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Article

# Polyphenols from blue honeysuckle (*Lonicera caerulea* L. var. *edulis*) berry inhibit lipid accumulation in adipocytes by suppressing lipogenesis

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**Abstract:** Polyphenols have been shown to possess outstanding anti-obesity properties. In this study, the effect of blue honeysuckle berry extract (BHBE) with high polyphenol content on lipid accumulation in adipocytes and the underlying mechanism were investigated for the first time. Composition analysis demonstrated that flavonoids (mainly flavonols and anthocyanins) were the primary polyphenols in BHBE, which contributed to its biological functions. The results of Oil Red O staining combined with triglyceride (TG) content determination showed that BHBE exhibited an obvious inhibitory effect on intracellular lipid accumulation in a dose-dependent manner. BHBE also reduced the protein level of fatty acid synthase (FAS) and increased the phosphorylation level of acetyl-CoA carboxylase (ACC), indicating that lipogenesis was suppressed by BHBE treatment. Moreover, BHBE was found to significantly promote the phosphorylation of AMP-activated protein kinase (AMPK) and further reduce the expression of key transcription factors (PPAR $\gamma$ , C/EBP $\alpha$ , and SREBP-1c) that regulate lipogenesis. In addition, the expression of beige adipocyte markers (*Tmem26* and *Cd137*) and uncoupling protein 1 (UCP1) was increased in BHBE-treated adipocytes. In summary, we consider that BHBE inhibits lipid accumulation in adipocytes by suppressing lipogenesis as well as by promoting beiging of adipocytes. These results support blue honeysuckle berry as a candidate functional food against obesity.

**Keywords:** blue honeysuckle berry; polyphenols; flavonoids; lipid accumulation; lipogenesis

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## 1. Introduction

Obesity, characterized by adipose tissue expansion, is a chronic disease resulting from energy metabolism imbalance [1]. In the past few decades, obesity has become a serious public health problem and is still spreading rapidly across the world [2]. Studies show that obese people are at an increased risk of numerous diseases such as type 2 diabetes, hypertension, atherosclerosis, and hepatic steatosis [3]. However, most anti-obesity drugs have been withdrawn because of their side effects [4]. Currently, the global anti-obesity strategies, beyond exercise, focus on diet improvement. As we know, there is a close relationship between diet and obesity. Nutrition researches have confirmed the positive effects of diet enriched with healthy foods against obesity [5].

Blue honeysuckle berry, the fruit of *Lonicera caerulea* L. var. *edulis*, is widely harvested in north China, Russia, and Japan [6]. According to some records, the berry has been used in folk medicine for the treatment of stomachache and infectious diseases in the Russian Far East for centuries [7]. There is also evidence of its medicinal value for improving diabetes mellitus, hypertension, and gastrointestinal disorders [8,9]. Due to

these health benefits, blue honeysuckle berry, like avocado, buckwheat, and strawberry, is categorized as a “superfood” and has been used to make processed foods and beverages. Previous studies have shown that blue honeysuckle berry contains vitamins, microelements, and bioactive compounds which contribute to its various biological functions, including anti-oxidation and anti-inflammation [10]. Moreover, the berry is considered to be one of the richest sources of polyphenols, especially anthocyanins, among fruits [11]. Although polyphenols have been shown to possess the potential to regulate lipid metabolism [12], the effects of blue honeysuckle berry on obesity have not been investigated to date.

At the cellular level, obesity results from the storage of excess energy in adipocytes as lipids. Several studies suggest that it is possible to inhibit the development of obesity by intervening in lipogenesis [13,14]. Adipogenesis is the essential process for the formation of lipid-laden mature adipocytes, in which lipogenesis begins at the middle stage and accelerates in the terminal stage. This process is regulated by a cascade of adipogenic transcriptional factors including peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), CCAAT/enhancer binding protein alpha (C/EBP $\alpha$ ), and sterol regulatory element binding protein-1c (SREBP-1c), which are considered to be the determinants of lipogenesis [15]. As a crucial energy sensor, AMPK is involved in cellular energy homeostasis by regulating glucose and lipid metabolism. AMPK activation can promote cellular glucose uptake, enhance fatty acid oxidation, and suppress lipogenesis through short-term regulation of protein phosphorylation or long-term effects on gene expression [16]. Studies indicate that AMPK agonists reduce the expression of lipogenic enzymes in adipocytes [17]. Thus, AMPK has emerged as a promising target for lipid metabolic disorders including obesity.

In the present study, *in vitro* experiments regarding the effect of BHBE on lipid accumulation in adipocytes were carried out using 3T3-L1 cells, which represented a well-characterized model of adipogenesis. Furthermore, in order to explore the underlying mechanism, we investigated the effects of BHBE on AMPK and its downstream adipogenic transcriptional factors that regulate lipogenesis, as well as on beige adipocyte markers.

## 2. Materials and Methods

### 2.1. Materials and reagents

Mature blue honeysuckle berries were provided by Heilongjiang Academy of Agricultural Sciences (Harbin, China). 3T3-L1 cells were obtained from National Infrastructure of Cell Line Resource (Beijing, China). Compound C (CC) was purchased from MedChemExpress (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Thermo Fisher Scientific (Beijing, China). Calf serum (CS) and fetal bovine serum (FBS) were purchased from Tianhang Biotechnology (Hangzhou, China). 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone (DEX) were purchased from Sigma-Aldrich (Shanghai, China). Insulin was purchased from Wanbang Biopharmaceuticals (Xuzhou, China). All other chemicals (analytical grade) were purchased from Kermel Chemical Reagent (Tianjin, China). Deionized water was produced by an ELGA PURELAB Flex 2 Polisher system (Shanghai, China).

### 2.2. BHBE preparation

Twenty grams (fresh weight, FW) of blue honeysuckle berries were crushed and extracted with 400 mL of 70% ethanol solution in an ultrasonic bath (KQ-500DE, Kunshan Ultrasonic Instruments Co., Ltd., China). The extraction was performed at room temperature for 2 hrs. Then, the extract was filtered, and the solution was concentrated to dryness using a vacuum rotary evaporator (RE-52AA, Yarong Biochemistry Instrument Factory, China). Afterwards, the extract was collected and stored at -20 °C.

### 2.3. Determination of total polyphenol content

The total polyphenol content of BHBE was determined according to the Folin-Ciocalteu colorimetric method. In brief, 100  $\mu\text{L}$  of Folin-Ciocalteu reagent (Yuanye Bio-Technology Co., Ltd., China) was added to 20  $\mu\text{L}$  of sample solution (10 mg/mL), and the mixture was incubated in dark for 5 min. Then, 80  $\mu\text{L}$  of 7.5% sodium carbonate solution was added to the mixture. After incubation in dark for 60 min, the absorbance of the mixture was measured at 765 nm using a microplate reader (Infinite 200 PRO, Tecan, Switzerland). A standard curve was established using gallic acid as the standard substance with concentrations range from 0 to 100  $\mu\text{g/mL}$ , and the result was expressed in gallic acid equivalent per gram of the test sample (mg GAE/g BHBE).

### 2.4. Analysis of polyphenolic compounds

The polyphenols in BHBE were analyzed using an UHPLC system (1290 Infinity II, Agilent Technologies, USA) coupled with Orbitrap MS (Q Exactive Focus, Thermo Fisher Scientific, USA). The chromatographic separation was performed on an ACQUITY UPLC HSS T3 column (2.1 mm  $\times$  100 mm, 1.8  $\mu\text{m}$ , Waters, USA). The column temperature was kept at 25  $^{\circ}\text{C}$  and the injection volume was 2  $\mu\text{L}$ . The mobile phase was 0.1% formic acid in water (A) and acetonitrile (B). The elution gradient was set as follows: 0-1 min, 1% B; 1-8 min, 1-99% B; 8-10 min, 99% B; 10-10.1 min, 99-1% B; 10.1-12 min, 1% B. The flow rate was maintained at 0.5 mL/min. The mass spectrometer was used to acquire MS/MS spectra on an information-dependent basis (IDA). In this mode, the acquisition software (Xcalibur 4.0.27, Thermo) continuously evaluates the full scan survey MS data as it collects and triggers the acquisition of MS/MS spectra depending on preselected criteria. ESI source conditions were set as follows: sheath gas flow rate as 45 Arb, aux gas flow rate as 15 Arb, capillary temperature 320  $^{\circ}\text{C}$ , spray voltage as 3.8 kV (positive) or -3.1 kV (negative), collision energy as 20/40/60 eV in NCE model.

### 2.5. 3T3-L1 cell culture and differentiation

3T3-L1 cells were grown in DMEM supplemented with 10% CS at 37  $^{\circ}\text{C}$  in 5%  $\text{CO}_2$  atmosphere. For differentiation, 2 days post confluence (day 0), the cells were incubated in differentiation medium (DMEM supplemented with 10% FBS, 1  $\mu\text{M}$  dexamethasone, 0.5 mM IBMX and 5  $\mu\text{g/mL}$  insulin). After 2 days (day 2), the cells were exposed to maintenance medium (DMEM supplemented with 10% FBS and 5  $\mu\text{g/mL}$  insulin), and the medium was refreshed every 2 days until experiments were performed on day 8. 3T3-L1 cells were treated with or without BHBE from day 2 to day 8.

### 2.6. Cell viability evaluation

The viability of 3T3-L1 cells was determined by CCK-8 (Dojindo Chemistry Technology Co., Ltd., China) assay. The cells were seeded in 96-well plates at an initial density of  $1 \times 10^5$  cells/well. When confluence reached 60–70%, the cells were treated with BHBE at different concentrations and incubated for 48 hrs. Afterwards, 10  $\mu\text{L}$  CCK-8 working solution was added into each well, and the cells were incubated at 37  $^{\circ}\text{C}$  for 2 hrs. The absorbance was measured at 450 nm using the microplate reader. The cell viability was calculated as follow:

$$\text{cell viability (\%)} = (A_s - A_b) / (A_c - A_b) \times 100\%$$

where  $A_s$ ,  $A_b$ , and  $A_c$  are the absorbance of sample, blank, and negative control, respectively.

### 2.7. Oil Red O staining

Lipid accumulation in adipocytes was characterized using Oil Red O staining kit (Solarbio Technology Co., Ltd., China). Cells were washed with PBS for twice and fixed with ORO fixative solution for 30 min. Then, the cells were washed with 60% isopropa-

nol and stained with freshly prepared ORO stain solution for 15 min. Thereafter, the cells were washed with deionized water until there was no residual staining solution. The imaging of the Oil Red O staining were performed using a microscope (DMI 4000B, Leica, Germany), and the stained lipid droplets, dissolved in isopropanol, were quantified by measuring the absorbance at 510 nm using the microplate reader.

### 2.8. Determination of TG content

TG content of adipocytes was determined using TG assay kit (Nanjing Jiancheng Bioengineering Institute, China). Cells were digested and centrifuged at 1000 rpm for 10 min. After removing the supernatant, the cells were sonicated in 0.2 mL PBS. Then, 1 mL working solution was added, and the cells were incubated at 37 °C for 30 min. The absorbance was measured at 510 nm using the microplate reader. TGs at a concentration of 2.26 mM were used as the standard substance, and the results were expressed as triglyceride per gram of cellular protein (mmol/g prot). The TG content was calculated as follows:

$$\text{TG content (\%)} = (A_s - A_b) / (A_c - A_b) \times 2.26 / C_p$$

where  $A_s$ ,  $A_b$ , and  $A_c$  are the absorbance of sample, blank, and positive control, respectively, and  $C_p$  is the protein concentration of sample.

### 2.9. RNA extraction, cDNA synthesis and real-time PCR

Total RNA was extracted from cells using RNA-easy Isolation Reagent (Vazyme, China) according to the manufacturer's protocol, and quantified by a Microvolume Spectrophotometers (NanoDrop 2000C, Thermo Fisher Scientific, USA). The cDNA was synthesized from 1 µg of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, China). The reaction mixture included 10 ng of cDNA, 0.4 µM of forward and reverse primers, and 10 µL of 2×AceQ Universal SYBR Master Mix (Vazyme, China). The expressions of target genes were detected using a Real-Time PCR Detection System (CFX96 Touch, Bio-Rad, USA). The cycling conditions were applied: 95 °C for 5 min; 39 cycles of 95 °C for 10 s and 60 °C for 30 s; and 65 °C for 5 s. Results were normalized using *Actb* as the reference. The primer sequences of target genes were listed in Table 1.

**Table 1.** The primer sequences of target genes for real-time PCR.

Gene	Sequence *	Accession
<i>Actb</i>	F: CAGATGCCACTACAGCAGC	NM_001243262
	R: CCTGCCGCTGCCATAGAAG	
<i>Lipe</i>	F: TGGCACACCATTTGACCTG	NM_010719
	R: TTGCGGTTAGAAGCCACATAG	
<i>Pnpla2</i>	F: GGGTGGCTATGTGGATGG	NM_001163689
	R: CTCTGCCTGAGAATGGGG	
<i>Tmem26</i>	F: TCCTGTTGCATTCCCTGGTC	NM_177794
	R: GCCGGAGAAAGCCATTGT	
<i>Cd137</i>	F: AGTGGGCTGTGAGAAGGTG	NM_011612
	R: ACTCCGCGTTGTGGGTAGA	
<i>Eva1</i>	F: GATGCGCTAACTGTGACGTG	NM_007962
	R: GGTCTTTGAACCGTCCGCT	
<i>Ebf3</i>	F: CAGGCCATCGTGTATGAAGG	NM_010096
	R: GTGTCTCGTTTCTATTGCCACA	

\* F: forward sequence, R: reverse sequence.

### 2.10. Western blot analysis

Cells were lysed with RIPA buffer containing 2% protease and phosphatase inhibitor cocktail (Beyotime, China) on ice for 30 min. Then, the lysates were centrifuged at

12000 rpm for 15 min, and the supernatants were collected. The protein concentration was quantified using BCA protein assay kit (Beyotime, China). The same amount of proteins (25  $\mu$ g) were separated by SDS-PAGE gel electrophoresis and transferred to PVDF membranes (Millipore, USA). The membranes were blocked in 5% skim milk for 1 hr and then incubated with the primary antibodies against FAS, ACC, p-ACC (Ser79), AMPK $\alpha$ , p-AMPK $\alpha$  (Thr172), PKC $\alpha$ , PPAR $\gamma$ , C/EBP $\alpha$ , UCP1 (Cell Signaling Technology, USA), SREBP-1c,  $\alpha$ -Tubulin and GAPDH (ZSGB-BIO, China) at 4 °C overnight. Afterwards, the membranes were washed and incubated with HRP conjugated secondary antibodies (ZSGB-BIO, China) at room temperature for 1 hr. The target bands were visualized using a chemiluminescence imaging system (5200, Tanon, China) combined with BeyoECL Plus kit (Beyotime, China) and quantified by Image J software (NIH, USA).

### 2.11. Statistical analysis

All data were expressed as means  $\pm$  SD from three independent experiments and analyzed by SPSS v19.0 software. Differences between groups were examined by one-way analysis of variance, followed by Tukey's multiple comparison test, and  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. The main polyphenols in BHBE

According to the test results, the total polyphenol content of BHBE was  $239.73 \pm 22.38$  mg GAE/g. Moreover, composition analysis showed that chlorogenic acid was the most abundant among the polyphenolic compounds detected; however, flavonoids, including flavonols and anthocyanins, were the primary type of polyphenols in BHBE (Table S1). The top 10 polyphenolic compounds, which account for 90.65% of the total relative content of polyphenols detected, are listed in Table 2.

**Table 2.** The main polyphenolic compounds detected in BHBE.

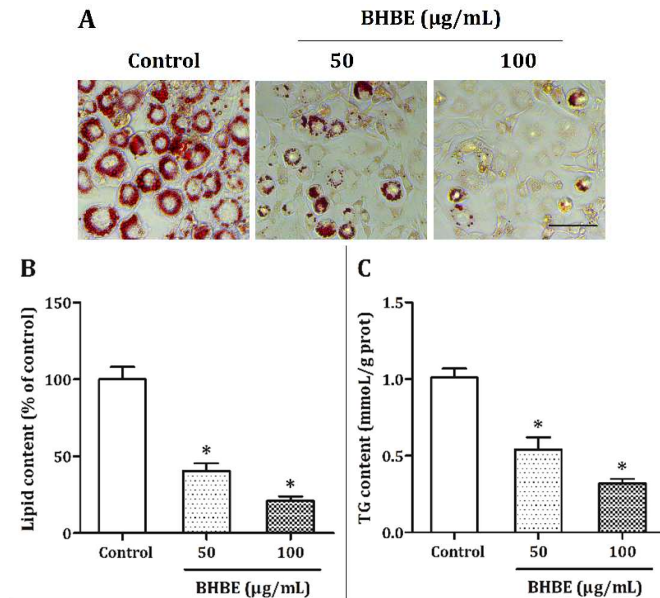
No.	Compound	Retention time (s)	m/z	Relative content (%)
1	Chlorogenic acid	146.34	353.0874	14.98
2	Cyanidin-3-glucoside	443.77	447.0928	14.53
3	Quercetin	274.44	301.0344	14.27
4	Astragalin	180.62	449.1063	12.36
5	Peonidin-3-glucoside	191.54	463.1227	11.98
6	Kaempferol	180.87	287.0533	10.61
7	Isorhamnetin	188.58	315.0509	4.53
8	Biorobin	181.12	595.1632	3.60
9	Panasenoside	163.11	611.1572	2.12
10	Peonidin-3-sophoroside	173.22	625.1730	1.68

### 3.2. BHBE inhibits lipid accumulation in adipocytes

CCK-8 assay was carried out to evaluate the cytotoxicity of BHBE, and the results showed that concentrations of BHBE up to 100  $\mu$ g/mL had no significant effect on the viability of 3T3-L1 cells (data not shown). To investigate the effect of BHBE on lipid accumulation in adipocytes, the intracellular lipid droplets were stained by Oil Red O and then quantified. It could be observed from Figure 1A that over 90% of 3T3-L1 cells were differentiated into mature adipocytes rich in lipid droplets, while BHBE caused an obvious decrease in both the number and size of lipid droplets with the increase of concentration. According to the quantified results, compared with the control, BHBE at concentrations of 50 and 100  $\mu$ g/mL reduced the intracellular lipid contents by 59.64% and 81.03%, respectively (Figure 1B). Coordinated with the staining assay, intracellular TG content was further determined. As shown in Figure 1C, the TG content of mature



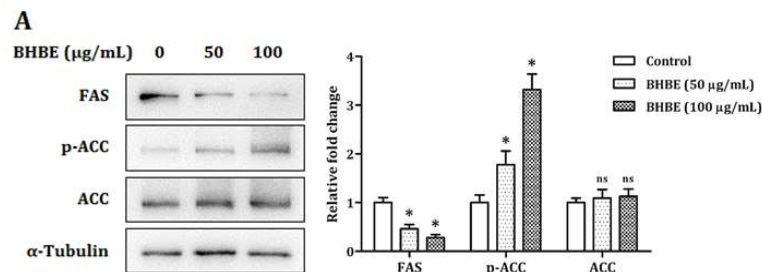
adipocytes was  $1.01 \pm 0.12$  mmol/g prot, while that of adipocytes treated with increasing concentrations of BHBE decreased to  $0.54 \pm 0.08$  and  $0.32 \pm 0.06$  mmol/g prot. These results indicated that BHBE significantly inhibited lipid accumulation in adipocytes in a dose-dependent manner.

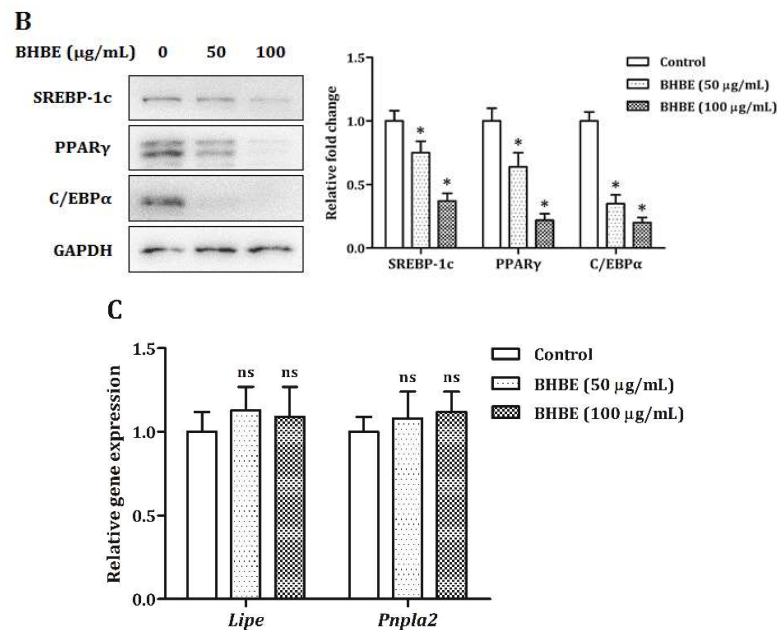


**Figure 1.** Effects of BHBE on lipid accumulation in adipocytes. (A) Microscopic images of intracellular lipid droplets stained with Oil Red O, scale: 100  $\mu\text{m}$ ; (B) quantified results of Oil Red O staining; (C) TG content of adipocytes. Data are expressed as mean  $\pm$  SD (n = 3). \*(p < 0.05) vs. Control.

### 3.3. BHBE suppresses lipogenesis in adipocytes

To explore the mechanism of BHBE inhibiting lipid accumulation in adipocytes, the effects of BHBE on the protein expression of ACC and FAS, the rate-limiting enzymes of lipogenesis, were determined by western blot analysis. As expected, BHBE significantly reduced the protein level of FAS and increased the phosphorylation level of ACC (Figure 2A), which indicated that BHBE suppressed lipogenesis. Furthermore, BHBE caused a significant decrease in the protein expression of SREBP-1c, PPAR $\gamma$ , and C/EBP $\alpha$  (Figure 2B). The present results suggested that BHBE suppressed lipogenesis in adipocytes at the transcriptional level. Additionally, we found that the gene expression of *Lipe* and *Pnpla2*, the major lipolytic enzymes, was not affected by BHBE treatment, indicating that luteolin had no significant effect on lipolysis (Figure 2C).

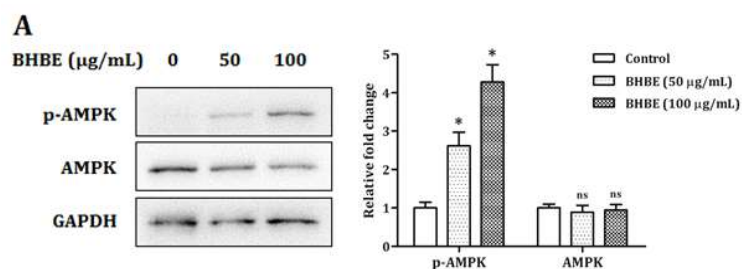


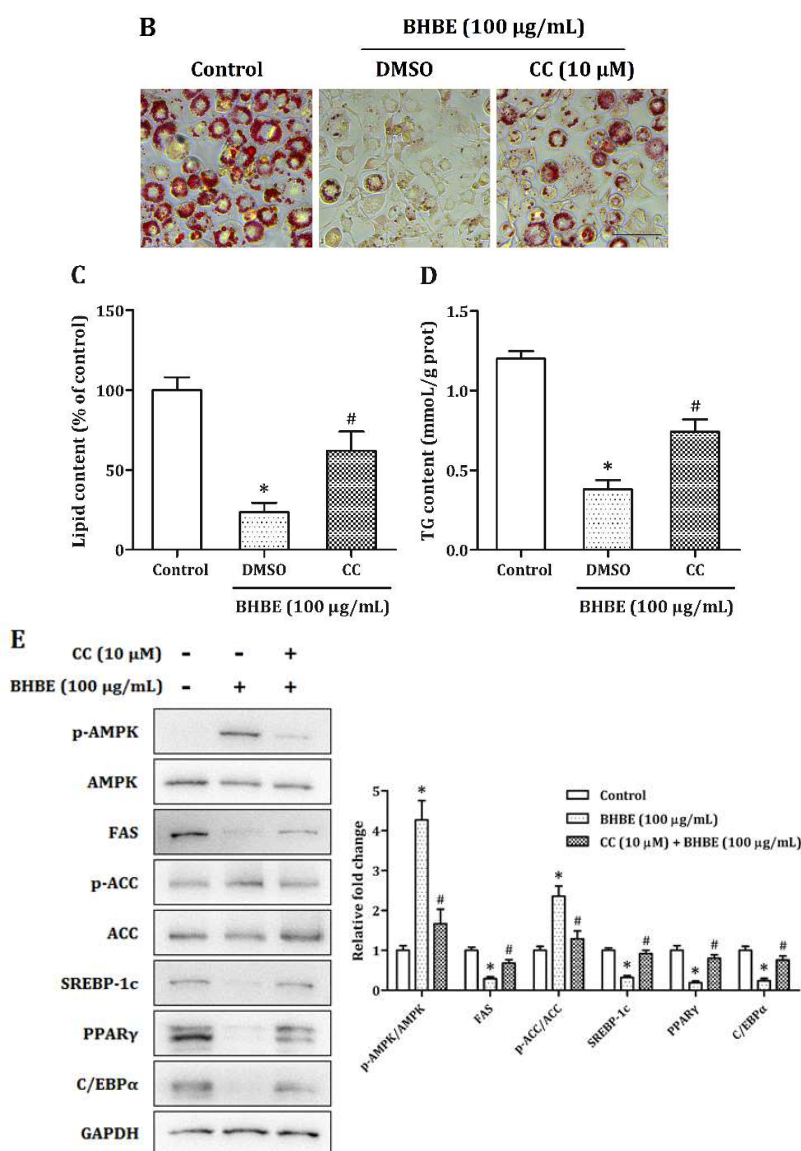


**Figure 2.** Effects of BHBE on lipogenesis and lipolysis of adipocytes. (A) Representative immunoblots and the relative protein levels of FAS, p-ACC, and ACC; (B) representative immunoblots and the relative protein levels of SREBP-1c, PPAR $\gamma$ , and C/EBP $\alpha$ ; (C) the relative mRNA levels of *Lipe* and *Pnpla2*. Data are expressed as mean  $\pm$  SD (n = 3). \*(p < 0.05) vs. Control; ns (not significant) vs. Control.

#### 3.4. BHBE suppresses lipogenesis through AMPK activation

As an energy metabolism sensor, we hypothesize that AMPK plays a central role in the regulation of lipogenesis. As expected, BHBE significantly increased the phosphorylation level of AMPK without affecting the total protein level of AMPK (Figure 3A). To investigate whether the inhibitory effect of BHBE on lipogenesis is AMPK signaling dependent, CC (10  $\mu\text{M}$ ), a selective AMPK inhibitor, was used to pretreat the cells before BHBE treatment. Oil Red O staining demonstrated that the decrease in both the number and size of lipid droplets caused by BHBE was attenuated by CC (Figure 3B). The quantified result showed that CC pretreatment led to a significant increase in the relative lipid content which was 41.65% higher than that of adipocytes treated with BHBE only (Figure 3C). Moreover, the result of TG content determination showed a similar trend with that of Oil Red O staining (Figure 3D). Western blot analysis indicated that CC effectively inhibited the increase in the phosphorylation level of AMPK caused by BHBE. Moreover, CC reversed the effects of BHBE on the protein expression of PPAR $\gamma$ , C/EBP $\alpha$ , and SREBP-1c, as well as on FAS and p-ACC (Figure 3E). Additionally, PKC, another upstream regulator of SREBP-1c, was not detected in the present study (data not shown). These results indicated that BHBE suppressed lipogenesis in adipocytes, at least in part, through AMPK activation.



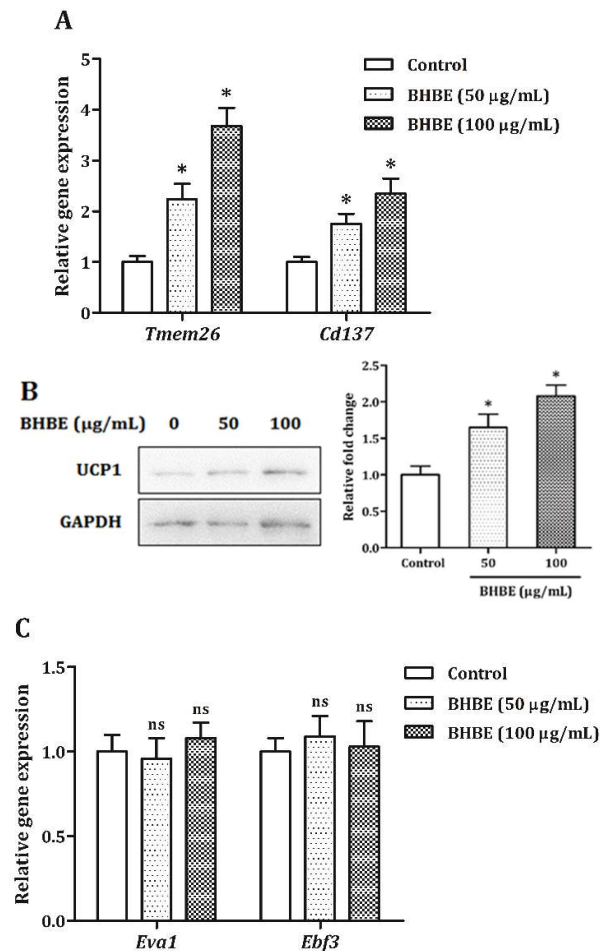


**Figure 3.** Effect of BHBE on lipogenesis is AMPK activation-dependent. (A) Representative immunoblots and the relative protein levels of p-AMPK and AMPK; (B) microscopic images of intracellular lipid droplets stained with Oil Red O, scale: 100 µm; (C) quantified results of Oil Red O staining; (D) TG content of adipocytes; (E) representative immunoblots and the relative protein levels of p-AMPK, AMPK, FAS, p-ACC, ACC, SREBP-1c, PPAR $\gamma$ , and C/EBP $\alpha$ . Data are expressed as mean  $\pm$  SD (n = 3). \* (p < 0.05) vs. Control; ns (not significant) vs. Control; # (p < 0.05) vs. BHBE treatment group.

### 3.5. BHBE increases the expression of beige adipocyte markers

In addition to suppressing lipogenesis, the effects of BHBE on the thermogenic potential of adipocytes were determined. As shown in Figure 4A, compared with the control, BHBE significantly increased the gene expression of *Tmem26* and *Cd137*. Moreover, the protein expression of UCP1 was increased in BHBE-treated adipocytes (Figure 4B). Additionally, we found that the gene expression of *Eva1* and *Ebf3*, the brown adipocyte markers, was not significantly affected by BHBE treatment (Figure 4C). These results suggested that BHBE had the potential to promote beiging rather than browning of adipocytes, which might play a role in its inhibition of intracellular lipid accumulation through thermogenesis.





**Figure 4.** Effects of BHBE on the expression of beige adipocyte markers and brown adipocyte markers. (A) The relative mRNA levels of *Tmem26* and *Cd137*; (B) representative immunoblots and the relative protein level of UCP1; (C) the relative mRNA levels of *Eva1* and *Ebf3*. Data are expressed as mean  $\pm$  SD (n = 3). \*(p < 0.05) vs. Control; ns (not significant) vs. Control.

#### 4. Discussion

Polyphenols are among the most abundant bioactive compounds in common foods and have been proven to possess the potential to inhibit the development of obesity. Several studies have identified the anti-obesity effects of foods rich in polyphenols, such as cranberry and pomegranate [18,19]. In this study, the extraction of polyphenols from blue honeysuckle berry was carried out. Notably, the total polyphenol content of blue honeysuckle berry (7.39 mg GAE/g FW) is much higher than that reported for blueberry (2.74 mg GAE/g FW). Bakowska-Barczak et al. found that blue honeysuckle berry contains the highest amount of polyphenols (11.11 mg/g) among all the berries grown in Canada, including bilberry (7.78 mg/g), raspberry (6.38 mg/g), and strawberry (4.37 mg/g) [20]. Raudsepp et al. also reported that blue honeysuckle berry contains twice as many polyphenols as black currant [21]. According to available research, blue honeysuckle berry is one of the richest sources of polyphenols among edible berries [22].

The results of composition analysis demonstrate that flavonoids are the main types of polyphenols in BHBE. As a large class of secondary metabolites, flavonoids are widely distributed in plants that exhibit various bioactivities. It was observed that quercetin, kaempferol, isorhamnetin, and their glycosides, belonging to flavonols, accounted for a

high proportion (46.29%) of the polyphenols detected. Seo et al. reported that quercetin was able to reduce the expression of PPAR $\gamma$ , C/EBP $\alpha$ , FABP4, and triglyceride-synthesis enzymes, which led to an anti-obesity effect in obese mice [23]. Moreover, both kaempferol and isorhamnetin have been shown to inhibit adipogenesis of adipocytes by causing a decrease in the expression of PPAR $\gamma$  and C/EBP $\alpha$  [24,25]. According to previous studies, blue honeysuckle berry contains a variety of anthocyanins, among which cyanidin-3-glucoside is the main one (accounting for over 80% of total anthocyanins) as well as the predominant polyphenol in blue honeysuckle berry, and its content is higher than that in other berries such as blackberry, blueberry, and raspberry [26,27]. However, our analysis showed that chlorogenic acid is the most abundant polyphenol in blue honeysuckle berry, which consists with the results described by Zadernowski et al. [28]. This may be due to the differences in species or growth location of the berries tested. Both cyanidin-3-glucoside and chlorogenic acid have been shown to suppress weight gain in high fat diet-induced obese mice by improving lipid metabolism [29,30]. As a result, we consider that flavonoids make a major contribution to the inhibitory effect of BHBE on lipid accumulation in adipocytes. In addition, it is worth noting that the effects of peonidin-3-glucoside, another major anthocyanin in BHBE, on lipid metabolism have not been elucidated.

Obesity caused by adipose tissue expansion is primarily due to lipid accumulation in adipocytes. Adipogenesis is the process whereby fibroblast-like precursor cells differentiate into lipid-laden mature adipocytes. This process can be divided into two stages. In the first stage, fibroblast-like precursor cells restrict themselves to the adipocyte lineage, forming preadipocytes. Then, in the second stage, the specified preadipocytes undergo growth arrest and differentiate into adipocytes with subsequent lipid accumulation resulting from lipogenesis [31]. Adipose tissue expansion occurs through hyperplasia or hypertrophy of adipocytes. However, the number of adipocytes in a certain depot is basically determined in adolescence and is almost stable through adulthood [32]. Therefore, it is more reasonable for anti-obesity strategies to focus on intervening in lipogenesis than inhibiting precursor cell proliferation and differentiation for adults. 3T3-L1 cells, which can be induced to differentiate into mature adipocytes using a chemical cocktail, have been widely used to study adipogenesis in vitro. As previously mentioned, 3T3-L1 cells are transformed into preadipocytes by treatment with differentiation medium during the first 2 days. Over the next 6 days, in maintenance medium, the preadipocytes differentiate into adipocytes and initiate lipogenesis, eventually becoming lipid-laden mature adipocytes. Accordingly, in this study, the intervention with BHBE on lipogenesis started on day 2, and the results showed that BHBE significantly reduced the protein expressions of PPAR $\gamma$ , C/EBP $\alpha$ , and SREBP-1c, which are the key adipogenic transcription factors regulating lipogenesis. PPAR $\gamma$  and C/EBP $\alpha$  are considered to be the master regulators of adipogenesis. PPAR $\gamma$  not only controls adipocyte differentiation, but is also sufficient for this process. To date, there are no identified factors that can restore adipogenesis in the absence of PPAR $\gamma$ . C/EBP $\alpha$  acts in concert with PPAR $\gamma$  to promote adipocyte maturation and plays an accessory role in maintaining the expression of PPAR $\gamma$  [33]. Several studies have indicated that PPAR $\gamma$  antagonists are able to effectively inhibit adipogenesis. SREBP-1c is primarily responsible for lipogenesis by regulating lipogenic enzymes, including ACC and FAS [34]. Therefore, we believe that the inhibition by BHBE of lipogenesis is achieved at the transcriptional level.

Furthermore, our results indicated that BHBE exerted the above-mentioned effects by promoting the phosphorylation of AMPK. As an upstream regulator of adipogenic genes, AMPK regulates lipid metabolism by inhibiting lipogenesis and stimulating fatty acid oxidation. Previous studies have demonstrated that AMPK agonists inhibit adipogenesis by reducing the expression of PPAR $\gamma$  in mouse adipocytes [35]. Moreover, chronic AMPK activation in hyperlipidemic rats resulted in decreased expression of li-

pogenic enzymes in white adipose tissue [36]. Soetikno et al. reported that phosphorylation of AMPK switched off fatty acid synthesis acutely by down-regulating of SREBP-1, which led to ACC inactivation, in STZ-induced type 1 diabetic rats [37]. In addition, although some AMPK agonists have been shown to promote lipolysis [38], in the present study, BHBE was found to have no significant effect on lipolytic enzymes.

Besides lipolysis, thermogenesis is also an effective way to consume intracellular accumulated lipids. Beige adipose tissue is a distinct type of brown adipose tissue that defends against obesity through thermogenesis. Wu et al. found that the expression of *Tmem26* and *Cd137* could distinguish beige adipocytes from both brown as well as white adipocytes, and elevated expression of *Ucp1* was found in both *Tmem26*-selected and *Cd137*-selected adipocytes separated from inguinal adipose tissue [39]. UCP1, which enables the separation of lipid oxidation from ATP production, thus inducing the conversion of stored lipids to heat, plays a key role in regulating the thermogenic capacity of adipose tissue [40]. Beige adipocytes resemble white adipocytes in having low basal expression of UCP1, but, like brown adipocytes, they respond to cAMP stimulation with high UCP1 expression and respiration rates [41]. Although it has been reported that polyphenolic compounds, such as epicatechin, quercetin, and resveratrol, facilitated browning of white adipose tissue, UCP1 was used as the indicator rather than brown adipocyte-specific markers in most of these studies, and the corresponding results were not so convincing [42]. Our results showed that BHBE significantly increased the expression of beige adipocyte markers, implying its potential to promote adipocyte beiging. Similarly, Tamura et al. found that apple polyphenols induced beiging of epididymal adipose tissue by increasing the gene expression of *Cd137* [43].

Based on the results of this study, we will further carry out *in vivo* experiments regarding the anti-obesity effect of blue honeysuckle berry using obese mice.

## 5. Conclusions

In this study, BHBE with high polyphenol content was found to exert an inhibitory effect on lipid accumulation in adipocytes. Our results indicated that BHBE suppressed lipogenesis, at least in part, by promoting the phosphorylation of AMPK and further reducing the expression of adipogenic transcription factor-regulated lipogenic enzymes. Moreover, the expression of beige adipocyte markers was elevated in BHBE-treated adipocytes. These findings suggest that BHBE plays a dual role in the inhibition of lipogenesis and the promotion of adipocyte beiging, which provides credible evidence for blue honeysuckle berry as a candidate functional food against obesity.

**Supplementary Materials:** S1: The polyphenolic compounds detected in BHBE.

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