyme complex; and a Rapidase press L + Rohament CL complex) were performed to determine the optimal conditions for processing *E. cava*. Of these treatments, the yields were highest for the 90°C hot water treatment (27.75%), 60% ethanol treatment (11.42%) and Rapidase press L + Rohament CL enzymatic treatment (21.87%). The total polyphenol and phlorotannin contents of the raw materials were 1,708.01 and 1,031.74 mg/g, respectively, for the 90°C hot water treatment; 1,059.54 and 575.57 mg/g, respectively, for the 60% ethanol treatment and 1,120.83 and 847.03 mg/g, respectively, for the Rapidase press L + Rohament CL enzymatic treatment.

These results indicated that the hot water treatment extract produced higher total polyphenol and phlorotannin contents than the enzymatic treatments; however, enzymatic treatment was more efficient in terms of stability and economy. The EEC

used in the present study included three components (eckol, dieckol and phlorofucofuroeckol A) with 17.5 mg/g of dieckol as an indicator substance, and high yields and high concentrations of polyphenols were obtained from *E. cava*. The previous study also investigated the inhibitory effects of EEC treatment on 3T3-L1 adipocyte differentiation and adipogenesis-associated gene expression (58). The expression levels of CCAAT/enhancer-binding protein α (C/EBP α), and the adipogenesis-associated genes sterol regulatory element-binding protein-1c (SREBP-1c), adipose fatty acid-binding protein (A-FABP) and fatty acid synthase (FAS) significantly decreased following treatment, which indicates that EEC may be a potential agent for the prevention of obesity at the cellular level.

In addition, *Garcinia cambogia* extract was used as a positive control, as the inhibitory effect of EEC on 3T3-L1 adipocyte differentiation and adipogenesis-associated gene expression have been demonstrated (57,59). Based on these data, the present study aimed to investigate the anti-obesity effects of EEC in C57BL/6N mice with high-fat diet (HFD)-induced obesity *in vivo*.

Materials and methods

Preparation of enzyme-treated EEC. *E. cava* was purchased in 2012 from Taekyug-nongsan (Jeju-do, Korea). For the present study, *E. cava* chips of ~5 cm in size were prepared by cutting the leaves and removing the stems and roots of the algae. Next, the EEC was prepared by placing 30 kg of *E. cava* chips in 750 l distilled water with enzymes (300 g Rapidase X-Press L and 300 g Rohament CL; BISION Co., Gyeonggi-do, Korea), stirring the suspension at 50°C for 24 h, centrifuging the solution at 3,000 x g and 4°C for 20 min, vacuum-filtering it, and then adding three volumes of 60% ethanol. After 18 h of stirring, the solution was filtered and concentrated using rotary evaporation to 6° Brix. The concentrated solution was made into a powder using a spray dryer; the final extracted material had a weight of 3.65 kg, which represents a yield of 12.2% (EEC; product no. JY202-MM130426R). The *G. cambogia* extract powder (main ingredient, hydroxycitric acid) was purchased from ES Ingredients (Gyeonggi-do, Korea).

Animal care and experimental design. The doses of EEC administered in the animal experiments were determined based on the concentration of EEC that was effective at the cellular level in preliminary experiments. The C57BL/6 mouse strain is the most studied experimental model of diet-induced obesity, as it is sensitive to HFD-induced weight gain (60). HFD intake promotes increases in body weight, adipose tissue weight, hyperlipidemia, and hyperglycemia in rodents (61). Therefore, for the present study, 60 male C57BL/6N TacSam mice (age, 4 weeks; weight, 17.7 \pm 0.73 g) were purchased from Samtako Bio Korea Co. (Gyeonggi-do, Korea), housed in standard cages under a 12-h light-dark cycle, and maintained in a room at 23 \pm 3°C with a relative humidity of 55 \pm 5%. All mice consumed a commercial diet *ad libitum* and had *ad libitum* access to tap water for 1 week prior to the start of the experiments. The mice were randomly divided into six groups (n=10/group) as follows: Mice receiving a normal diet (ND group), mice receiving a HFD (HFD group), mice fed a

HFD with 25 mg/kg *G. cambogia* extract (GHD group), mice fed a HFD with 5 mg/kg/day EEc (EHD5 group), mice fed a HFD with 25 mg/kg/day EEc (EHD25 group) and mice fed a HFD with 150 mg/kg/day EEc (EHD150 group).

The ND group was fed a purified diet with added corn oil that was based on the composition of the AIN-76 semi-purified diet (MP0290545220; MP Biomedicals, LLC, Solon, OH, USA). The HFD was identical to the ND, except that it contained 220 g fat/kg (170 g lard and 50 g corn oil) and 1% cholesterol, which was intended to induce obesity in 10 weeks. Therefore, the HFD was more calorie-dense than the ND (5,380 vs. 3,850 kcal/kg). The diet of the EHD groups was identical to that of the HFD but with the addition of 5, 25 or 150 mg/kg/day of EEc to the diet. The mice were weighed every 7 days and their food intake was recorded daily during the feeding period. At the end of the experimental period, the mice were fasted for 12 h, blood was collected from the abdominal vena cava, and the white adipose tissue and liver were removed, weighed and frozen in liquid nitrogen. The feed efficiency ratio (FER) was calculated as the ratio between weight gain and total feed intake.

The animal protocol of the present study was approved by the Institutional Animal Care and Use Committee of Pukyong National University (Busan, Republic of Korea; approval no. 2012-02).

Biochemical analysis. Blood was collected from the abdominal vena cava and serum was obtained by centrifuging the blood at 2,500 x g for 15 min at 4°C. Serum samples were stored at -70°C and subsequently, serum concentrations of total cholesterol (TC; cat. no. AM202), high-density lipoprotein cholesterol (HDL-C; cat. no. AM203), TG (cat. no. AM157), glutamic oxaloacetic transaminase (GOT; cat. no. AM103), glutamic pyruvic transaminase (GPT; cat. no. AM102), glucose (cat. no. AM201; Asan Pharmaceutical Co., Ltd., Gyeonggi-do, Korea), insulin (cat. no. 80-INSMS-E01; Alpc Diagnostics, Windham, NH, USA) and leptin (cat. no. ADI-900-019A; Enzo Life Sciences, Inc., Farmingdale, NY, USA) were determined enzymatically using commercial kits. The liver tissue samples (0.2 g) were homogenized in 1 ml PBS, centrifuged at 2,500 x g for 15 min at 4°C and stored at -70°C. All serum sample levels were measured using enzyme kits according to the manufacturer's instructions.

Western blot analysis. The liver tissue was washed with PBS and lysed with extraction buffer (1% Nonidet P-40, 1 mM EDTA, 50 mM Tris, pH 7.4, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM sodium orthovanadate, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM NaF and 1 mM phenylmethane sulfonyl fluoride). Subsequently, the extracts were centrifuged at 9,750 x g for 10 min and the supernatants were used for western blot analysis.

The total protein (40 µg) was subjected to 8-15% SDS-PAGE and then transferred to a polyvinylidene fluoride transfer membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 1% bovine serum albumin (BSA; GenDepot Inc., Barker, TX, USA) in a buffer of 5 mM Tris-HCl, 20 mM sodium chloride, pH 7.4, and 0.1% Tween-20 (TBS-T) and incubated with primary antibodies (1:1,000 dilution) in 1% BSA in TBS-T with gentle agitation overnight

at 4°C. Membranes were washed twice for 15 min in TBS-T, incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000 dilution) for 2 h at room temperature and then washed again.

The immunoreactive bands were detected using an enhanced chemiluminescence substrate (Advansta, Menlo Park, CA, USA) and visualized using the GeneSys imaging system (SynGene Synoptics, Ltd., London, UK). The following primary antibodies obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) were used: Anti-C/EBPα (cat. no. sc-9314; anti-goat), anti-peroxisome proliferator activated receptor γ (PPARγ; cat. no. sc-1984; anti-goat), anti-phosphorylated AMP-activated protein kinase (p-AMPK; cat. no. sc-33524; anti-rabbit), anti-AMPK (cat. no. sc-74461; anti-mouse), anti-p-acetyl-CoA carboxylase (p-ACC; cat. no. sc-271965; anti-mouse), anti-ACC (cat. no. sc-30212; anti-rabbit), anti-SREBP-1c (cat. no. sc-366; anti-rabbit), anti-A-FABP (cat. no. sc-18661; anti-goat), anti-fatty acid synthase (FAS; cat. no. sc-55580; anti-mouse), anti-glucose transporter type 4 (GLUT4; cat. no. sc-1606; anti-rabbit), anti-adiponectin (cat. no. sc-26497; anti-goat), anti-leptin (cat. no. sc-842; anti-rabbit) and anti-GAPDH (cat. no. sc-25778; anti-rabbit). The secondary antibodies used were HRP-conjugated anti-mouse immunoglobulin (Ig) G (cat. no. sc-2031; Santa Cruz Biotechnology, Inc.), anti-rabbit IgG (cat. no. A-0545; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and anti-goat IgG (cat. no. A50-101P; Bethyl Laboratories Inc., Montgomery, TX, USA).

Statistical analysis. Results were expressed as the mean ± standard deviation for each group (n=10 animals). Significant differences among multiple mean values were assessed using one-way analysis of variance followed by Bonferroni's multiple comparison test using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

EEc supplementation decreases body weight, liver weight and adipose tissue weight in C57BL/6N mice with HFD-induced obesity. Preliminary experiments were conducted to set the concentrations for the present study. Various concentrations of EEc (5, 12.5, 25, 50, 150, 200, 250, 500 and 1,000 mg/kg) were determined by preliminary experiments. Based on the results, the concentration of the EEc group was set with similar results to the GHD group. Therefore, in the present study, the concentration of EEc was set to 5, 25 and 150 mg/kg. After 10 weeks of feeding, the HFD group had a significantly higher final body weight and more cumulative body weight gain than the ND group. The EEc-supplemented groups had significantly lower final body weights than those observed in the HFD group (HFD, 1.13-; GHD, 1.02-; EHD5, 1.13-; EHD25, 1.05-; and EHD150, 0.82-fold difference compared with the ND group), and less body weight gain (HFD, 1.44-; GHD, 1.14-; EHD5, 1.35-; EHD25, 1.17-; EHD150, 0.46-fold difference compared with the ND group) (Fig. 1A and B). However, despite the increased body weight of the HFD group, daily food intake (3.2-3.4 g/day) did not differ among the experimental groups (data not shown).

As the HFD was more calorie-dense than the ND (5,380 vs. 3,850 cal/kg), the FER over 10 weeks was 44% greater in the HFD group than that in the ND group (ND, 4.66±0.48%; HFD, 6.72±0.48%; GHD 5.29±0.48%; EHD5, 6.30±0.48%; EHD25, 5.46±0.48%; EHD150, 2.28±0.35%). The absolute and relative weights of the livers were significantly greater in the HFD group than those in the ND group, while treatment with EEC resulted in a significant reduction in the absolute liver weight compared with that of the untreated HFD mice (ND, 1.28±0.18 g; HFD, 2.14±0.31 g; GHD 1.93±0.29 g; EHD5, 1.79±0.28 g; EHD25, 1.52±0.15 g; EHD150, 0.97±0.14 g) (Fig. 1C).

To examine whether the reduced body weight gain in the EEC-treated groups was associated with decreased fat accumulation, the epididymal, perirenal and mesenteric white adipose tissues were weighed; they were significantly reduced in the EHD group (Fig. 1D-F). The mean epididymal adipose tissue weights were as follows: ND, 0.21±0.03 g; HFD, 0.29±0.02 g; GHD, 0.20±0.03 g; EHD5, 0.26±0.04 g; EHD25, 0.25±0.03 g; and EHD150, 0.20±0.02 g (Fig. 1D). The mean perirenal adipose tissues weights were as follows: ND, 0.47±0.05 g; HFD, 0.63±0.08 g; GHD, 0.44±0.09 g; EHD5, 0.48±0.08 g; EHD25, 0.48±0.05 g; and EHD150, 0.33±0.05 g (Fig. 1E). The mean mesenteric adipose tissues were as follows: ND, 0.46±0.16 g; HFD, 1.03±0.05 g; GHD, 0.43±0.17 g; EHD5, 0.35±0.20 g; EHD25, 0.34±0.03 g; and EHD150, 0.21±0.08 g (Fig. 1F).

In addition, the livers of the HFD group were lighter in colour than those of the ND and EHD groups (Fig. 1G); they were enlarged and had a pinkish colour, which is indicative of liver steatosis. However, supplementation with EEC reversed these effects as evidenced by the livers of the EHD groups, which were small and dark red and similar to those of the ND group. Notably, the liver of the EHD 150 group was observed to be brownish. The original colour of the EEC powder used in the present study was dark brown. The discolouration of the liver may have been due to the powder.

EEC supplementation decreases serum TC and HDL-C levels in C57BL/6N mice with HFD-induced obesity. To determine whether the effects of EEC supplementation were associated with changes in the serum levels of obesity-associated markers, serum TC and HDL-C were measured in each group. The serum TC levels in the GHD group (59.9±16.6) were reduced compared with those in the HFD group (vs. 83.6±6.10 mg/dl), but the TC levels of the EHD groups did not significantly differ from those in the ND group (48.1±8.36 mg/dl) (Fig. 2A). The EHD-25 and -150 groups had significantly increased serum HDL-C levels compared with those in the HFD group (ND, 30.8±4.41; HFD, 38.4±3.87; GHD, 53.3±9.72; EHD5, 40.9±9.39; EHD25, 57.2±11.06; and EHD150, 61.2±7.39 mg/dl; Fig. 2B). In addition, the HDL-C/TC ratio in the EHD groups increased in a dose-dependent manner (ND, 64.0%; HFD, 46.0%; GHD, 88.9%; EHD5, 49.4%; EHD25, 74.8%; and EHD 150, 81.9%). The HDL-C/TC ratio in the EHD 150 group was similar to that in the GHD group (Fig. 2C).

EEC supplementation decreases serum insulin and leptin levels in C57BL/6N mice with HFD-induced obesity. A HFD induces NAFLD in animal models as well as humans, which is important as NAFLD is closely associated with obesity, diabetes and insulin resistance (62,63). To determine

whether the effects of EEC supplementation were correlated with changes in serum levels of NAFLD-associated markers, serum glucose, insulin and leptin levels were determined in each group. As demonstrated in Fig. 3, all treatment groups had decreased blood glucose levels compared with those in the HFD group (Fig. 3A), but these were not dose-dependent. The mean blood glucose levels in the HFD group were 176.1±13.9 mg/dl and those of the other groups ranged from 136.3±4.2 to 147.5±16.6 mg/dl.

The serum insulin levels in the EHD groups (0.64±0.16, 0.31±0.03 and 0.20±0.10 ng/ml for EHD5, -25 and -150, respectively) were significantly lower than those in the HFD group (0.92±0.15 ng/ml). The insulin levels in the ND group were 0.20±0.10 ng/ml and those in the GHD group were 0.37±0.18 ng/ml. The leptin levels in the EHD groups (5.4±0.27, 4.6±0.41 and 1.4±1.17 ng/ml for EHD5, -25 and -150, respectively) were significantly lower than those in the HFD group (5.6±0.02 ng/ml) (Fig. 3C), and the leptin levels in the GHD group were 4.8±0.23 ng/ml. The serum glucose, insulin and leptin levels in the EHD25 group were similar to those in the GHD group.

EEC supplementation decreases serum GOT and GPT levels in C57BL/6N mice with HFD-induced obesity. The consumption of a HFD may induce hepatic steatosis (64). Thus, to determine whether EEC supplementation had any effect on the serum levels of hepatic steatosis-associated markers, the concentrations of GOT and GPT were measured. All treatment groups exhibited decreased GOT and GPT levels compared with those in the HFD group (Fig. 4A and B), but these differences were not dose-dependent. The GOT and GPT levels in the HFD group were 58.3±13.6 IU/l and 12.6±1.56 IU/l, respectively. In the other groups, the GOT levels ranged between 22.3±8.9 and 41.4±10.9 IU/l and the GPT levels ranged between 7.6±0.73 and 9.8±0.16 IU/l. These results supported the protective effect of EEC against the development of hepatic steatosis.

EEC supplementation decreases serum and hepatic TG in C57BL/6N mice with HFD-induced obesity. To determine whether EEC supplementation affected the serum and hepatic levels of obesity-associated markers, TG levels were determined in each group. All treatment groups exhibited decreased serum TG levels compared with those in the HFD group and the effects of EHD were dose-dependent (Fig. 5A). The serum TG concentrations in the ND, HFD, GHD, EHD5, EHD25 and EHD150 groups were 46.4±11.67, 57.9±12.16, 40.1±5.04, 56.7±11.40, 43.4±9.06 and 38.0±5.05 mg/dl, respectively. The TG levels in the EHD5, EHD25 and EHD150 groups were 1, 24 and 34% lower than those in the HFD group. The EHD groups also exhibited significantly lower hepatic TG levels than the HFD group (Fig. 5B). The hepatic TG levels in the HFD, ND and GHD groups were 61.9±12.4, 29.6±3.73, and 46.1±3.11 mg/g. Supplementation with EEC resulted in significant dose-dependent decreases in hepatic TG levels such that the levels of the EHD5, EHD25 and EHD150 groups were 46.5±4.69, 36.5±4.46 and 20.8±6.79 mg/g, respectively; these levels were 25, 41 and 66% lower, respectively, than those in the HFD group.

EEC supplementation modulates the expression of genes involved in lipid metabolism and activates AMPK in mice.

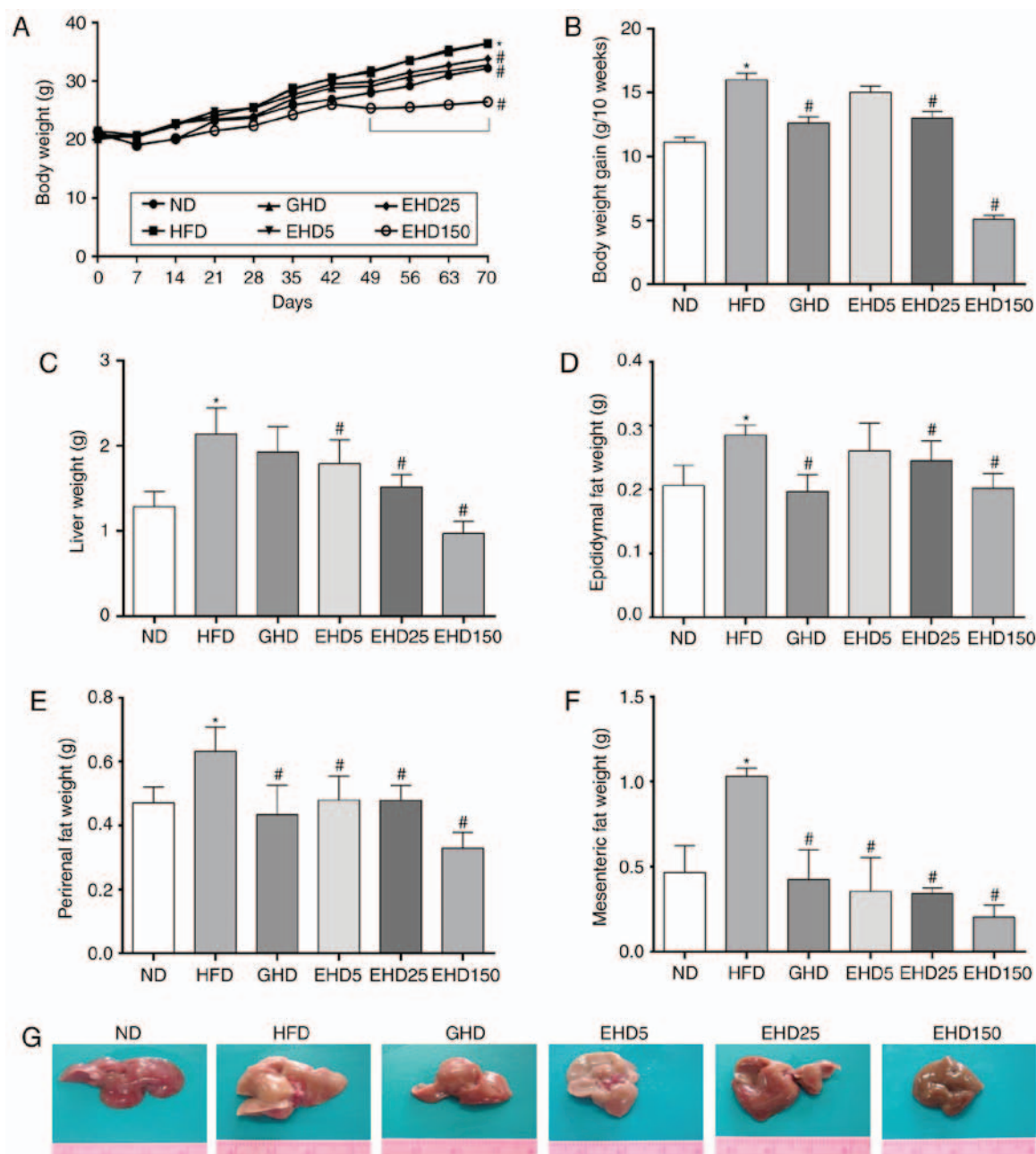


Figure 1. Effects of enzyme-treated *E. cava* extracts on the body, liver and adipose tissue weight of experimental mice. Changes in (A) body weight, (B) body weight gain, (C) liver weight, (D) epididymal fat weight, (E) perirenal fat weight and (F) mesenteric fat weight. (G) Representative images of livers of the ND-, HFD-, GHD- and EHD-fed mice (magnification, x100). Values are expressed as the mean \pm standard deviation (n=10). Data were analysed using one-way analysis of variance. * $P < 0.05$ vs. ND between 49-70 days; # $P < 0.05$ vs. HFD group between 49-70 days. Groups: ND, normal diet group; HFD, high fat diet group; GHD, mice fed a HFD and 25 mg/kg/day *Garcinia cambogia* extract; EHD 5, mice fed a HFD and 5 mg/kg/day EEC; EHD 25, mice fed a HFD and 25 mg/kg/day EEC; EHD150, mice fed a HFD and 150 mg/kg/day EEC; EEC, enzyme-treated *Ecklonia cava* extract.

Adipogenesis and lipogenesis are accompanied by changes in the sequential activation of several pro-adipogenic transcription factors, including C/EBP α / β / δ and PPAR γ . Thus, the present study examined whether the observed reductions in hepatic lipid accumulation were due to the downregulation of these transcription factors. The expression of C/EBP α and PPAR γ in the liver tissues obtained from the EEC-supplemented groups (EHD25 and -150) exhibited a decrease compared with those in the HFD group (Fig. 6A and B). Among them, all treatment groups had decreased C/EBP α expression levels compared with those in the HFD group, apart from those in the EHD5 group (Fig. 6A and B); however, these decreases were not

dose-dependent. The C/EBP α expression levels in the HFD, GHD, EHD25 and EHD150 groups were 2.25-, 1.3-, 1.4- and 1.7-fold higher, respectively, than those in the ND group.

Activated AMPK is able to phosphorylate and regulate hydroxymethylglutaryl-CoA reductase and ACC *in vivo*; these enzymes are key regulatory factors involved in sterol synthesis and fatty acid synthesis, respectively. The p-AMPK/AMPK and p-ACC/ACC ratios were significantly increased in liver tissue obtained from the EHD5, -25, and -150 groups compared with those in the HFD group (Fig. 6C and D). The hepatic p-AMPK/AMPK ratio in the HFD, GHD, EHD5, EHD25 and EHD150 groups was increased by 1.25-, 0.51-, 2.77-, 3.01- and

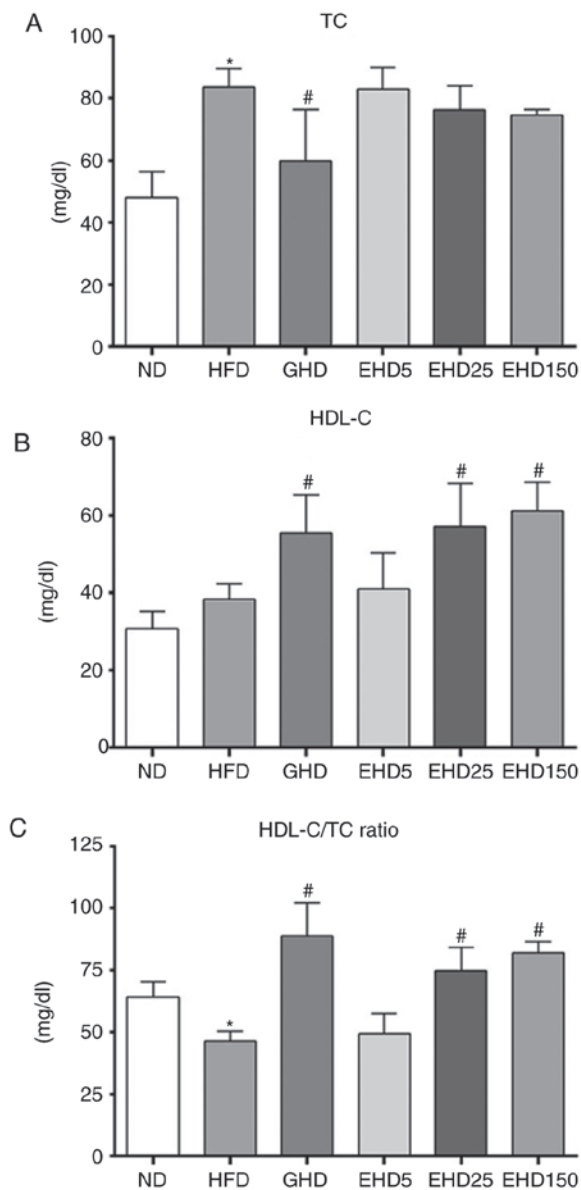


Figure 2. Effects of EEc treatment on serum TC and HDL-C levels of mice fed a HFD for 10 weeks. (A) Serum TC levels, (B) serum HDL-C levels and (C) serum HDL-C/TC ratio. Values are expressed as the mean \pm standard deviation (n=10). Data were analysed using one-way analysis of variance. *P<0.05 vs. ND; #P<0.05 vs. HFD group. Groups: ND, normal diet group; HFD, high fat diet group; GHD, mice fed a HFD and 25 mg/kg/day *Garcinia cambogia* extract; EHD 5, mice fed a HFD and 5 mg/kg/day EEc; EHD 25, mice fed a HFD and 25 mg/kg/day EEc; EHD150, mice fed a HFD and 150 mg/kg/day EEc. EEc, enzyme-treated *Ecklonia cava* extract; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol.

2.61-fold, respectively, compared with that in the ND group. The p-ACC/ACC ratio in the HFD, GHD, EHD5, EHD25 and EHD150 groups was increased by 3.09-, 3.04-, 4.11-, 6.17- and 7.76-fold of that in the ND group. Thus, EEc supplementation resulted in a restoration of AMPK activity as evidenced by the inhibition of HFD-induced increases in the phosphorylation of AMPK and its target ACC.

EEc supplementation reduces the expression of proteins involved in adipogenesis in mice. To examine the effects of EEc on lipid metabolism, the levels of adipogenesis-associated proteins in the liver tissues of the mice were measured. EEc

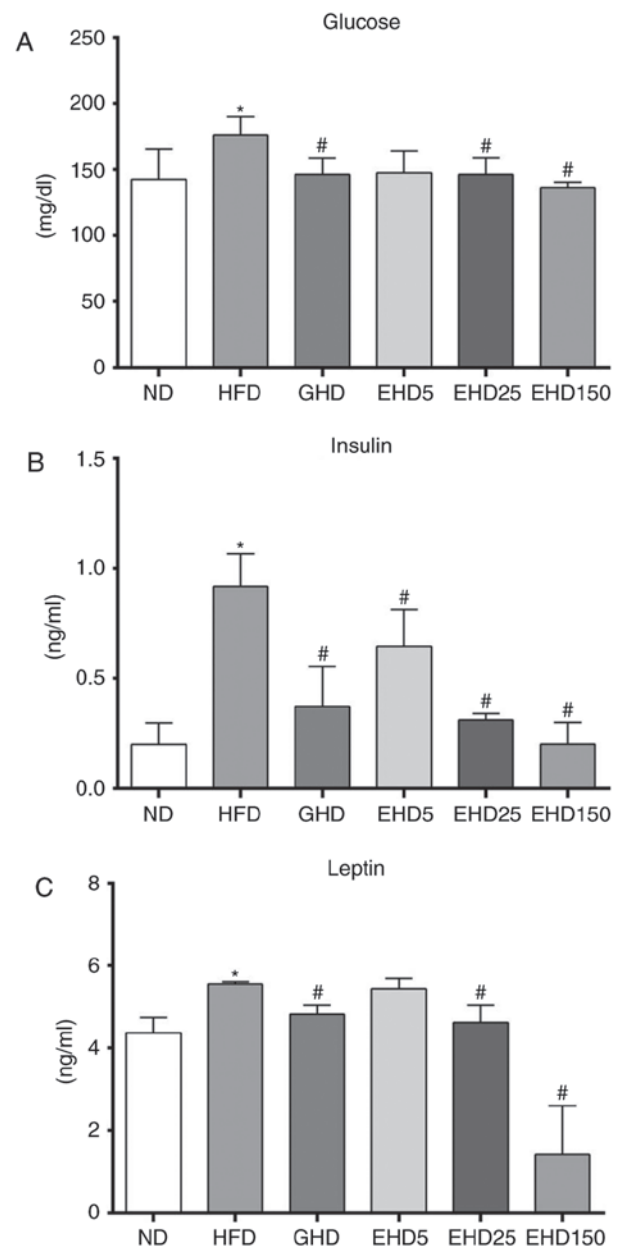


Figure 3. Effects of EEc treatment on the serum concentrations of glucose, insulin and leptin of mice fed a HFD for 10 weeks. (A) serum glucose, (B) serum insulin and (C) serum leptin levels. Values are expressed as the mean \pm standard deviation (n=10). Data were analysed using one-way analysis of variance. *P<0.05 vs. ND; #P<0.05 vs. HFD group. Groups: ND, normal diet group; HFD, high fat diet group; GHD, mice fed a HFD and 25 mg/kg/day *Garcinia cambogia* extract; EHD 5, mice fed a HFD and 5 mg/kg/day EEc; EHD 25, mice fed a HFD and 25 mg/kg/day EEc; EHD150, mice fed a HFD and 150 mg/kg/day EEc; EEc, enzyme-treated *Ecklonia cava* extract.

supplementation inhibited the expression of several hepatic adipogenesis-associated transcription factors. The HFD group exhibited a significantly increased expression of the target genes of PPAR γ involved in the adipogenesis pathway, including A-FABP, FAS and leptin (Fig. 7). By contrast, EEc supplementation significantly decreased the protein levels of SREBP-1c, A-FABP, FAS, and leptin compared with those in the HFD group. The GLUT4 protein levels increased significantly in liver tissue obtained from the EHD5, -25 and -150 groups compared with those in the HFD group (Fig. 7). The GLUT4 protein levels in the HFD, GHD, EHD5, EHD25 and

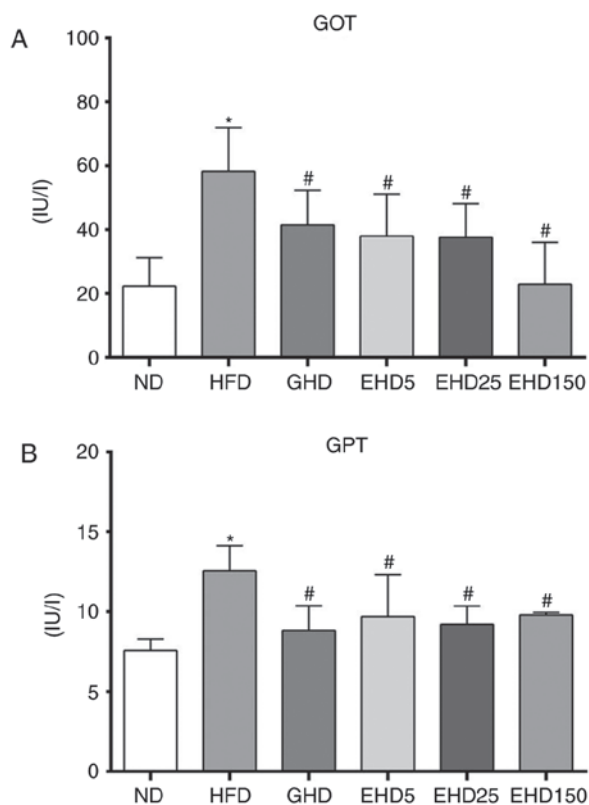


Figure 4. Effects of EEc treatment on the serum concentration of GOT and GPT of mice fed a HFD for 10 weeks. (A) Serum GOT and (B) serum GPT levels. Values are expressed as the mean \pm standard deviation (n=10). Data were analysed using one-way analysis of variance. *P<0.05 vs. ND; #P<0.05 vs. HFD group. Groups: ND, normal diet group; HFD, high fat diet group; GHD, mice fed a HFD and 25 mg/kg/day *Garcinia cambogia* extract; EHD 5, mice fed a HFD and 5 mg/kg/day EEc; EHD 25, mice fed a HFD and 25 mg/kg/day EEc; EHD150, mice fed a HFD and 150 mg/kg/day EEc; EEc, enzyme-treated *Ecklonia cava* extract; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase.

EHD150 groups increased by 1.22-, 1.33-, 2.49-, 2.99- and 3.42-fold, respectively, compared with those in the ND group. The adiponectin protein levels increased significantly in liver tissue obtained from the EHD-25 and -150 groups compared with those in the HFD group.

Discussion

Inhibition of obesity by *E. cava* extract has been previously studied (51-53). The phlorofucofuroeckol A (51), polyphenol-rich fraction (52,53) of *E. cava* extract has been examined for its anti-obesity effect in a murine model fed on a high-fat diet. The present study investigated the *in vivo* anti-obesity effects of EEc in C57BL/6N mice with HFD-induced obesity by treating them with doses of 5, 25 and 150 mg/kg/day for 10 weeks. After 10 weeks, HFD-fed mice that received EEc supplementation exhibited significant decreases in body, liver, as well as epididymal, perirenal and mesenteric fat weight compared with the values in HFD-fed mice that did not receive EEc supplementation. The reduction in body weight was in parallel with decreased adipose tissue weight. In addition, EEc significantly decreased the weight of the epididymal, perirenal and mesenteric adipose tissue, reduced overall body fat stores and lowered the FER compared with those in the HFD group.

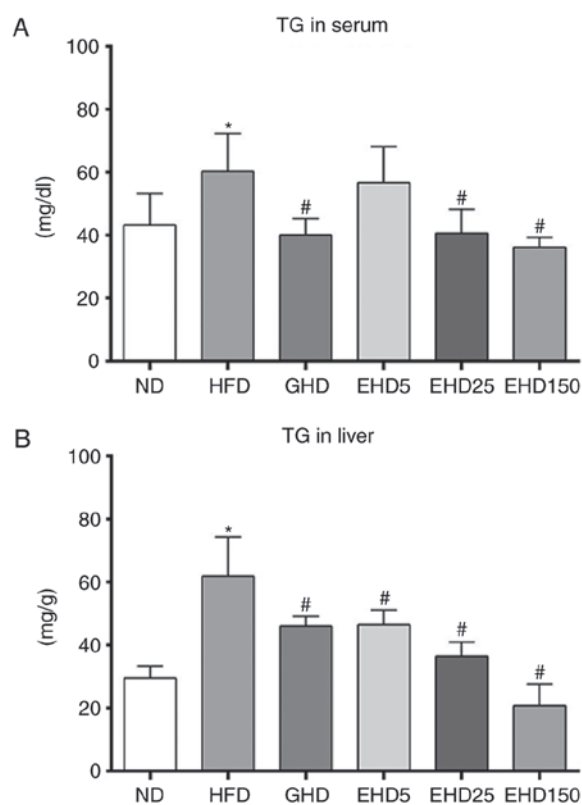


Figure 5. Effects of EEc treatment on serum and hepatic TG concentrations in mice fed a HFD for 10 weeks. (A) Serum TG and (B) liver TG levels. Values are expressed as the mean \pm standard deviation (n=10). Data were analysed using one-way analysis of variance. *P<0.05 vs. ND; #P<0.05 vs. HFD group. Groups: ND, normal diet group; HFD, high fat diet group; GHD, mice fed a HFD and 25 mg/kg/day *Garcinia cambogia* extract; EHD 5, mice fed a HFD and 5 mg/kg/day EEc; EHD 25, mice fed a HFD and 25 mg/kg/day EEc; EHD150, mice fed a HFD and 150 mg/kg/day EEc; EEc, enzyme-treated *Ecklonia cava* extract; TG, triglyceride.

The present study also investigated changes in the serum levels of obesity-associated factors, including TC, HDL-C, glucose, insulin, leptin, GOT, GPT and TG. EEc supplementation had no effect on TC levels but increased the levels of HDL-C. HDL-C and low-density lipoprotein cholesterol (LDL-C) carry cholesterol in the body. In particular, LDL-C causes the accumulation of cholesterol in tissues throughout the body, which results in an increased risk of cardiovascular disease (65). By contrast, epidemiologic studies have reported an inverse correlation between HDL-C content and the risk of cardiovascular disease (66). HDL-C removes cholesterol from the bloodstream and relocates it to liver tissues, where it is eliminated. High levels of HDL-C appear to protect against cardiovascular disease, whereas low levels of HDL-C are an important risk factor for cardiovascular disease. In addition, epidemiological and clinical studies have reported that low HDL-C levels are linked to coronary events (65,66). In the present study, treatment with EEc was expected to reduce the risk of obesity-induced hyperlipidemia and cardiovascular disease by increasing HDL-C levels, regardless of its effects on TC.

EEc supplementation also significantly decreased insulin and leptin levels. The serum insulin levels in the HFD group were significantly higher than those in the EHD groups, which decreased by 29.8, 66.1 and 78.2% in the EHD5, -25, and -150 groups, respectively, compared with those in the HFD group.

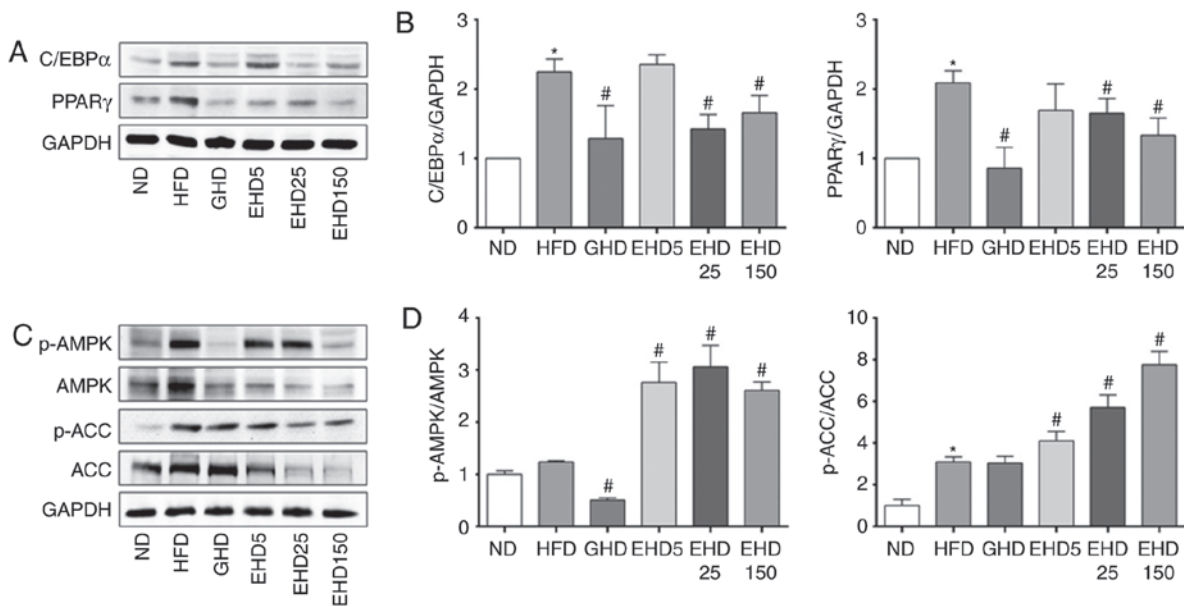


Figure 6. Effects of dietary EEC supplementation on the hepatic expression of AMPK and the regulation of lipid metabolism toward lipid catabolism in HFD-fed mice. (A) The protein expression levels of C/EBP α and PPAR γ in the livers of the experimental mice at 10 weeks were measured by western blotting. (B) Protein bands were quantified with normalization to the internal control GAPDH and the relative expression levels were determined with the ND group set as 1. (C) Protein expression levels of p-AMPK, AMPK, p-ACC and ACC in the livers of the experimental mice at 10 weeks were examined using western blot analysis. (D) Protein bands were quantified with normalization to the internal control GAPDH and the relative expression levels were determined with the ND group set as 1. The ratios of p-AMPK/AMPK and p-ACC/ACC are presented. Values are expressed as the mean \pm standard deviation. Data were analysed using one-way analysis of variance. * $P < 0.05$ vs. ND; # $P < 0.05$ vs. HFD group. Groups: ND, normal diet group; HFD, high fat diet group; GHD, mice fed a HFD and 25 mg/kg/day *Garcinia cambogia* extract; EHD 5, mice fed a HFD and 5 mg/kg/day EEC; EHD 25, mice fed a HFD and 25 mg/kg/day EEC; EHD150, mice fed a HFD and 150 mg/kg/day EEC; EEC, enzyme-treated *Ecklonia cava* extract; p-AMPK, phosphorylated AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; C/EBP α , CCAAT/enhancer-binding protein α ; PPAR γ , peroxisome proliferator activated receptor γ .

The serum leptin levels in the HFD group (5.6 ± 0.02 ng/ml) were higher than those in the ND group (4.4 ± 0.38 ng/ml) and all three EHD groups (5.4 ± 0.27 , 4.6 ± 0.41 and 1.4 ± 1.17 ng/ml for the EHD5, -25 and -150 groups, respectively). Increased insulin levels in the blood promote lipid synthesis by increasing free fatty acid transfer from fat cells to the liver while inhibiting lipid oxidation (67). Leptin, which is a hormone produced primarily by white adipose tissue, is associated with obesity and metabolic syndrome. Leptin affects body weight, food intake and energy balance by suppressing appetite and promoting satiety (68). Leptin levels are closely associated with body fat mass and thus exhibit an increase in HFD-fed experimental animals and in obese patients (69). In the present study, serum insulin and leptin levels were lower in mice treated with EEC and were proportional to body fat mass. The production levels of insulin and leptin are stimulated by increased levels of these hormones (70). Therefore, reductions in serum insulin and leptin levels in the EEC-supplemented groups were likely due to the inhibition of body fat accumulation by EEC.

GOT and GPT are enzymes used as indicators of liver tissue damage. In the present study, GOT and GPT activities decreased in the EHD groups compared with those in the HFD group. In the pathologies of HFD-induced fat liver, alcohol-induced fatty liver or liver injury, GOT and GPT are released into the bloodstream, which increases their activity (71,72). In the present study, the activities of GOT and GPT were attenuated by the addition of EEC, but only the activity of GOT enzyme significantly differed from that the HFD group. EEC supplementation also significantly decreased serum and hepatic TG levels. Hyperlipidemia is a metabolic

disorder that may be caused by HFDs and is characterized by elevated serum TG levels (73,74). In addition, abdominal obesity is associated with elevated blood levels of TG (75). In the present study, serum TG levels were elevated in the HFD group, which had the highest body fat weight.

In the present study, the hepatic expression levels of the lipid metabolism-associated genes AMPK and ACC were investigated with western blot analysis. In a previous study, EEC treatment in 3T3-L1 adipocytes reduced C/EBP $\alpha/\beta/\delta$ and PPAR γ levels (58). In the present study, the EHD groups had lower expression levels of C/EBP α and PPAR γ compared with those in the HFD group. AMPK is a key enzyme involved in intracellular energy balance, glucose uptake and lipid metabolism via its effects on transcriptional factors, lipogenesis and fatty acid oxidation-associated proteins (76). The phosphorylation of AMPK inhibits PPARs and C/EBP, which are the main transcription factors involved in adipocyte differentiation (77). In addition, activated AMPK inhibits downstream ACC activity and prevents the production of malonyl-CoA from acetyl-CoA (78). In the present study, the EHD groups exhibited a decreased body weight and increased protein expression of p-AMPK protein and p-ACC compared with that in the HFD group. Similarly, *E. cava* polyphenol extract induced anti-obesity effects through the activation of AMPK in HFD-fed mice (79).

Next, the hepatic expression levels of adipogenesis-associated proteins, including C/EBP $\alpha/\beta/\delta$, PPAR γ , SREBP-1c, A-FABP, FAS, GLUT4, adiponectin and leptin were assessed by western blot analysis. In a previous study, EEC treatment of 3T3-L1 adipocytes reduced C/EBP $\alpha/\beta/\delta$ and PPAR γ

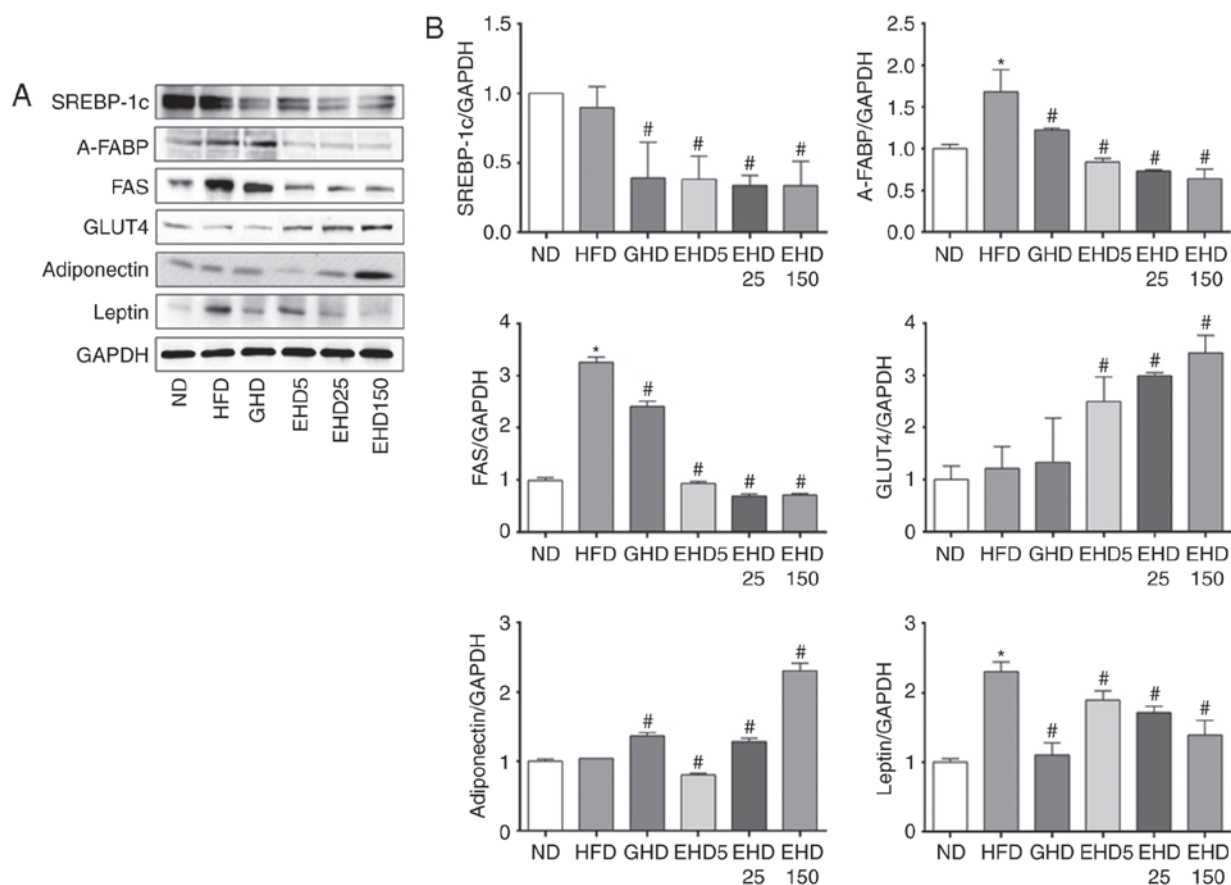


Figure 7. Effects of dietary EEC supplementation on the hepatic expression of adipogenesis-associated proteins in HFD-fed mice. (A) Hepatic protein levels of SREBP-1c, A-FABP, FAS, GLUT4, adiponectin and leptin in the experimental mice after 10 weeks were examined using western blot analysis. (B) Bands were normalized to an internal control (GAPDH) and the relative expression levels were determined with the ND group set as 1. Values are expressed as the mean \pm standard deviation. Data were analysed using one-way analysis of variance. * $P < 0.05$ vs. ND; # $P < 0.05$ vs. HFD group. Groups: ND, normal diet group; HFD, high fat diet group; GHD, mice fed a HFD and 25 mg/kg/day *Garcinia cambogia* extract; EHD 5, mice fed a HFD and 5 mg/kg/day EEC; EHD 25, mice fed a HFD and 25 mg/kg/day EEC; EHD150, mice fed a HFD and 150 mg/kg/day EEC; EEC, enzyme-treated *Ecklonia cava* extract; SREBP-1c, sterol regulatory element-binding protein-1c; A-FABP, adipose fatty acid-binding protein; FAS, fatty acid synthase; GLUT4, glucose transporter type 4.

levels (58). In the present study, the C/EBP α and PPAR γ levels in the EHD groups were lower than those in the HFD group. Previously, EEC treatment in 3T3-L1 adipocytes was reported to lower the levels of adipogenesis-associated proteins, including SREBP-1c, A-FABP, FAS and adiponectin (58). In the present study, EEC treatment resulted in significantly reduced SREBP-1c, A-FABP, FAS and leptin levels, but in increased GLUT4 and adiponectin levels. Activation of C/EBP α promotes the differentiation of pre-adipocytes in cooperation with PPAR γ , which, in turn, causes the trans-activation of adipogenesis-specific genes including A-FABP and FAS (80,81). In addition, although the liver has generally been regarded as being void of significant expression of GLUT-4, a previous study suggested the expression of GLUT-4 mRNA in porcine liver (82). Various isoforms of GLUT (GLUT-1-6 and -8-12) were identified to be expressed in the human liver tissue. Particularly GLUT2 and GLUT4 were identified as the main GLUTs responsible for glucose transport into hepatocytes. Among them, GLUT4 is the major insulin-dependent glucose transporter and is known to be involved in the rate-limiting role of glucose utilization in insulin-sensitive tissue, including brown and white adipose tissues, as well as skeletal and cardiac muscles (83). However, GLUT-4 mRNA was decreased in the liver tissue of model mice with diet-induced obesity.

The present study investigated the expression of GLUT-4 in HFD-induced liver tissue. The results demonstrated that the expression of GLUT-4 was increased in the EHD groups compared with that in the HFD group.

In most of the experiments of the present study, the EHD25 group (treated with EEC at 25 mg/kg/day) exhibited results similar to those of the GHD group (25 mg/kg/day of *G. cambogia* extract), including reduction in body weight, activation of AMPK and ACC, reduction in the levels of obesity-associated factors in serum and the liver, and alterations in the expression of lipid metabolism- and adipogenesis-associated proteins.

The anti-obesity effect observed in present study is not likely to be due to an enzyme (digestive enzymes pectinase; Rapidase X-Press L and cellulase; Rohament CL) that may be partly contained in the EEC. Following the treatment of *E. cava* chips with enzymes for 24 h, the supernatant was collected via centrifugation and immersed in ethanol. Through this process, the enzymes are mostly filtered out. Taken together, the results of the present study suggested that EEC supplementation reduces HFD-induced obesity in C57BL/6N mice. Thus, EEC may prevent and treat obesity, NAFLD and obesity-associated diseases, and may be a suitable candidate of dietary supplements and/or anti-obesity nutraceutical agents that prevent and/or treat obesity-associated diseases.

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