

ORIGINAL ARTICLE

# Blue honeysuckle rich in cyanidin-3-*O*-glucoside inhibited adipogenic differentiation by modulation of the adipogenesis pathway in 3T3-L1 adipocytes

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## Popular scientific summary

- This is the first study reporting the content of Cyanidin-3-*O*-glucoside (C3G) from Blue honeysuckle (BH) extract and its potential anti-obesity effect.
- BH extracts decreased lipid accumulation and formation of free glycerol, and also suppressed expression of PPAR $\gamma$ 1/2, ADRP, C/EBP $\alpha$ , and ACC proteins in a dose-dependent manner.
- These results could imply that BH extracts inhibit adipogenesis by regulating the transcriptional factors in 3T3-L1 cells.

## Abstract

**Background:** Blue honeysuckle (BH; *Lonicera caerulea* L.), which is a traditional medicinal plant, is known to be a rich source of anthocyanins and phenolic acids due to its strong antioxidant and anti-inflammatory activities. Its anti-obesity effects, which are a result of attenuating abnormal lipid and glucose metabolisms, have also been reported.

**Aim:** The purpose of this study is to investigate the effect of BH on genes and proteins that are involved in the adipocyte differentiation using 3T3-L1 cells.

**Methods:** The effects of the water extracts of the BH were examined on adipogenesis and lipolysis using a biochemical and molecular analysis of the 3T3-L1 cells.

**Results:** Cyanidin-3-*O*-glucoside (C3G) from the BH extract was determined in order to contain 1.67 mg/g by the high-performance liquid chromatography analysis. The lipid accumulation in the adipocytes was reduced, which ranged from 58 to 26% in the BH (500 and 1,000  $\mu$ g/mL) compared to the control group. The lipolysis that was measured by the glycerol content was not affected by the BH at 1,000  $\mu$ g/mL. The BH downregulated the expression of the main transcription factors of the adipogenesis pathway, such as peroxisome proliferator-activated receptor  $\gamma$ , 1, 2, adipose differentiation-related protein, CCAAT/enhancer binding protein  $\alpha$ , and acetyl CoA carboxylase, while increasing the expression of the Adenosine monophosphate (AMP)-activated protein kinase  $\alpha$ .

**Conclusion:** These findings suggest that the BH is a good source of C3G, and it could be effective in regard to inhibiting the adipogenesis as opposed to the lipolysis, which indicates the potential for natural anti-obesity ingredients.

Keywords: blue honeysuckle; cyanidin-3-*O*-glucoside; 3T3-L1 cells; adipogenesis; anti-adipogenic

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The worldwide increase of obesity that is related to a metabolic syndrome results in many harmful consequences, which include cancer, infertility,

accelerated aging, and inflammation (1). It is predicted that the population with this syndrome may increase two-fold to over 300 million by the year 2025 (2). Currently, the

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existing pharmacological agents who treat this metabolic syndrome have several limitations, which include various side effects and high rates of secondary failure (3). The research about the complementary or substitute approaches for the prevention of metabolic syndrome is being progressively investigated due to these limitations (4). For instance, conjugated linoleic acid (CLA) was developed as a representative anti-obese medicinal agent for type II diabetes (5). However, other studies found that it induced hyperinsulinemia and insulin resistance, which is primarily in ob/ob mice (6, 7). Thus, the development of natural materials, which are harmless to humans, should be developed for the effective control of obesity and diabetes (8).

Blue honeysuckle (BH; *Lonicera caerulea* L.) is a traditional plant which is known as a folk medicine in Northern Russia, China, and Japan (9). Its edible berries are a rich source of anthocyanins (10, 11) that have multiple biological activities, which include strong antioxidant activity and anti-inflammation (12, 13). There is evidence that supports the pharmacological effects of BH in regard to improving abnormal lipid, glucose metabolism, diabetes, and obesity (9, 14). A previous study reported that the dietary intake of BH dose-dependently reduced hepatic fat deposition and obesity in high fat diet (HFD) mice (15). Also, in regard to anti-obesity, it was found that the administration of BH to HFD mice upregulated the AMP-activated protein kinase (AMPK) on lipid metabolism (16). However, more transcription factors that are involved in lipid metabolism are needed to be further study for elucidating the anti-obesity effect of BH.

Adipogenesis is the process where the precursor stem cells in adipose tissue differentiate into lipid laden adipocytes (17). A previous study also mentioned that adipogenesis also involves changes in the cells morphology, which is regulated by a complex gene expression program. In this study, the key transcription factors and proteins that regulate the potential adipogenic or anti-adipogenic bioactivity are addressed.

As a result, the present study's aims are 1) to investigate the effect of BH on lipid accumulation and lipolysis and 2) to elucidate the mechanism via measuring the gene modulation of the transcription factors, which include the peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) 1, 2, adipose differentiation-related protein (ADRP), CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ), AMPK $\alpha$ , and acetyl CoA carboxylase (ACC) genes.

## Materials and methods

### Chemicals and reagents

The Dulbecco's modified Eagle's Medium (DMEM), Dulbecco's Phosphate Buffer Saline, fetal bovine serum (FBS), and trypsin solution were purchased from Corning Inc. (NY, USA). The newborn bovine calf serum

(New Zealand) was obtained from Thomas Scientific Inc. (NJ, USA), and the penicillin–streptomycin was purchased from Biotechnic Research, Inc. (CA, USA). The EZ-Free Glycerol Assay kit was obtained from DoGen-Bio Co. Ltd (Seoul, Republic of Korea). The antibodies for the western blot analysis, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), PPAR $\gamma$ , C/EBP $\alpha$ , AMPK $\alpha$ 1, AMPK $\alpha$ 2, ACC, and adiponectin were purchased from Cells Signaling Technology, Inc. (MA, USA). The ADRP, sterol regulatory element-binding protein 1 (SREBP1), and phospho-SREBP-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA), Abcam (California, USA), and Afinit (Pennsylvania, USA), respectively.

### Sample preparations

The BH extracts were prepared and supplied by Aribio H&B (Jecheon, Republic of Korea), and they were stored at room temperature without exposure to light and humidity until further analysis. After grinding the raw material of the BH, water was added to the raw material at a 5:1 volume ratio for extraction at room temperature for 3–5 h. The extraction was squeezed and filtered, which was followed by centrifugation at 12,000 rpm using a high-speed centrifuge. The mixture was then lyophilized at 55–65°C after being concentrated to be 10 brix.

### Analysis of cyanidin-3-O-glucoside by high-performance liquid chromatography

Cyanidin-3-O-glucoside (C3G) from BH was identified and quantified using a high-performance liquid chromatography (HPLC) with a photodiode-array detector that consisted of an isocratic pump, a degasser, and an automatic injection system (Model Ultimate 3000; Thermo Science, MA, USA), with the separation conducted using a Shiseido capcell pak C<sub>18</sub> UG120 column (5  $\mu$ m, 4.6 mm x 250 mm). The analytical column was continued with an injection volume of 10  $\mu$ L at 30°C. The mobile phase that contained 5% formic acid in water (A) and acetonitrile (B) of HPLC grade was used. The flow rate was 1.0 mL/min with a ultraviolet (UV) absorbance of 516 nm, and the gradient condition was as conducted by following the procedure that is described next: 0–15 min, 90% A; 15–16 min, 0% A; 16–22 min, 0% A; 22–23 min, 90% A; 23–30 min, 90% A.

### Cells culture and differentiation

The 3T3-L1 cells were initially maintained in DMEM with 10% newborn bovine calf serum and 1% penicillin/streptomycin at 37°C with 5% CO<sub>2</sub>. For the cells differentiation, the 3T3-L1 pre-adipocytes were seeded in a T175 flask to 1.5  $\times$  10<sup>5</sup> cells per well and incubated for 48 h. After 100% confluence, which is called day 0, the adipocyte differentiation was induced in a DMEM that contained 10% FBS with 3-isobutyl-1-methylxanthin (IBMX) of 0.5 mM, insulin of 5  $\mu$ g/mL, and dexamethasone of 1

$\mu\text{M}$ . After 3 days of seeding, the medium was changed to DMEM that contained FBS (10%) and insulin (5  $\mu\text{g}/\text{mL}$ ). The medium that was composed of DMEM with 10% FBS was then replaced with a new medium every 2 days. The BH was treated with various concentrations, which included 0, 250, 500, and 1,000  $\mu\text{g}/\text{mL}$ . The medium was replaced every other day until the cells were differentiated.

#### Cells viability assay

The cells viability was investigated using a Cell Counting Kit (CCK)-8 assay with a CCK from Dojindo Molecular Technologies, Inc. (Maryland, USA). After 2 days of cells differentiation, the 3T3-L1 cells were treated with various BH concentrations, which included 0, 39, 78, 156, 313, 625, 1,250, 2,500, and 5,000  $\mu\text{g}/\text{mL}$  for 48 h. The DMEM/F12 medium with 1% penicillin/streptomycin that contained 10  $\mu\text{L}$  of CCK-8 (10%, v/v) was added to the well (120  $\mu\text{L}/\text{well}$ ), and the plates were cultured at 37°C for 2 h. The absorbance was analyzed at 450 nm using a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific), and the percentage of cells viability was calculated and expressed as a % of the control.

#### Lipid accumulation by Oil Red O staining

Oil Red O staining was performed in mature adipocytes in order to confirm the excessive lipid accumulation. Differentiated mature adipocytes were washed with 1X Phosphate-buffered saline twice and fixed with 4% paraformaldehyde for 1 h. The fixed cells were washed twice with distilled water (DW) and 60% isopropanol, and they were then stained using an Oil Red O working solution for 30 min. The stained cells were washed with DW four times and then imaged using a microscope (OLYMPUS model CKX41SF, Tokyo, Japan), which was used in order to capture the lipid droplet morphology, and it was coupled with a digital camera (SONY DSLR-A500, Japan) with a 200 $\times$  magnification. In order to quantify the lipid accumulation content, the stained cells were dissolved using 100% isopropanol and measured with an ELISA plate reader (Varioskan Flash, ThermoFisher Scientific, San Jose, CA, USA) at 500 nm. The cumulative percentage was calculated by using the following formula:

$$\text{Lipid accumulation (\%)} = \left( 1 - \frac{\text{Negative control} - \text{Sample}}{\text{Negative control} - \text{Control}} \right) \times 100$$

#### Free Glycerol Assay

The 3T3-L1 cells were seeded in 6-well culture plates at  $1 \times 10^6$  cells/well and incubated to differentiate for 12 days as accounted in the cells culture method. After the 3T3-L1 cells differentiated, the cells were treated with BH, which included 0, 250, 500, and 1,000  $\mu\text{g}/\text{mL}$  for 48 h. The free glycerol contents were measured using an EZ-Free Glycerol Assay kit (DoGenBio Co. Ltd, Seoul) and

a Multi-plate Reader (Thermo Fisher Scientific) at 535 nm/595 nm.

#### RNA isolation and real-time-polymerase chain reaction analysis

The total RNA was extracted using an Easy-BLUE™ Total RNA Extraction Kit (iNtRON, Gyeonggido, Republic of Korea). For the real-time polymerase chain reaction (RT-PCR) quantification, the cDNA was synthesized using a PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Seoul, Republic of Korea), according to the manufacturer's instructions. The RT-PCR was performed using 100 ng of cDNA in a 20  $\mu\text{L}$  reaction volume with Emerald Amp® PCR Master Mix (TaKaRa, Seoul, Republic of Korea) in a 96-well plate. The fluorescent products that were detected at the end of each cycle were determined using a real-time reverse transcriptase PCR thermocycler, which used the genomic growth of the fluorescence that was associated with the product in order to determine the threshold cycle (Ct) of each cycle using the formula  $2^{-\Delta\Delta\text{Ct}}$ . The housekeeping gene that encodes the  $\beta$ -actin was used as the standard for all the samples. The primers that were used in the current study are listed in Table 1, and all the real-time experiments were recorded in triplicate.

#### Western Blot Analysis

The protein lysates of the differentiated 3T3-L1 cells were lysed using a Radioimmunoprecipitation assay (RIPA) lysis buffer (Bio-Rad, California, USA) that was supplemented with phosphatase and protease inhibitors. The protein concentration was determined using a Bovine serum albumin (BSA) analysis, and an aliquot amount (50  $\mu\text{g}$ ) of the total protein from each sample was separated by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred to a polyvinylidene difluoride membrane, and it was treated with 5% skim milk for 1 h at room temperature for the blocking. The membranes were then cultured for 16 h at 4°C with the primary antibody. After that, they were cultured with the corresponding secondary antibodies (1:10,000) for 1 h at room temperature. Finally, the protein bands were scanned using the image analysis system (WSE 6200 Lumino Graph Chemidoc, Atto) with ultra-sensitive enhanced chemiluminescent (ECL, Super Signal™ West FEMTO Maximum Sensitivity Substrate, Thermo Scientific). The intensity of the visualized western blot band was quantified using the ImageJ (NIH, USA) software.

#### Statistical analysis

All the data were recorded in triplicate, which were indicated as mean  $\pm$  standard error of the mean (SEM). In regard to measuring the significance between the two distributions, the Student *t*-test was applied. The differences between the mean values of the multiple groups were analyzed using one-way analysis of variance, which was followed by the

**Table 1.** List of primers for the sequences of the transcription factors

Target gene	Direction	Sequence (5'-3')
PPAR $\gamma$	F	CAG GCC TCA TGA AGA ACC TTCT
	R	GCT TCA ATC GGA TGG TTC TTC G
CEBP $\alpha$	F	GAC ATC AGC GCC TAC ATC GA
	R	CCC GGG TAG TCA AAG TCA CC
SREBP1c	F	TGT GAT CTA CTT CTT GTG GCC C
	R	GAA TAC CCT CCT CAT AGC AGG C
ACC	F	AGG ATG AAG GGG AAT CCA GGT A
	R	GACA AG GTA AGC CCC AAT ACC A
AMPK $\alpha$ 1	F	ATT GTC ACA GGC ATA TGG TGG T
	R	GGT CAT CAT CAA AAG GGA GGG T
AMPK $\alpha$ 2	F	AAT TAT GCA GCA CCT GAG GTC A
	R	CAG ACT TCC TTC ACA GCC TCA T
ADRP	F	GGT GGA TAA GAC CAA AGG AGC A
	R	CCT CCT GAG TGA GAG GGA AGT A
Adiponectin	F	GGT CTT CTT GGT CCT AAG GGT G
	R	TTC ATG TAC ACC GTG ATG TGG T
$\beta$ -Actin	F	AGA AGC TGT GCT ATG TTG CTCT
	R	AGT TTC ATG GAT GCC ACA GGAT

Tukey test using the Graphpad Prism 3.0 software (Graphpad Software, CA, USA). The statistical significance was considered as  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ .

## Results and discussion

### Identification of bioactive component in the extracts of the BH

Figure 1 illustrates the HPLC-UV chromatograms of the standard solution of the C3G, which is shown in Fig. 1a, and BH extracts, which is shown in Fig. 1b. A major peak eluted at 10.115 min of the retention time, which was identified by matching it with the retention time for the LC chromatogram of the standard stock solution, which was the C3G at 10.196 min. The BH turned out to have 16.7 mg/g (1.67%), which was based on the calibration curve of the standard stock solution.

### Effect of the BH on the cells viability in the 3T3-L1 cells

The effect of the BH on the cells viability in the 3T3-L1 adipocytes was estimated by the 3T3-L1 preadipocytes that were treated with varying concentrations, which included 0, 39, 78, 156, 313, 625, 1,250, 2,500, and 5,000  $\mu\text{g}/\text{mL}$  of the BH for 48 h, which are shown in Fig. 2. The cells viability appeared to have a decreasing pattern from 156 to 2,500  $\mu\text{g}/\text{mL}$  of the BH, which showed a cells viability that was less than 83% of the control group without the cytotoxic effect. However, 57% of the cells viability occurred at 5,000  $\mu\text{g}/\text{mL}$  concentration of the BH. An inhibitory concentration ( $\text{IC}_{50}$ ) of the BH was observed to be 5,884.82  $\mu\text{g}/\text{mL}$ . Based on this result, the following experiment was conducted by adding non-cytotoxic concentrations that included 250, 500, and 1,000  $\mu\text{g}/\text{mL}$  of the BH.

### Effect of the BH on the lipid accumulation in the 3T3-L1 adipocyte

The effect of the BH on the lipid accumulation in the mature 3T3-L1 cells was evaluated by measuring the intracellular lipid droplets that turned into a red color, which were imaged by using Oil Red O staining, which is illustrated in Fig. 3a, and the quantitated lipid accumulation was then expressed as a percent of the control group, which is shown in Fig. 3b. It was observed that 250, 500, and 1,000  $\mu\text{g}/\text{mL}$  of the BH decreased by 1.2, 1.7, and 3.7 times in a dose-dependent manner, respectively. The treatment of the BH at 500 and 1,000  $\mu\text{g}/\text{mL}$  seemed to suppress the size of the lipid accumulation in the differentiated cells. According to the quantitative data that were obtained by quantifying this red section, the lipid contents in 500 and 1,000  $\mu\text{g}/\text{mL}$  of the BH were  $58 \pm 20.32\%$  and  $26 \pm 6.31\%$  of the control cells, respectively, which is shown in Fig. 3b. In particular, the 1,000  $\mu\text{g}/\text{mL}$  of the BH was found to show a significant inhibition in the lipid accumulation as opposed to the 250 and 500  $\mu\text{g}/\text{mL}$  of the BH ( $P < 0.01$ ), which is illustrated in Fig. 3b.

### Effect of the BH on the free glycerol formation

In order to investigate whether the BH could increase the release of the glycerol in the 3T3-L1 cells or not, the intracellular glycerol concentration (nmol/protein mg) was measured. After that, it was converted to a percent of the control group, which is shown in Fig. 4. The results showed that IBMX robustly increased the free glycerol contents up to 254% of the control, whereas the treatment with the BH decreased the free glycerol formation in a concentration-dependent manner. The content of the free glycerol was 109% at 250, 110% at 500, and 71% at 1,000  $\mu\text{g}/\text{mL}$  of the BH.

### Effects of the BH on the mRNA expression of the lipogenic gene in the 3T3-L1 cells

The gene expressions of the PPAR $\gamma$ , C/EBP $\alpha$ , ADRP, AMPK $\alpha$ 1, AMPK $\alpha$ 2, ACC, SREBP1, and adiponectin, which are responsible for the molecular regulation of the adipogenesis during the adipocyte differentiation, were investigated using quantitative RT-PCR. All the mRNA levels were measured after inducing the differentiation process of the 3T3-L1 cells in the presence of the BH at concentrations of 250, 500, and 1,000  $\mu\text{g}/\text{mL}$ . The results showed that the BH suppressed the expression of the PPAR $\gamma$ , ADRP, C/EBP $\alpha$ , and ACC genes while inducing the expression of the AMPK $\alpha$ 2 compared to the control adipocytes, which is illustrated in Fig. 5. However, the mRNA expression of the SREBP1 and adiponectin was not affected in a dose dependent in the BH treatment. The PPAR $\gamma$  mRNA expressions significantly decreased, which showed  $0.81 \pm 0.06$ ,  $0.68 \pm 0.25$ ,

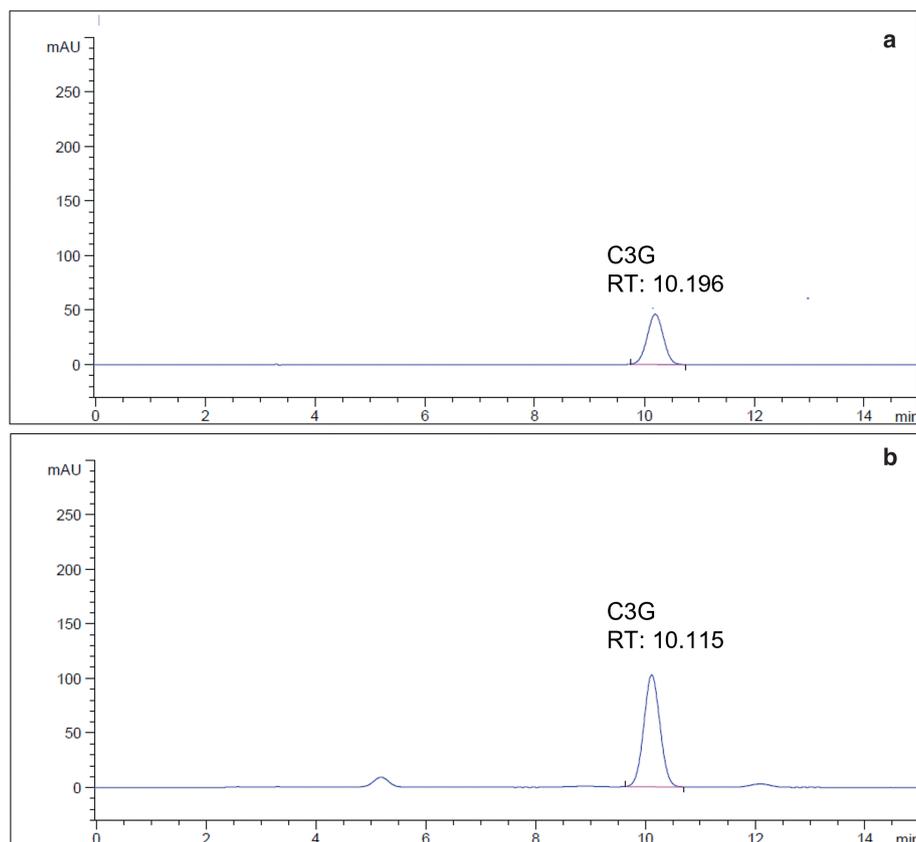


Fig. 1. The HPLC analysis of specific ingredients (C3G) of the BH extracts. (a) Standard solution and (b) BH solution.

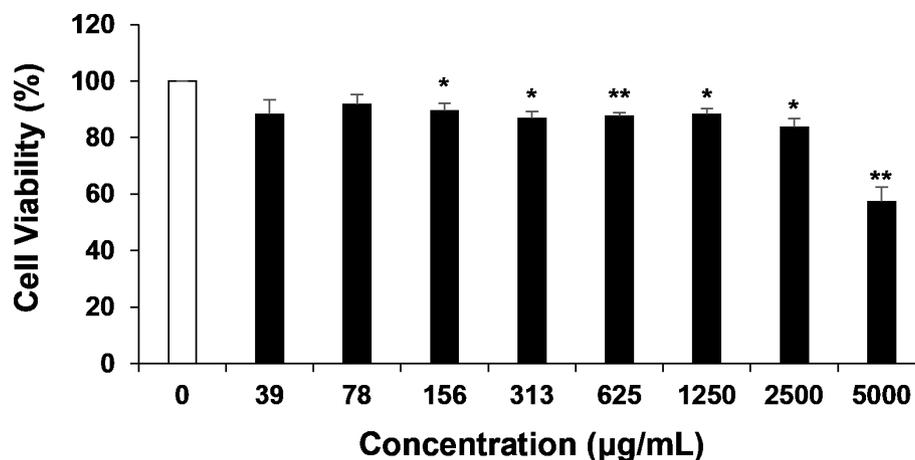
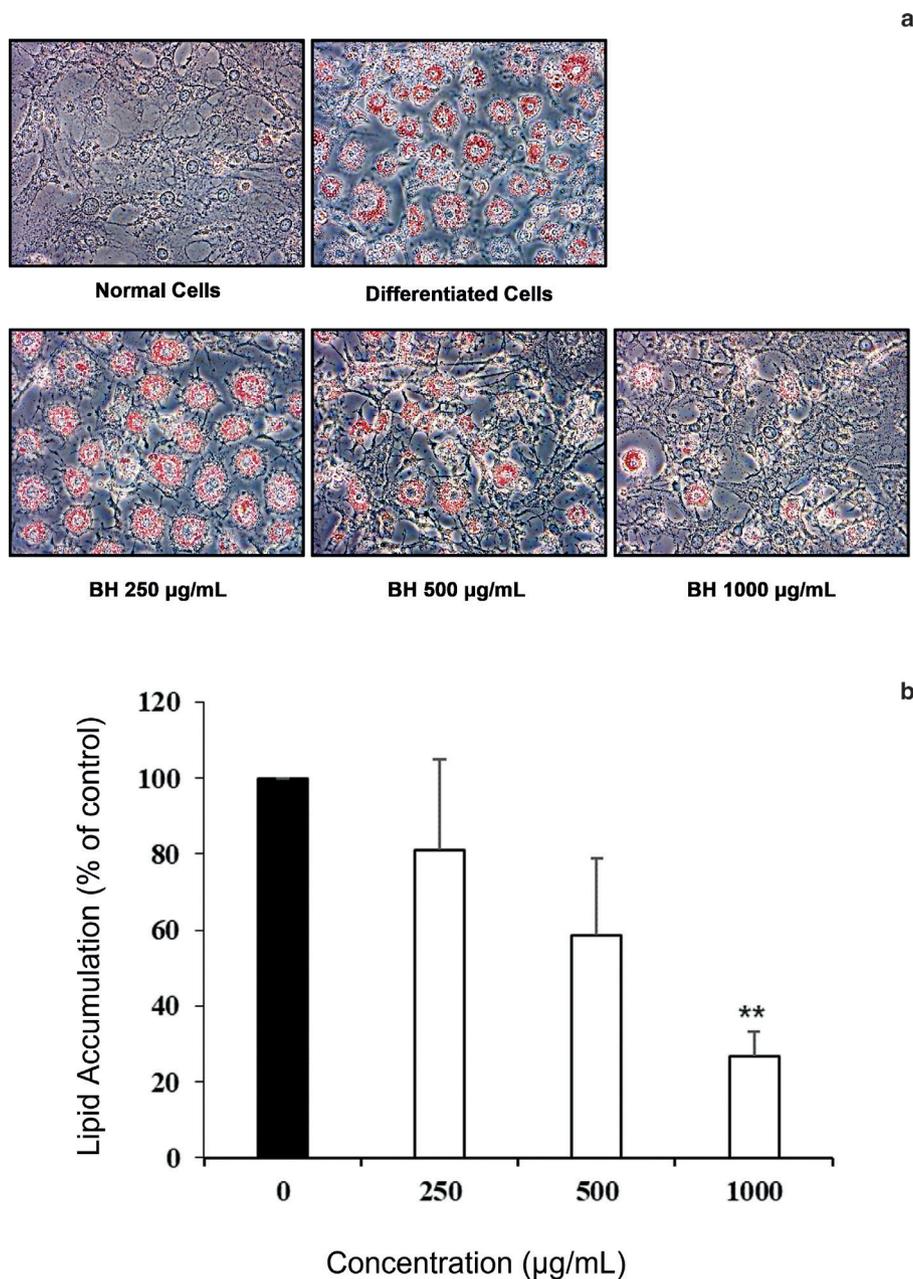


Fig. 2. The cells viability of the BH doses according to the concentration (0–5,000 µg/mL) in the 3T3-L1 cells line. The data results are shown as mean  $\pm$  SEM,  $n = 3$ . \* $P < 0.05$  and \*\* $P < 0.01$  as compared with the negative control group.

and  $0.25 \pm 0.21$ -fold in 250, 500, and 1,000 µg/mL of the BH, respectively, compared to the control. The C/EBP $\alpha$  mRNA expressions were concentration-dependently downregulated in 250, 500, and 1,000 µg/mL of the BH to  $0.79 \pm 0.08$ ,  $0.52 \pm 0.08$ , and  $0.32 \pm 0.12$ , respectively. The mRNA expression of the ADRP gene significantly decreased in the BH 1,000 µg/mL, which was 2.34-fold

lower than the control ( $P < 0.001$ ). The treatment of the BH (250, 500, and 1,000 µg/mL) upregulated the mRNA expressions of the AMPK $\alpha 1$  and AMPK $\alpha 2$  in a concentration-dependent manner. The ACC mRNA expressions were reduced with the increase in the BH concentrations, which significantly lowered at both 500 µg/mL (1.73-fold) and 1,000 µg/mL (2.90-fold) compared to



**Fig. 3.** The effects of the BH on lipid accumulation. (a) Histology of the typical 3T3-L1 adipocytes by Oil Red O staining (original magnification 200×) and (b) the quantification value of the Oil Red O stained 3T3-L1 cells. The data results are shown as mean  $\pm$  SEM,  $n = 3$ . \*\* $P < 0.01$  as compared with the negative control group.

the control. In regard to the SREBP, 250, 500, and 1,000  $\mu\text{g/mL}$  of the BH induced up to 0.9, 0.8, and 0.9-fold, respectively, compared to the control cells. In regard to the treatment with the BH at various doses, which included 250, 500, and 1,000  $\mu\text{g/mL}$ , the adiponectin was not significantly affected.

#### *Effects of the BH on the protein expression of the lipogenic gene in the 3T3-L1 cells*

The expression of the PPAR $\gamma$ 1, PPAR $\gamma$ 2, ADRP, C/EBP $\alpha$  (p42 and p30), AMPK $\alpha$ , and ACC genes at the protein

levels was further evaluated, which is shown in Fig. 6. Both the PPAR $\gamma$ 1 and PPAR $\gamma$ 2 mRNA expressions were reduced, and the BH at 1,000  $\mu\text{g/mL}$  significantly decreased to  $0.03 \pm 0.06$  and  $0.01 \pm 0.02$ -fold for the PPAR $\gamma$ 1 and PPAR $\gamma$ 2, respectively ( $P < 0.01$ ). Significant changes on the ADRP mRNA expressions were observed at 500 and 1,000  $\mu\text{g/mL}$  of BH, which showed  $0.82 \pm 0.10$  and  $0.43 \pm 0.02$ -fold lower than the control, respectively. In regard to both the C/EBP $\alpha$ (p42) and C/EBP $\alpha$ (p30) mRNA expressions, its tremendous reduction was observed at both 500 and 1,000  $\mu\text{g/mL}$  of the BH. The mRNA expressions

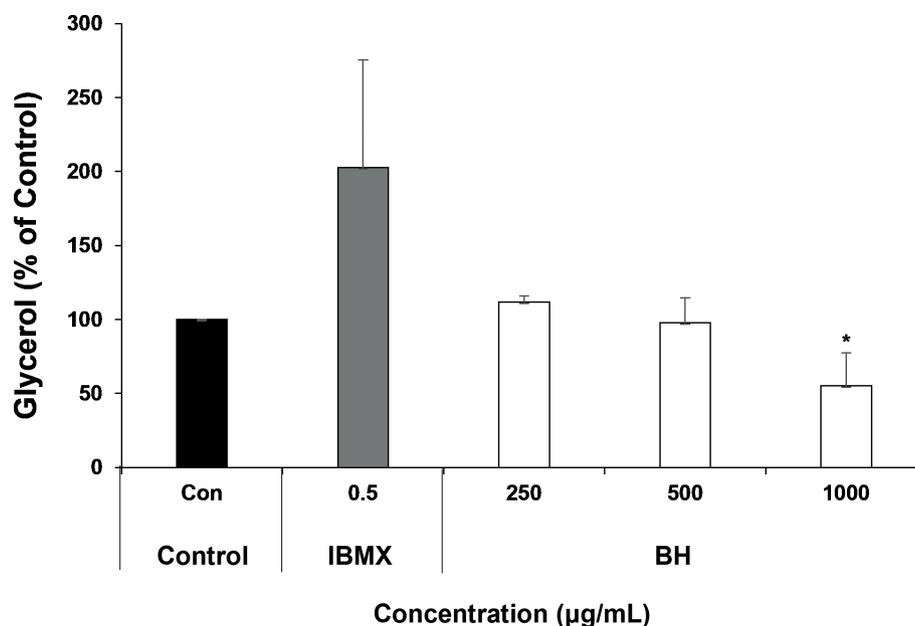


Fig. 4. Effect of the BH on the glycerol release in the 3T3-L1 cells. The data results are shown as mean  $\pm$  SEM,  $n = 3$ .

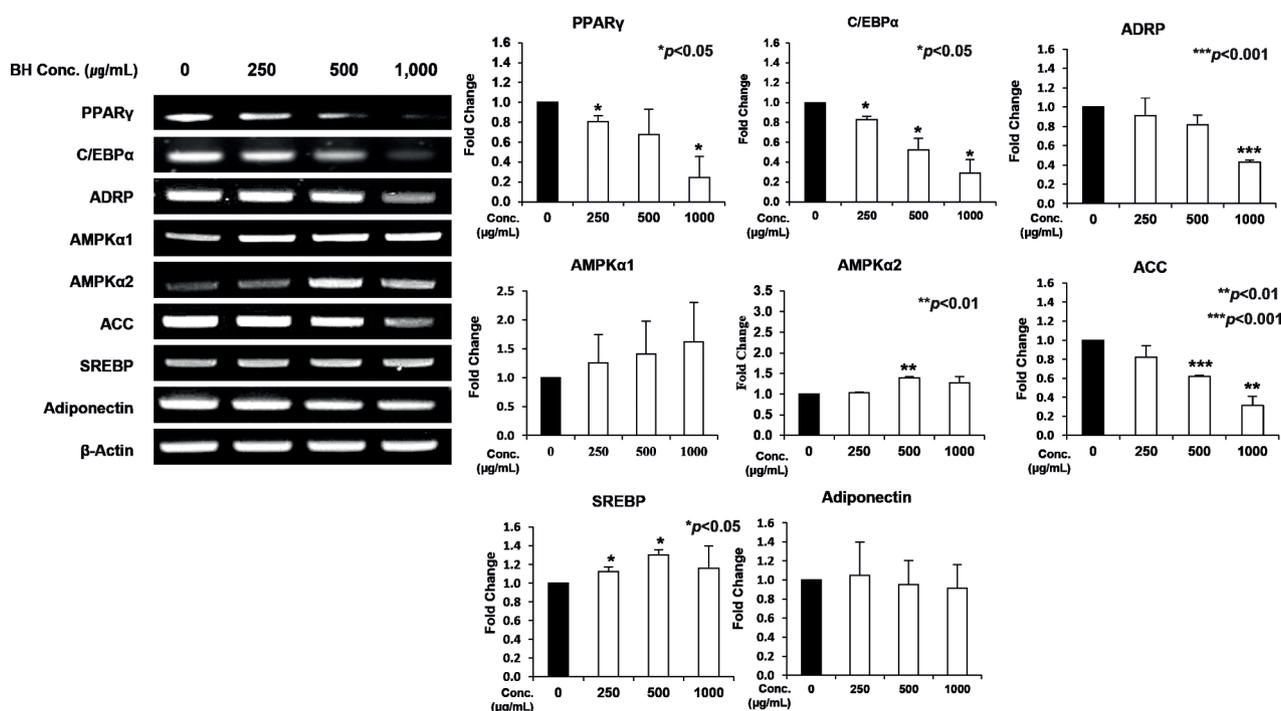
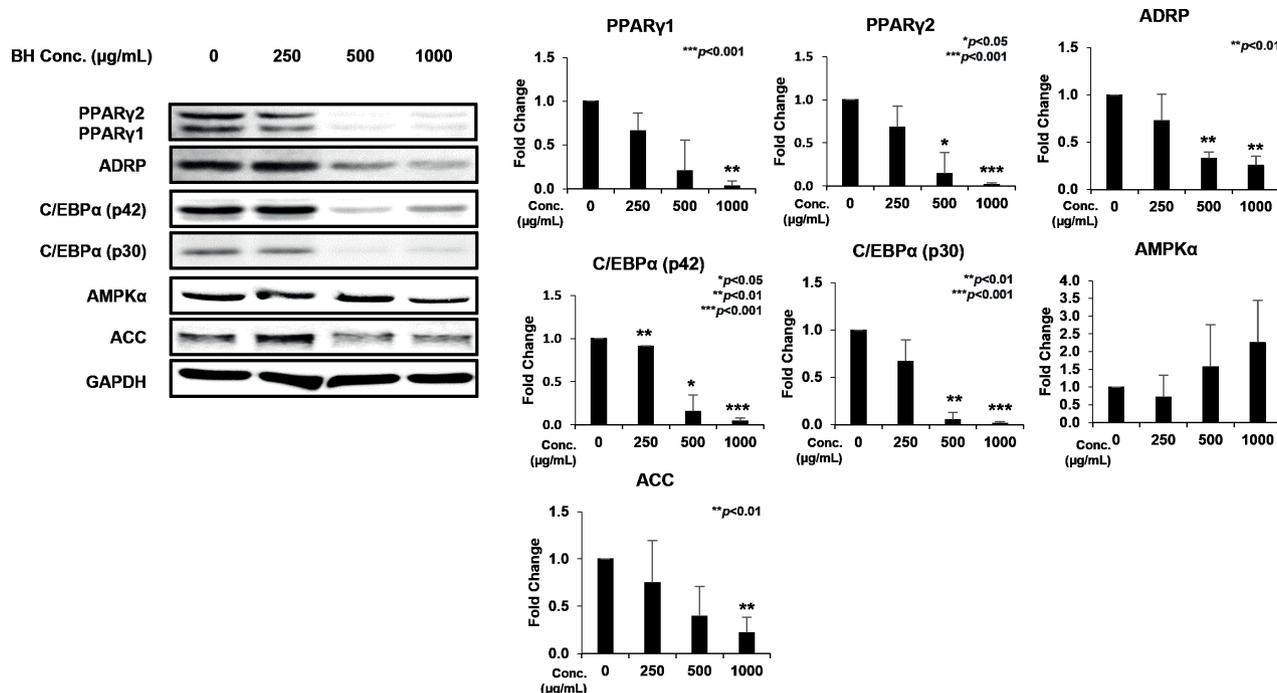


Fig. 5. Effects of the BH on the mRNA expression of the adipogenesis-related markers. The PPAR $\gamma$ , C/EBP $\alpha$ , ADRP, AMPK $\alpha$ 1, AMPK $\alpha$ 2, ACC, SREBP1, and adiponectin mRNA were determined by RT-PCR and normalized by the  $\beta$ -actin expression. The data results are shown as mean  $\pm$  SEM,  $n = 3$ . \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  as compared with the negative control.

of the AMPK $\alpha$ 2 were found to be  $1.56 \pm 1.20$  and  $2.16 \pm 1.24$ -fold of the control for 500 and 1,000  $\mu\text{g/mL}$  of the BH, respectively, which indicated it was upregulated by treating the BH, but it was not significantly effected ( $P > 0.05$ ). 1,000  $\mu\text{g/mL}$  of BH significantly downregulated the ACC mRNA expressions, which has  $0.22 \pm 0.16$ -fold compared to the control.

## Discussion

The contents of the C3G in the fresh BH and pomace were within the range of 2.3–21.8 mg/g according the raw material forms in a previous study (18). Another study found that the content of the anthocyanins from the BH ranged from 1.16 to 5.93 mg/g, and the C3G was the major one, which occupied 79–88% (10). The previous reports did



**Fig. 6.** Effects of the BH on the protein expression of the adipogenesis-related markers. The PPAR $\gamma$ 1, PPAR $\gamma$ 2, ADRP, C/EBP $\alpha$ , AMPK $\alpha$ , and ACC protein levels were measured using a western blot and normalized with a GAPDH expression. The data results are shown as mean  $\pm$  SEM,  $n = 3$ . \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  as compared with the negative control.

not show a significant decrease on the cells viability in the BH treatment up to 1,000  $\mu\text{g/mL}$  (19), which is similar to our results.

The results from our study imply that the BH could be effective in regard to preventing obesity by inhibiting the lipid accumulation, even though the size and the percentage of lipid accumulation between 500 and 1,000  $\mu\text{g/mL}$  were not correlated. A previous study reported that the BH reduced body fat, the abdominal fat density, the total cholesterol, and triglycerides under HFD mice (20). According to a recent study, the BH at 1 mg/mL reduced both adipocyte lipid contents up to 82% and the lipid droplet size (19). This study also confirmed that the BH suppressed adipogenesis by the down regulating of the transcription factors, such as PPAR $\gamma$ , C/EBP $\alpha$ , and SREBP1, so that key transcriptional genes would be influenced from the certain concentration of BH extract.

Adipocyte, which is differentiated into mature lipid cells, tends to accumulate excess triglycerides, which results in storing excess lipid. Therefore, suppressing lipid production is one way in order to prevent obesity. Meanwhile, another way is the hydrolysis of the triglycerols that proportionally release glycerol and free fatty acids from the adipocytes, which causes lipolysis (21). This study further investigated the effect of the BH on the lipolysis.

According to the previous reports, the triglyceride accumulation was reduced to 77.42% in the 1% BH supplemented group of the HFD-induced obese mice, but the

results for the free glycerol study could not be found (19). The results from the current study indicated that the treatment of the BH did not induce the lipolysis, but it inhibited the adipogenesis. Adipogenesis is a complex process that includes a cascade of transcription factors that regulate the differentiation and a gene expression network that is associated with the adipogenesis (22). The differentiation of the preadipocytes into mature adipocyte generally occurs in several phases from early to late, and it is regulated by several transcription factors (23). In order to elucidate this mechanism, this study further evaluated the expressions of the genes of the transcription factors that are related with both the adipogenesis and the lipolysis.

During the adipogenic differentiation of the 3T3-L1 preadipocyte, the late stage genes, which include the PPAR $\gamma$  and C/EBP $\alpha$ , are the most crucial lipogenic markers (24). The advent of the PPAR $\gamma$  and C/EBP $\alpha$  enhanced the expression of most of the genes that are related to the adipocyte phenotype (22, 25). It was discovered that the expression profile of the SREBP1c was similar to the PPAR $\gamma$ , which is where the overexpression of the SREBP1c in the 3T3-L1 cells increased the lipid accumulation and the adipocyte marker expression compared to the control cells (26). In contrast to the down-regulation of the SREBPs reduced lipid accumulation in the white adipocytes and the adipose tissue (27), the ADRP was upregulated in a parallel manner to the lipids that were conserved during the lipid droplet formation,

and the exogenous expression of the ADRP increased the mass of the neutral lipids and the overaccumulation of the droplets (28). The AMPK is a key regulator of the cellular and body energy balance, and the adiponectin was the hormones that were secreted by the adipose organization, which regulated the energy expenditure throughout the control glucose and lipid metabolism (29). The inhibition of the adipocyte differentiation occurred with the treatment of the anthocyanins that were isolated from the fruit of *Vitis coignetiae* Pulliat by activating the AMPK signaling pathway (30). In the current study, the BH that mainly contained the C3G significantly decreased the gene expression of the PPAR $\gamma$ , ADRP, C/EBP $\alpha$ , and ACC in the 3T3-L1 differentiation, whereas it induced the SREBP, AMPK $\alpha$ 1, and AMPK $\alpha$ 2 gene expression. This is consistent with our findings, which reduced the nuclear SREBP-1c binding in the target DNA and the mRNA expression of the lipogenic genes, which included the SREBP-1c, PPAR $\gamma$ , and C/EBP $\alpha$ , which the ACC was dose-dependently reduced (27). However, this study did not observe the down-regulation of the SREBP1. Overall, the results from this study suggest that the anti-obesity mechanism of the BH could prevent the adipogenesis by activating the AMPK as well as down-regulating the PPAR $\gamma$ , ADRP, C/EBP $\alpha$ , and ACC gene expressions.

PPAR $\gamma$  can be divided into two protein isomers, such as PPAR $\gamma$ 1 and PPAR $\gamma$ 2. PPAR $\gamma$ 1 is expressed at the main levels by the adipocytes along with the pre-adipocytes, whereas PPAR $\gamma$ 2 is the explicit protein on the adipocyte (22). The protein level of PPAR $\gamma$  upregulated genes working on the lipid absorption and storage from adipose tissue was reduced (31), which is similar to our results. The ADRP, which is an important role in the qualitative tissue of the intracellular lipid droplets, was downregulated (32). Several members of the C/EBP family, which include C/EBP $\alpha$  (p42 and p30) and C/EBP $\beta$ , tended to be over-expressed, and they induced the differentiation of the mature fat cells (26, 33). Our results confirmed that the protein levels of C/EBP $\alpha$  (p42 and p30) in the adiposity cells were reduced by the BH, which lead to the adipogenesis inhibition. This is consistent with the results from the level of mRNA, which is shown in Figs. 5 and 6. The previous studies showed that the activation of the AMPK in the adipocytes improved the insulin sensitivity and the fat accumulation (34). The activation of the AMPK was informed in order to suppress the adipogenesis by inhibiting the ACC in both the fatty acid oxidation and the fatty acid synthesis (35). The protein expression levels in the AMPK were matched with the gene mRNA level in the AMPK, which was found to increase in a dose-dependent manner, whereas the ACC protein expression levels were downregulated, which is illustrated in Fig. 6. These results suggest that the BH could regulate a set of multifold transcriptional factors, which are associated with the adipogenesis.

## Conclusions

In this study, our purpose was to investigate the effect of BH that is rich in C3G on the lipid accumulation and formation of free glycerol. BH treatments at various concentrations did not affect the free glycerol content, but they significantly inhibited the lipid accumulation. The BH extracts at 500 and 1,000  $\mu$ g/mL were further elucidated by the measuring transcription factors of the signaling pathways for both the adipogenesis and lipolysis. Treatment of the BH inhibited the expression of the adipogenesis in the related genes, which included the PPAR $\gamma$ , ADRP, and ACC proteins in a dose-dependent manner. It simultaneously induced the AMPK1, AMPK2, and SREBP expressions. The PPAR $\gamma$ 1, PPAR $\gamma$ 2, ADRP, C/EBP $\alpha$ , and ACC were consistently downregulated except for AMPK $\alpha$  in the protein levels. Our data suggest that the treatment of the BH could not induce lipolysis, but it could inhibit the adipogenesis by attributing to modulating the transcriptional factors in the signaling pathways of the adipogenesis. In order to map the proteins that were involved in the adipocyte differentiation, several genome-array studies that used *in vitro* models of adipocyte differentiation were applied. However, more studies are required for use of the BH that contains C3G as a biomolecule in order to prevent obesity that is linked to metabolic disorders.

## Authors' contributions

Hyun Jeong Lee: writing – original draft and formal analysis. Eun-Hye Choi: writing – original draft. Yoon-Seok Chun: investigation and resources. Jong-Kyu Kim: investigation and project administration. Jung-Ok Lee: funding acquisition and project administration. Jin-Seol Rhee: methodology and data curation. Youn-Bi Jang: methodology and data curation. Tae-Gyu Lim: validation and supervision. Soon-Mi Shim: supervision and writing – review and editing.

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The authors declare that there are no conflicts of interest. The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

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