Gelidium amansii ethanol extract suppresses fat accumulation by down-regulating adipogenic transcription factors in *ob/ob* mice model

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Abstract The purpose of this study was to determine the anti-obesity effects of *Gelidium amansii* extract (GAE) in the C57BL/6J-*ob/ob* mice. The *ob/ob* mice were fed GAE at 0.5% for 4 weeks, after which body weight, epididymal adipose tissue weight, plasma triglycerides, and hepatic lipid accumulation were significantly reduced in GAE-fed mice compared with *ob/ob* control mice. Plasma adiponectin levels were significantly higher in GAE-fed mice than in *ob/ob* control mice. These findings were supported by the expression levels of enzymes and proteins related to lipid metabolism assessed by western blotting: protein expression levels of the peroxisome proliferator-activated receptor γ and CCATT/enhancer binding protein α decreased significantly, while hormone-sensitive lipase and phospho-AMP-activated protein kinase levels increased in the GAE-fed mice compared with *ob/ob* control mice. These findings demonstrate that GAE regulates plasma lipid profiles and increasing high-density lipoprotein cholesterol levels as well as by regulating the expression levels of lipid metabolic factors, resulting in reduced weight gain in *ob/ob* mice.

Keywords: *Gelidium amansii* extract, C57BL/6J *ob/ob* mice, adipogenesis, lipolysis, anti-obesity

Introduction

Obesity is a major cause to the burden of chronic disease and disorder in developed countries and is considered a risk factor associated with the progress of various chronic diseases including insulin resistance, type 2 diabetes, dyslipidemia, cardiovascular disease, heart failure, hypertension, and fatty liver (1,2). The accumulation of visceral fat furthermore leads to abnormalities in lipid metabolism, carbohydrate metabolism, and blood pressure according to excess energy in the body and increases susceptibility to arteriosclerotic diseases because of abnormal adipocytokine. Such states are referred to as metabolic syndrome (3). Obesity leads to excessive growth and amplification of fat tissue due to an imbalance between energy intake and expenditure (4). Several pharmacological approaches to the treatment and prevention of obesity have been indicated and obesity therapies include the inhibition of nutrient absorption and the administration of drugs that control lipid management (5). Currently available therapeutic agents include sibutramine, orlistat, phentermine, and diethylpropion (6). Some of these drugs, however, have been reported serious adverse effects such as headaches, vomiting, heart attack and stomach pain (7). The

development of natural products for the treatment of obesity is a challenging task; however, a natural product treatment may require less time and cost to develop compared with conventional single-entity pharmaceuticals (8).

Seaweeds are rich in bioactive compounds including polyphenols, carotenoids, phycocyanins, phycobilins, and polysaccharides, many of which have known beneficial effects on human health (9). *Gelidium amansii*, a well-known red algae, has been commonly consumed for many years and has recently been included in diet foods owing to its high dietary fiber content in the form of agarose and agaropectin. According to several studies, *G. amansii* furthermore has anti-oxidative, immunomodulatory, anti-tumor, and cytotoxic effects (10–12). The effects of *G. amansii* on obesity have not been examined *in vivo*, and the purpose of this study was therefore to examine the effects of *G. amansii* extract (GAE) on body weight gain, fat mass, lipids profiles in plasma and liver, plasma adiponectin levels, and the expression of lipid metabolic factors such as hormone-sensitive lipase (HSL), phospho-AMP-activated protein kinase (pAMPK), peroxisome proliferator-activated receptor γ (PPAR γ), and CCATT/enhancer binding protein α (C/EBP α) in the C57BL/6J *ob/ob* mouse model of obesity.

Materials and Methods

Preparation of *G. amansii* extract *G. amansii* was collected in Jeju Island, Korea. The plant samples were washed three times to remove sand and salt, after which they were dried at room temperature and ground into a powder. *G. amansii* powder was extracted with 80% ethanol for 24 h at 40°C, after which the GAE was concentrated in a rotary vacuum evaporator, freeze-dried to a powder, and stored at 4°C. The GAE was found to have a total polyphenolic content of 1.81 ± 0.08 mg/mL and a total flavonoid content of 1.55 ± 0.16 mg/mL.

Determination of total phenolic content The total polyphenol content of GAE was estimated colorimetrically using Folin–Ciocalteu reagent (13). The appropriately diluted extract (200 μ L) was mixed with 15 mL distilled water in a 25 mL volumetric flask; subsequently, 1.25 mL undiluted Folin–Ciocalteu reagent was added. After 1 min, 3.75 mL 20% sodium carbonate was added to the mixture. After incubation for 30 min at room temperature, the absorbance was measured at 760 nm using an ultraviolet and microplate reader. The phenolic content of the samples was calculated based on the standard curve for gallic acid.

Determination of total flavonoid content The total flavonoid content of GAE was determined as described previously with slight modifications (14). GAE (2.5 mL) was diluted with distilled water (10 mL), followed by the addition of 5% NaNO₂ solution (0.75 mL). Thereafter, 10% AlCl₃ solution (0.75 mL) was added, and the reaction mixture was incubated at room temperature for 5 min. After incubation, 5 mL 1 M NaOH was added, and distilled water was added to a final volume of 25 mL. The mixture was shaken vigorously, and the absorbance was measured at 510 nm using a microplate reader.

Animals and diets Male 5-week-old C57BL/6J lean mice ($n=7$) and C57BL/6J-*ob/ob* mice ($n=14$) were purchased from Jung-Ang Lab Animal Inc. (Seoul, Korea). The mice were housed in a temperature-controlled room (24°C) with a 12 h light/dark cycle and were acclimated for two weeks before being divided into three experimental groups ($n=7$ each). The C57BL/6J lean mice (lean control group) and the *ob/ob* mice (*ob/ob* control group) were fed a standard semi-synthetic diet (AIN-93G) for 4 weeks, while the *ob/ob* mice in the *ob/ob*-GAE group were fed a standard AIN-93G diet supplemented with GAE (0.5 g/100 g diet) for 4 weeks. During the experimental period, animals had free access to water and food, and their body weight and food intake were measured weekly. At the end of the experimental period, the mice were sacrificed with ether after withholding food for 12 h, and blood samples were taken from the inferior vena cava for assessments of plasma adiponectin and lipid levels. After blood sample collection, the livers, kidneys, and adipose tissues were collected, rinsed, weighed, and stored at -80°C . The animal handling and care procedures used in this study were in

accordance with current international laws and policies (PNU Guide for the Care and Use of Laboratory Animals).

Assays for plasma lipids and adiponectin Plasma levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) were determined using commercial assay kits (Asan Pharm., Seoul, Korea) according to the manufacturer's instructions, while low-density lipoprotein cholesterol (LDL-C) levels were calculated using the Friedewald formula: $\text{LDL-C} = \text{TC} - \text{HDL-C} - (\text{TG}/5)$ (15). Plasma adiponectin levels were measured using a commercial mouse ELISA kit (Shibayagi Co. Ltd, Gumma, Japan).

Assay for hepatic lipids Hepatic lipids were extracted using a procedure developed by Folch *et al.* (16), while hepatic cholesterol and triglyceride concentrations were analyzed using the enzymatic kit used for the plasma analyses.

Western blot analysis Epididymal adipose tissue and liver homogenates in lysis buffer were centrifuged at $20,000 \times g$, (4°C, 15 min) and the protein concentrations in the resulting supernatants were measured using a protein assay kit (Bio-Rad, Hercules, CA, USA). Protein samples (30 μ g) were separated on 12% SDS-PAGE gels. Separated proteins were transferred to nitrocellulose membranes that were subsequently blocked in 5% skim milk in Tris-buffered saline (0.1% Tween-20) for 1 h at room temperature. Blocked membranes were incubated with primary antibodies against PPAR γ , C/EBP α , HSL, and phospho-AMPK α [p-AMPK] (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. The membranes were washed then and incubated with secondary antibodies for 1 h at room temperature, after which each antigen-antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence using an LAS-1000 plus analyzer (Fujifilm, Tokyo, Japan). Band densities were determined using an image analyzer (Multi Gauge V3.1, Fujifilm) and were normalized to β -actin levels.

Data and statistical analyses Data are reported as mean $\pm$ SD of triplicate experiments and statistical analyses were carried out using SAS 9.1 software. Differences among groups were evaluated by one-way analysis of variance (ANOVA) analysis followed by Duncan's multiple range tests. Differences with $p < 0.05$ were considered statistically significant.

Results and Discussion

Effects of GAE supplementation on body weight, food intake, and food efficiency ratio To determine whether GAE supplementation for 4 weeks has a regulatory effect on obesity, the body weights of the animals were measured (Table 1). Body weight gain in the GAE-supplemented group (4.60 ± 1.03 g) was significantly lower than that

Table 1. Effects of GAE supplementation on body weight gain, food intake, and food efficiency ratio

	Lean ¹⁾	<i>ob/ob</i>	<i>ob/ob</i> -GAE
Initial body weight (g)	20.70±0.55 ^{b2)}	32.60±1.80 ^a	32.85±1.57 ^a
Final body weight (g)	23.00±0.51 ^b	39.88±1.71 ^a	37.45±1.36 ^a
Weight gain (g/4 weeks)	2.30±0.45 ^c	7.28±1.06 ^a	4.60±1.03 ^b
Food intake (g/day)	2.33±0.23 ^b	3.37±0.69 ^a	2.93±0.34 ^{ab}
FER ³⁾	3.53±0.25 ^b	7.72±0.65 ^a	4.36±0.34 ^b

¹⁾Lean (lean control group): C57BL/6J lean mice fed an AIN-93G diet; *ob/ob* (*ob/ob* control group): C57BL/6J-*ob/ob* mice fed an AIN-93G diet; *ob/ob*-GAE: C57BL/6J-*ob/ob* mice fed a diet supplemented with *Gelidium amansii* extract (0.5 g/100 g diet).

²⁾Values represent means±SD; *n*=7. ^{a-b}values denoted by different letters differ significantly among groups (*p*<0.05).

³⁾FER: Food efficiency ratio=[weight gain (g)]/[total food intake (g)]×100.

in the *ob/ob* control group (7.28±1.06 g) after the 4-week experimental period and the food intakes of the *ob/ob* control group and the GAE-supplemented group were 3.37±0.69 and 2.63±0.34 g/day, respectively. Mice whose diet was supplemented with GAE exhibited a decreased food efficiency ratio (FER; calculated as [weight gain (g)]/[total food intake (g)]×100) compared to *ob/ob* control group mice (4.36±0.34 and 7.72±0.65, respectively). The body weight gains and FERs measured for the different animal groups indicate that obesity was reduced by supplementation with GAE.

In the present study, GAE supplementation significantly lowered weight gain and FER in *ob/ob* mice. Based on the FER equation, change in body weight is the most important index affecting the FER and FER can therefore be applied as an indicator for obesity: a low FER value effectively predicts the avoidance of obesity (17). The FER findings reported here indicate that the mice administered the GAE were less efficient in transforming the nutrients they were taking in into biomass, suggesting that GAE may have potential as an anti-obesity agent.

Effects of GAE supplementation on liver, kidney, and adipose tissue mass

The masses of the epididymal adipose tissues (1.95±0.06 vs. 2.30±0.23 g) of GAE-fed mice were significantly lower than those of *ob/ob* control mice. The mesenteric adipose tissue weights in the *ob/ob* control group and the GAE supplementation group were 0.42±0.08 and 0.25±0.05 g, respectively (Table 2).

Reduced body weight gain can be associated with reduced fat mass, and reduced fat mass may therefore lead to reduced body weight (18). The higher liver and fat tissue masses in the *ob/ob* control mice in this study relative to the lean control mice indicate that fat tissue mass of this animal model reflects excess energy consumption; however, liver and adipose tissues masses in the GAE-supplemented group were significantly lower than those in the *ob/ob* control group. Excess energy intake and reduced energy consumption results in excessive abnormal growth of white adipose tissue, which may contribute to the occurrence of obesity. Loncar *et al.* (19) reported that epididymal adipose tissue in the rat is usually

Table 2. Effects of GAE supplementation on liver, kidney, and adipose tissue masses

	Lean ¹⁾	<i>ob/ob</i>	<i>ob/ob</i> -GAE
Liver (g)	0.60±0.09 ^{b2)}	2.40±0.40 ^a	1.90±0.38 ^a
Kidney (g)	0.26±0.01 ^a	0.29±0.02 ^a	0.28±0.03 ^a
Epididymal AT ³⁾ (g)	0.42±0.07 ^c	2.30±0.23 ^a	1.95±0.06 ^b
Mesenteric AT (g)	0.08±0.03 ^c	0.42±0.08 ^a	0.25±0.05 ^b

¹⁾Lean (lean control group): C57BL/6J lean mice fed an AIN-93G diet; *ob/ob* (*ob/ob* control group): C57BL/6J-*ob/ob* mice fed an AIN-93G diet; *ob/ob*-GAE: C57BL/6J-*ob/ob* mice fed a diet supplemented with *Gelidium amansii* extract (0.5 g/100 g diet).

²⁾Values represent means±SD; *n*=7; ^{a-c}values denoted by different letters differ significantly among groups (*p*<0.05)

³⁾AT, adipose tissue

considered white adipose tissue with a function and characteristic structure. Mesenteric adipose tissue is also considered to play an essential role in the development of obesity-related diseases (20). Seaweeds are considered to be a rich source of phenolic compounds with a number of important biological activities such as phlorotannins. It was recently reported that treatment with phlorotannins results in decreased adipogenesis and lipogenesis in 3T3-L1 preadipocytes (21). *G. amansii*, red seaweed, also has many different bioactive compounds including phycocolloids, pigments, flavonoids and polyphenol compounds. The studies reported that plant-derived polyphenols such as flavonoids exerted the anti-obesity effect (22). We guessed that the anti-obesity effect of GAE might come from the polyphenols and flavonoids, because GAE contained 1,810 µg/mL polyphenols and 1,550 µg/mL flavonoids. GAE also contained pheophorbide A as a main bioactive compound, which exerted anti-obesity effect. In this study, GAE supplementation suppressed fat accumulation in epididymal and mesenteric adipose tissue in mice, resulting in reduced body weight gain. These results suggest that the anti-obesity effect of GAE may come from pheophorbide A as well as polyphenol compounds.

Effect of GAE supplementation on plasma and hepatic lipid levels

The effects of 4 weeks of GAE supplementation on plasma and hepatic lipid levels were examined. Compared with the lean control animals, the *ob/ob* control mice exhibited higher levels of TG, TC, and LDL-C but lower levels of HDL-C (Table 3). Mice supplemented with GAE were found to reduce the levels of plasma TG, and LDL-C (154.62±10.56, and 93.26±3.21 mg/dL, respectively) compared to *ob/ob* control mice (188.20±22.51, and 136.45±19.20 mg/dL, respectively). The GAE supplementation group also exhibited higher HDL-C levels compared with the *ob/ob* control group (74.38±11.12 and 47.61±15.35 mg/dL, respectively). Hepatic TG and TC levels showed comparable tendencies to those in the plasma: the GAE supplementation group had significantly reduced hepatic TG and TC levels (22.27±3.38 and 5.12±0.13 mg/g tissue, respectively) compared with the *ob/ob* control group (31.44±1.70 and 7.54±0.21 mg/g tissue, respectively).

Table 3. Effects of GAE supplementation on plasma and hepatic lipid levels

	Lean ¹⁾	<i>ob/ob</i>	<i>ob/ob</i> -GAE
Plasma (mg/dL)			
Triglyceride	127.60±11.54 ^{b2)}	188.20±22.51 ^a	154.62±10.56 ^b
Total cholesterol	156.64±22.05 ^b	221.70±21.10 ^a	198.56±19.00 ^a
HDL-C	62.20±7.61 ^{ab}	47.61±15.35 ^b	74.38±11.12 ^a
LDL-C	68.92±5.41 ^b	136.45±19.20 ^a	93.26±3.21 ^b
Hepatic (mg/g tissue)			
Triglyceride	9.54±2.43 ^c	31.44±1.70 ^a	22.27±3.38 ^b
Total cholesterol	2.21±0.28 ^c	7.54±0.21 ^a	5.12±0.13 ^b

¹⁾Lean (lean control group): C57BL/6J lean mice fed an AIN-93G diet; *ob/ob* (*ob/ob* control group): C57BL/6J-*ob/ob* mice fed an AIN-93G diet; *ob/ob*-GAE: C57BL/6J-*ob/ob* mice fed a diet supplemented with *Gelidium amansii* extract (0.5 g/100 g diet).

²⁾Values represent mean±SD; *n*=7; ^{a-c}values denoted by different letters differ significantly among groups (*p*<0.05).

Plasma and hepatic TG, TC, and LDL-C levels were significantly lower in the GAE-supplemented group than in the *ob/ob* control group, while HDL-C levels were higher in the GAE-supplemented group than in the *ob/ob* control group (Table 3). Triglycerides are synthesized in the liver, released into the blood as very low-density lipoproteins, and then carried to the adipose tissue (23). High-density lipoprotein cholesterol (HDL-C) plays a key role in the process of reverse cholesterol transport, transporting cholesterol esters and TGs from peripheral tissues to the liver for removal (24). Supplementation with GAE was shown in this study to improve lipid profiles and to correlate with reduced body weight gain and adipose tissue weight. Various bioactive components in algae including polysaccharides, carotenoids, and polyphenols, have been suggested to be involved in indicating biological activities. Red algae, in particular, are known to contain polyphenols such as chlorophyll α , β -carotene, lutein, and zeaxanthin (25). Some studies reported that seaweed extracts containing polyphenols treatment inhibits adipocyte differentiation, evidenced by reduced lipid accumulation and decreased expression of adipogenic markers via mitogen-activated protein kinase (MAPK) signaling pathways (26,27). According to Seo *et al.* (28), GAE inhibits lipid accumulation in 3T3-L1 adipocytes. Taken together, the present findings suggest that polyphenol-rich GAE improves lipid profiles and attenuates adipose tissue weight gain and hepatic lipid accumulation in *ob/ob* mice.

Effect of the GAE supplementation on plasma adiponectin levels

Adiponectin levels were found to be significantly lower in the *ob/ob* control animals than in the lean control animals (Fig. 1), and GAE supplementation was shown to significantly increase adiponectin levels in the *ob/ob* mice. Adiponectin is a protein hormone, regulates many metabolic processes, including fatty acid catabolism and glucose modulation (29). Plasma adiponectin levels are known to be diminished in obesity, diabetes, and atherosclerosis (30); in present study, plasma adiponectin levels were higher in the GAE-supplemented

Fig. 1. Effect of GAE supplementation on plasma adiponectin levels. Lean (lean control group): C57BL/6J lean mice fed an AIN-93G diet; *ob/ob* (*ob/ob* control group): C57BL/6J-*ob/ob* mice fed an AIN-93G diet; *ob/ob*-GAE: C57BL/6J-*ob/ob* mice fed a diet supplemented with *Gelidium amansii* extract (0.5 g/100 g diet). Data represent means±SD; *n*=7; ^{a-b}values denoted by different letters differ significantly among groups (*p*<0.05).

animals than in the *ob/ob* control animals (Fig. 1). Weight loss has previously been shown to be associated with increased adiponectin and HDL-C levels (31) and porphyran, a dietary fiber from red algae, may improve glucose metabolism via the enhancement of adiponectin expression (32). Based on these previous findings and on the present data, GAE provided prevention against excess weight gain and obesity.

Effects of GAE supplementation on HSL, pAMPK, PPAR γ , and C/EBP α expression

The expression levels of HSL, PPAR γ , and C/EBP α in adipose tissue and pAMPK in the liver were assessed. Supplementation with GAE was found to significantly increase the protein expression levels of HSL and pAMPK, both proteins associated with lipolysis. As shown in Fig. 2A, HSL expression in adipose tissue was significantly increased by GAE supplementation, reaching levels 2.2-fold higher than that measured in the *ob/ob* control group. The expression of pAMPK was also significantly increased by GAE supplementation: the GAE-supplemented group animals exhibited 3.4-fold higher pAMPK expression levels compared to the *ob/ob* control group animals.

The effects of GAE on the expression levels of the adipogenesis-related genes PPAR γ and C/EBP α were investigated, and it was shown that GAE supplementation significantly reduced the expression levels of PPAR γ and C/EBP α by ~0.49 and ~0.64-fold, respectively, compared to those in the *ob/ob* control group. These findings suggest that GAE regulates the expression of lipid metabolic factors.

Lipid accumulation in adipocytes is regulated by the balance between lipolysis and lipogenesis, where lipolysis in adipocytes is modulated in a step-wise fashion by HSL, the major lipase in adipocytes with significant hydrolytic action against triacylglyceride and diacylglyceride, resulting in the release of fatty acids and glycerol

Fig. 2. Effects of GAE supplementation on HSL, pAMPK, PPAR γ , and C/EBP α expression. Western blot signal intensities were determined by densitometric analysis using Multi Gauge V3.1 software. Representative blots of HSL, pAMPK, PPAR γ , and C/EBP α protein expression are shown with protein expression levels quantified relative to the expression level observed in *ob/ob* control mice. Data represent means $\pm$ SD; $n=7$; ^{a-c}values denoted by different letters differ significantly ($p<0.5$) from one another (Duncan's multiple range test). HSL, hormone-sensitive lipase; pAMPK, phosphorylated AMP-activated protein kinase α ; PPAR γ , peroxisome proliferator-activated receptor γ ; C/EBP α , CCAAT/enhancer-binding protein α .

(33,34). HSL catalyzes the rate-limiting step in this hydrolysis of triacylglycerol. HSL is also engaged in the mobilization of fatty acid from intracellular lipid stores in tissues. Thus, the activity of HSL seems to be essential for the regulation of fatty acid metabolism (35,36). Supplementation with GAE was shown to result in increased HSL expression in the adipose tissue (Fig. 2), which indicates that GAE reduces lipid accumulation by stimulating lipolysis via HSL elevation.

Adiponectin is known to activate the AMPK pathway, which may provide the mechanical connection between adiponectin levels and the related hepatic steatosis, fatty acid oxidation, and lipid synthesis (37). The AMPK enzyme plays a major role in lipid and glucose metabolism and controls metabolic diseases such as obesity (38). Our data show that pAMPK expression in the GAE-supplemented group was significantly higher than that in the *ob/ob* control group (Fig. 2A), demonstrating that GAE inhibits hepatic lipid accumulation through the activation of AMPK, thereby inhibiting the lipogenic enzyme and increasing the hepatic fatty acid transport or oxidation.

The AMPK enzyme is furthermore involved in the regulation of PPAR γ and C/EBP α , which are the central regulators of adipogenesis (39,40) and are distributed in white adipose tissue. These regulatory factors play a crucial role in maintaining adipose expansion and adiposity. Both C/EBPs and PPAR γ target genes were responsible for lipid metabolism, which increased lipid accumulation and leads to the development of a mature lipid laden adipocyte (41). PPAR γ is a ligand-activated transcription factor that plays an important role in the development of obesity. Adipocyte differentiation provokes PPAR γ activity, and it transfers hormonal stimulation to its target genes such as C/EBP α (42). As shown in Fig. 2B, the protein expression levels of PPAR γ and C/EBP α were significantly lower in the GAE-supplemented group than in the *ob/ob* control group. This finding is in agreement with a report demonstrating that the red algae *Gracilaria verrucosa* led to decreased mRNA levels of PPAR γ and C/EBP α (43). Plant-derived polyphenols such as flavonoids and resveratrol play a suggested protective role against obesity (44); the GAE in this study was shown to contain 1,810 $\mu\text{g/mL}$ polyphenols and 1,550 $\mu\text{g/mL}$ flavonoids. These compound classes may therefore be responsible for the anti-obesity activity of GAE observed in this study, suggesting that GAE contains relatively enriched anti-obesity polyphenol compounds. The polyphenol-rich GAE treatment in this study was shown to suppress both PPAR γ and C/EBP α expression, which may have led to reduced fat gain.

In conclusion, this study is to investigate the anti-obesity effects of GAE in C57BL/6J *ob/ob* mice. We demonstrate here that supplementation with GAE for 4 weeks significantly decreases body weight gain, adipose tissue mass, and the levels of TG, TC, LDL-C, as well as increasing lipolysis via the activation of adiponectin and HSL. Supplementation of diet with GAE was furthermore shown to decrease the expression levels of the adipogenic transcription factors PPAR γ and C/EBP α as well as increasing pAMPK expression. Taken together, our findings suggest that GAE may be a potential pharmacologic agent for the treatment of obesity.

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