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Hypoglycemic and hypolipidemic effects of blueberry anthocyanins by AMPK activation: *In vitro* and *in vivo* studies

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ABSTRACT

Blueberries are rich in bioactive anthocyanins, with a high level of malvidin, which is associated with antioxidant benefits that contribute to reducing the risk of diabetes. The objective of this study was to investigate the hypoglycemic and hypolipidemic effects of blueberry anthocyanin extract (BAE), malvidin (Mv), malvidin-3-glucoside (Mv-3-glc), and malvidin-3-galactoside (Mv-3-gal) in both human hepatocarcinoma cell line HepG2 and in a high-fat diet combining streptozotocin-induced diabetic mice. High glucose treatment significantly increased hepatic oxidative stress up to 6-fold and decreased HepG2 cell viability. Pretreatment with BAE, Mv, Mv-3-glc and Mlv-3-gal significantly mitigated these damages by lowering the reactive oxygen species (ROS) by 87, 80, 76, and 91%, and increasing cell viability by 88, 79, 73, and 98%, respectively. These pretreatments also effectively inhibited hyperglycemia and hyperlipidemia, respectively by reducing the expression levels of enzymes participating in gluconeogenesis and lipogenesis and enhancing those involved in glycogenolysis and lipolysis, via adenosine monophosphate-activated protein kinase (AMPK) signaling pathway in HepG2 cells. To determinate the role of AMPK in BAE-induced reaction of glucose and lipid metabolism *in vivo*, doses of 100 mg/kg (blueberry anthocyanin extracts – low concentration, BAE-L) and 400 mg/kg (blueberry anthocyanin extracts – high concentration, BAE-H) were administrated per day to diabetic mice for 5 weeks. BAE treatments had a significant ($P < 0.05$) effect on body weight and increased the AMPK activity, achieving the decrease of blood- and urine-glucose, as well as triglyceride and total cholesterol. This research suggested that anthocyanins contributed to the blueberry extract-induced hypoglycemia and hypolipidemia effects in diabetes and BAE could be a promising functional food or medicine for diabetes treatment.

1. Introduction

Diabetes mellitus (DM) is a well-known disease typically characterized by an inability to maintain normal blood glucose levels. This chronic metabolic disorder causes serious harm to human health and imposes a heavy financial burden on worldwide health care systems [1]. This disease is classified into two main types based on the cause of blood glucose dysregulation. Type 1 DM is caused by an autoimmune destruction of beta-cells leading to an inability to produce insulin whereas type 2 diabetes is characterized by insulin resistance insulin receptors, having reduced function, do not allow cells to respond adequately to rising blood glucose.

In addition to hyperglycemia, diabetics tend to have hyperlipidemia both conditions of which can result in damage to organs and tissues via increased glycoxidative stress [2]. Insulin production and insulin receptor damage are among factors that cause hyperglycemia in diabetics. In the liver, over-expression of gluconeogenesis and glycogenolysis releases glucose into the bloodstream [3] whereas in the small intestine, over expression of glucose transporter 2 (GLUT2) causes an increase in glucose transport [4]. Hyperlipidemia caused by the over-expression of lipogenesis in adipose tissue [5] may also cause the impaired insulin-stimulated glucose uptake and glycogen synthesis leading to insulin resistance and thus contributing to the progression of diabetes

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Abbreviations

ACC	acetyl-co-enzyme A carboxylase
ANOVA	analysis of variance
AMPK	adenosine monophosphate-activated protein kinase
AMPKα	adenosine monophosphate-activated protein kinase alpha
BAE	blueberry anthocyanin extract
BAE-L	blueberry anthocyanin extracts – low concentration, dose of 100 mg/kg
BAE-H	blueberry anthocyanin extracts – high concentration, dose of 400 mg/kg
BCA	bicinchoninic acid
BSA	bovine serum albumin
DCFH-DA	dichloro-dihydro-fluorescein diacetate
DM	diabetes mellitus
DMEM	dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FOXO1	forkhead box O1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLUT2	glucose transporter 2
GS	glycogen synthase
GSK	glycogen synthase kinase
GSH-PX	glutathione peroxidase
G6Pase	glucose-6-phosphatase
HepG2	hepatocarcinoma cells
HG	high glucose (30 mM)
NG	normal glucose (5.5 mM)
HMGCR	3-hydroxy-3-methylglutaryl-coenzymeA reductase
HRP	horseradish peroxidase
HSL	hormone-sensitive triglyceride lipase
i.g.	intragastrically
Mv	malvidin
Mv-3-glc	malvidin-3-glucoside
Mv-3-gal	malvidin-3-galactoside
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide
OD	optical density
PEPCK	phosphoenolpyruvate carboxykinase
PGC	peroxisome-proliferator-activated receptorγcoactivator
PGC-1α	peroxisome-proliferator-activated receptorγcoactivator-1α
p-GS	glycogen synthase-phosphorylation
p-GSK3β	glycogen synthase kinase-3 beta-phosphorylation
ROS	reactive oxygen species
SD	standard deviation
SOD	superoxide dismutase
SREBP	sterol regulatory element-binding protein
STZ	streptozotocin
TCH	total cholesterol
TG	triglyceride
T2DM	type 2 diabetes mellitus

[6]. Long-term complications related to diabetes affect various organs and include hypertension, atherosclerosis, retinopathy, nephropathy, foot ulcers and peripheral neuropathy [7].

There is currently no cure for diabetes, however, its effects can be alleviated [8]. For people diagnosed with type 1 diabetes, a combination of diet regulation, and appropriate intake of insulin according to carbohydrates consumed is the common treatment. Current challenges for type 1 diabetics include inability or difficulties in estimating total carbohydrates and appropriate insulin dose; however, near ideal glycemic levels are possible given technological progress including insulin pumps [9]. For patients suffering from type 2 diabetes, treatment may be more complex including combinations of diet regulation, exercise, insulin and other drugs with a variety of mechanisms of actions that work to lower blood glucose [10]. Common dietary recommendation is to follow a diet of low carbohydrates [11,12].

Blueberry is one of the fruits that diabetic patients can consume and could diminish the risk of type 2 diabetes mellitus (T2DM) [13,14]. Blueberries are rich in a wide variety of compounds beneficial to human health including minerals, fiber organic acids, phenolic acids and flavonoids including flavonols and anthocyanins [15,16]. However, typical anthocyanin profiles include the galactosides, glucosides and arabinosides of delphinidin, malvidin, cyanidin, petunidin and peonidin [17] with malvidins and delphinidins usually being the major contributor to total anthocyanin content [18,19]. In our previous study [20], malvidin was the most abundant anthocyanidin found in rabbiteye blueberry fruits extract along with delphinidin, cyanidin, petunidin and peonidin which is in agreement with other authors who also found malvidin to be an important contributor to total anthocyanin content in different types of blueberries [17,21].

Blueberry anthocyanins may improve insulin sensitivity through antioxidant capacity and through other regulatory interactions as their effects cannot be explained entirely by the former [13,22]. Studies have also found benefits of blueberry anthocyanins for other diabetes-related concerns. In an *in vitro* study, Huang et al. [23] found that blueberry anthocyanins had anti-inflammatory and antioxidant effects on human retinal cells in high glucose conditions. Cells exposed to high glucose had 64% viability whereas when exposed to blueberry anthocyanin extract, malvidin, malvidin-glucoside or malvidin-galactoside, their viability was 7–9%, 83%, 91-% or 86%, respectively. On the other hand, Song et al. [24] found that blueberry anthocyanins reduced oxidative stress and inflammation in diabetic rat retina. Diabetic rats were given 20, 40, and 80 mg/kg of blueberry anthocyanins orally for 12 weeks. Rats with high doses of blueberry anthocyanins had lower blood glucose, higher antioxidant capacity and lower inflammation in the retina and lower reactive oxygen species compared to those without blueberry anthocyanins.

Although blueberry anthocyanins deliver health benefits to the liver and other organs for diabetics, hypoglycemic and hypolipidemic actions of blueberry anthocyanin extract (BAE) in human hepatic cells are unclear. In the present study, it was speculated that BAE, as well as Mv, Mv-3-glc, and Mv-3-gal have hypoglycemic and hypolipidemic activities in human hepatocarcinoma cells (HepG2) and mice and that they could maintain glucolipid homeostasis thus alleviating the development of diabetes.

2. Materials and methods

2.1. Chemical and reagents

Brightwell rabbiteye blueberries (*Vaccinium ashei*) were harvested from Fujiabian Orchard Picking (Nanjing, China) and their anthocyanin extracts were then obtained and stored in the dark at −18 °C. The reagent 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide (MTT) and the standards malvidin (Mv), malvidin-3-glucose (Mv-3-glc), and malvidin-3-galactose (Mv-3-gal) were procured from Sigma Aldrich (Shanghai, China). HepG2 primary cells, bicinchoninic acid (BCA) protein assay kit, and enhanced chemiluminescence (ECL) western blotting detection reagents were acquired from CW Biotechnology (Beijing, China). Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), and penicillin-streptomycin were procured from

Gibco (Auckland, New Zealand). BioFroxx streptozotocin (STZ) was purchased from Saiguo Biotech (Guangzhou, China). Dichloro-dihydro-fluorescein diacetate (DCFH-DA) detection kit was bought from Beyotime Institute of Technology (Shanghai, China). Bovine serum albumin (BSA) was acquired from Shyuanye (Shanghai, China). The high-fat and high-sugar feed (fat 35.5%, protein 20%, carbohydrate 36.4%, 0.1% cellulose) and the normal feed (fat 4.5%, protein 23%, carbohydrate 31.9%, 3.7% fructose, and 5.3% cellulose) for mice were obtained from Xietong Bioenginering Co., Ltd (Nanjing, China). The insulin, triglyceride (TG), total cholesterol (TCH), glutathione peroxidase (GSH-PX), and superoxide dismutase (SOD) assay kits were bought from Jiancheng Bioengineering Research Institute (Nanjing, China). AndyGene human Forkhead Box O1 (FOXO1), glucose-6-phosphatase (G6Pase), glycogen synthase-phosphorylation (p-GS), glycogen synthase kinase-3 beta-phosphorylation (p-GSK3β), glucose transporter 2 (GLUT2), acetyl coenzyme A carboxylase (ACC), hormone-sensitive triglyceride lipase (HSL), 3-hydroxy-3-methylglutaryl-coenzymeA reductase (HMGCR) and mouse fasting insulin enzyme-linked immunosorbent assay (ELISA) kits were all purchased from Bluegene Biotech (Shanghai, China). The chemicals and reagents used in this study were all pure analytical grade.

2.2. Antibodies

Primary antibodies against GS, p-GS (Ser641), sterol regulatory element-binding protein-1c (SREBP-1c), and phosphoenolpyruvate carboxykinase (PEPCK) were acquired from Abcam (Cambridge, United Kingdom). Primary antibodies against adenosine monophosphate-activated protein kinase alpha (AMPKα), p-AMPKα (Thr172), peroxisome-proliferator-activated receptor-γ-coactivator-1α (PGC-1α), ACC, p-ACC (Ser79), and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Nanjing Beidi Biomed Technology (Nanjing, China) while antibody against β-actin was bought from Sigma Aldrich (St. Louis, MO, USA). Primary antibodies were used at 1:1000 dilutions whereas 1:4000 dilutions were used for secondary antibodies.

2.3. Preparation of blueberry anthocyanin extract (BAE)

The extraction of blueberry anthocyanins was performed using our previous method of Huang et al. [25]. The frozen rabbiteye blueberries were held at room temperature until defrosted. Then, they were beaten at 10 000 rpm for 30 s using a T18 basic ULTRA-TURRAX homogenizer (IKA Works Guangzhou, China). An amount of 250 g of blueberries was then soaked in solution of 1000 mL of methanol containing 1% HCl for 24 h. The extract was then collected following centrifugation at 5000×*g* for 15 min. After evaporation of the solvent at 40 °C in a vacuum, the residue was extracted with 1:1 (v/v) ethyl acetate three times. The water phase containing anthocyanins was collected and concentrated using in vacuo evaporation to obtain the crude anthocyanin extract. The extract was further purified with AB-8 macroporous resin (Sigma Aldrich, Shanghai, China). To remove fructose and protein, the extract was subjected to column chromatography on 1000 g AB-8 macroporous resin for 24 h absorption and then eluted with double distilled water. The anthocyanin fraction was eluted with 80% ethanol containing 1% HCl solution, concentrated in vacuo, and then dried using an Eyela FDU-1200 freeze dryer (Tokyo Rikakikai, Japan) in order to get a blueberry anthocyanin extract (BAE) powder.

2.4. Cell culture and treatments

HepG2 cell lines, derived from a specific-distinguished human hepatocellular carcinoma, have a high proportion of liver-specific proteins. For this reason, they are commonly used as laboratory models for human liver cell studies [26]. HepG2 cells were grown in DMEM containing normal D-glucose (5.5 mM) supplemented with 10% FBS and 1% penicillin-streptomycin and kept at 37 °C and 5% CO_2 atmosphere incubator (Thermo Scientific, Waltham, MA, USA). After reaching 70–80% confluence, the HepG2 cells were subcultured. Four hours before the experiment, HepG2 cells were quiesced in a reduced serum medium. After pretreatment with 5 μg/mL of Mv, Mv-3-glc, Mv-3-gal, or BAE for 24 h, the cells were exposed to normal (5.5 mM) or high (30 mM) glucose concentrations to mimic normal and diabetic conditions. The cells were prepared for Western Blot analysis and the supernatants were collected for ELISA analysis.

2.5. Animals and experimental design

C57BL/6J healthy male mice (20 ± 2 g) from GemPharmatech (Nanjing, Jiangsu, China) were kept in standard laboratory conditions, including a constant temperature of 25 °C and a 12-h day, and night alternation. The Jiangsu Academy of Agricultural Sciences Subcommittee on Research Animal Care and Use Committee had previously approved all animal experimental procedures. A high-fat diet and streptozotocin (STZ) was used to induce T2DM in the animal models according to the method used by Islam and Loots [27]. All mice were fed with high-fat and high-sugar diet except for the control group which consumed a normal diet. After 4 weeks, the mice were fasted for 12 h and injected intraperitoneally with 100 mg/kg STZ dissolved in sodium citrate buffer solution (pH 4.2–4.5). One week after STZ induction, blood samples were taken from the tail vein of the mice who had fasted overnight. Mice which presented diabetic symptoms including polyuria, polydipsia, and hyperglycemia (fasting blood glucose level > 11.1 mmol/L) were recognized as T2DM and used for future study. To check the model's T2DM stability, all mice were fed with a normal diet for one week. The normal mice were used as the control group. The diabetic mice were then randomly divided into three groups: model, low-dose BAE (100 mg/kg), and high-dose BAE (400 mg/kg). For 6 consecutive days, intragastric administration of either the same volume of solvent (100 μL), 100 mg/kg or 400 mg/kg of BAE were given to the control group, the model group, the low dose BAE and high-dose BAE respectively. On the seventh day, body weight and fasting blood glucose were measured. The same intragastric administration continued for 5 weeks. Mice were then fasted overnight, and blood samples were collected for serum preparation from the inferior vena cava and stored at −20 °C. After blood sampling, all the mice were anesthetized and sacrificed. The mice liver, spleen, kidney, and thymus tissues were removed, weighed, and stored at −80 °C for further experiments.

2.6. Cell viability detection

The cell viability was determined by MTT method. Five μg/mL of Mv, Mv-3-glc, Mv-3-gal, or BAE were used for pretreatment of cells for 24 h. The cells were then treated with 5 mM or 30 mM glucose for 24 h and 10 μL 0.5% (5 mg/mL) of MTT was added to the cells which were then cultured again. After 4 h, the MTT solution was removed, and 100 μL dimethyl sulfoxide (DMSO) was added before the mixture was shaken slowly for 10 min to dissolve the cell crystal. The absorbance at 490 nm was measured on a StatFax-2100 microplate reader (Awareness Technology Inc., Plam, FL, USA) to obtain the optical density (OD) values. Cells cultured with normal glucose levels (5.5 mmol/L) were used as the control group, whereas wells without cells were used as the blank. The following formula was used to determine cell viability: Cell viability (%) = (sample group OD value - blank group OD value)/(control group OD value - blank group OD value) x 100%.

2.7. ROS fluorescence visualization

The reactive oxygen species (ROS) in HepG2 cells were assessed using the DCFH-DA detection kit. After an initial treatment with the 5 μg/mL of Mv, Mv-3-glc, Mv-3-gal, or BAE for 24 h which was later

followed by a 5 mM or 30 mM glucose treatment for 24 h, the cells were washed with PBS, and then 10 μM DCFH-DA was added to each well and allowed to react for 20 min at 37 °C. The cells were again washed thoroughly with PBS and then a group of these cells was immediately observed under an IX53 inverted fluorescent microscope (Olympus, Tokyo, Japan) at 530 nm emission and 485 nm excitation filters. The images are presented under 200× magnification. After dissociation, another group of cells was collected and their fluorescence was recorded by a Synergy H4 multi-mode microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The total fluorescence intensity of cells in each well was noted, and ROS generation was measured as a fold of the control.

2.8. ELISA

ELISA kits were used to quantify proteins involved in gluconeogenesis (FOXO1, G6Pase, p-GS), glycogenolysis (p-GSK3β), glucose transporter (GLUT2), lipogenesis (ACC and HMGCR), and lipolysis (HSL) in the supernatants of HepG2 cells. The quantity of GLUT2 in mice livers was also determined. The BCA protein assay kit was used to quantify the total cell protein of the supernatant. The absorbance at 450 nm was measured at 37 °C on a StatFax-2100 microplate reader (Awareness Technology Inc., Plam, FL, USA) to determine protein levels.

2.9. Western blotting analysis

Western blotting was performed on HepG2 lysates in order to measure the protein level of AMPK, p-AMPK, gluconeogenesis (PGC1, PEPCK), glycogenolysis (GS, p-GS), and lipolysis (ACC, p-ACC, SREBP-1c) in the cells. Western blotting was also performed to determine the quantities of AMPK, p-AMPK on the livers of diabetes induced mice. Either GAPDH or β-Actin was used as a loading control. LAS-3000 imaging system (Fuji, Tokyo, Japan) was used to observe the protein bands, and their density was quantified using Bio Profile 1D++ (Vilbert Lourmat, Marne La Vallée, France) software. All data were expressed as a fold change to the control.

2.10. Fasting blood glucose and glucose tolerance assay

Fasting blood glucose was measured once a week for a period of 5 weeks after a 12-h fasting period. For glucose tolerance assay, mice were fasted for 16 h and fed with 2 g/kg of 20% glucose (200 μL) intragastrically (i.g.) to determine the blood glucose at 0, 0.5, 1, 1.5, and 2 h. The blood was collected from the tail vein and the glucose levels were measured using a glucometer (Sinocare, Changsha, China). The glucose level in the urine of mice who received i.g. was also measured for a period of five weeks.

2.11. Estimation of serum biochemical indexes and enzyme activity in mice liver

The quantity of insulin, triglyceride, and total cholesterol in the serum was measured, while SOD and GSH-PX enzyme activity was estimated using the commercial kits according to the manufacturer's protocol. Absorbance at 500 nm for triglycerides (TG) and total cholesterol (TCH), and 450 nm for the others (insulin, SOD, and GSH-PX) was measured at 37 °C on a StatFax-2100 microplate reader (Awareness Technology Inc., Plam, FL, USA).

2.12. Statistical analysis

All data are presented as the mean value ± standard deviation (SD) of at least three independent experiments. The figures were obtained using GraphPad Prism Version 8 (GraphPad Software, Inc., CA, USA). One-way analysis of variance (ANOVA), t-tests or Sidak's multiple comparisons test were performed to determine statistical differences among different groups. Differences were considered significant at $P < 0.05$.

3. Results

3.1. In vitro studies

3.1.1. Effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on cell viability in high glucose-stimulated HepG2 cells

High glucose causes hepatic oxidative stress and contributes to cellular cytotoxicity [28]. According to our study, high glucose (HG, 30 mM: 35.67 ± 1.90%) stimulation for 24 h significantly lowered the cell vitality to 35.67% in comparison with normal glucose (NG, 5.5 mM: 100%) stimulated cells. The decrease of cell viability explained that high glucose disturbed the hepatic cellular homeostasis and led to apoptosis that caused degradation of glucose consumption and uptake, thus aggravated insulin resistance. Pretreatment with 5 μg/mL malvidin (Mv), malvidin-3-glucoside (Mv-3-glc), malvidin-3-galactoside (Mv-3-gal), and blueberry anthocyanin extract (BAE) ameliorated the glucose consumption and uptake after 24 h of high glucose incubation by significantly enhancing the cell vitalities at 99.04%, 97.76%, 96.47%, and 110.72%, respectively. Blueberry anthocyanin extract (BAE: 110.72 ± 14.00%) improved HepG2 cell vitality more than malvidin and malvidin derivatives. Malvidin (Mv: 99.04 ± 1.49%) could protect the cell vitality slightly more than its derivatives. Anti-diabetic effect of malvidin-3-glucoside (Mv-3-glc: 97.76 ± 1.82%) was slightly but not significantly higher than malvidin-3-galactoside (Mv-3-gal: 96.47 ± 1.71%) (Fig. 1).

3.1.2. Antioxidant effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on ROS levels in high glucose-stimulated HepG2 cells

The intensity of fluorescence of ROS in HepG2 cells treated with normal glucose was low. After stimulation with high glucose, the fluorescence intensity of ROS was significantly enhanced (Fig. 2). ROS level was significantly increased 6-fold when exposed to high glucose for 24 h (HG: 3309.62 ± 23.82 A U.) in comparison with normal glucose (NG: 560.41 ± 26.38 A U.). This indicated that the level of reactive oxygen

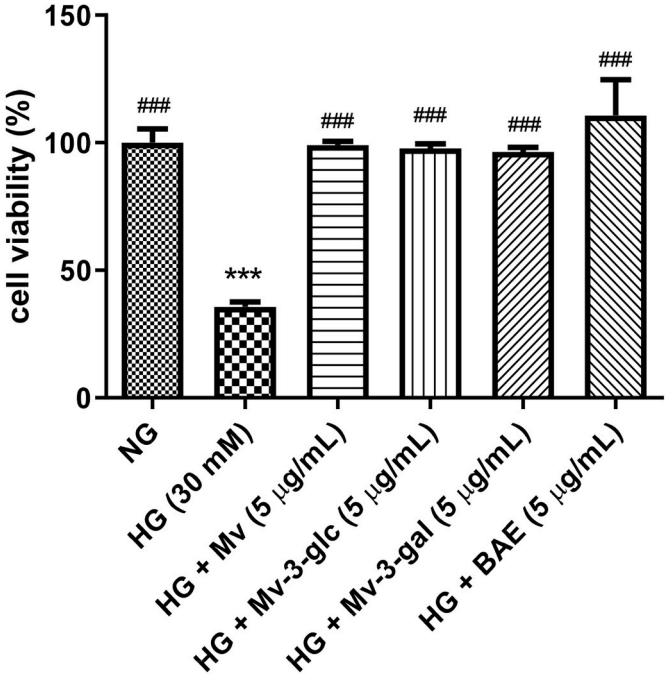

Fig. 1. Effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on cell viability in HepG2 cells exposed to high glucose for 24 h. Bars represent mean values ± SD (n = 3). *** indicate $P < 0.001$ compared with the control (normal glucose group, NG); ### indicate $P < 0.001$ compared with the model (high glucose group, HG).

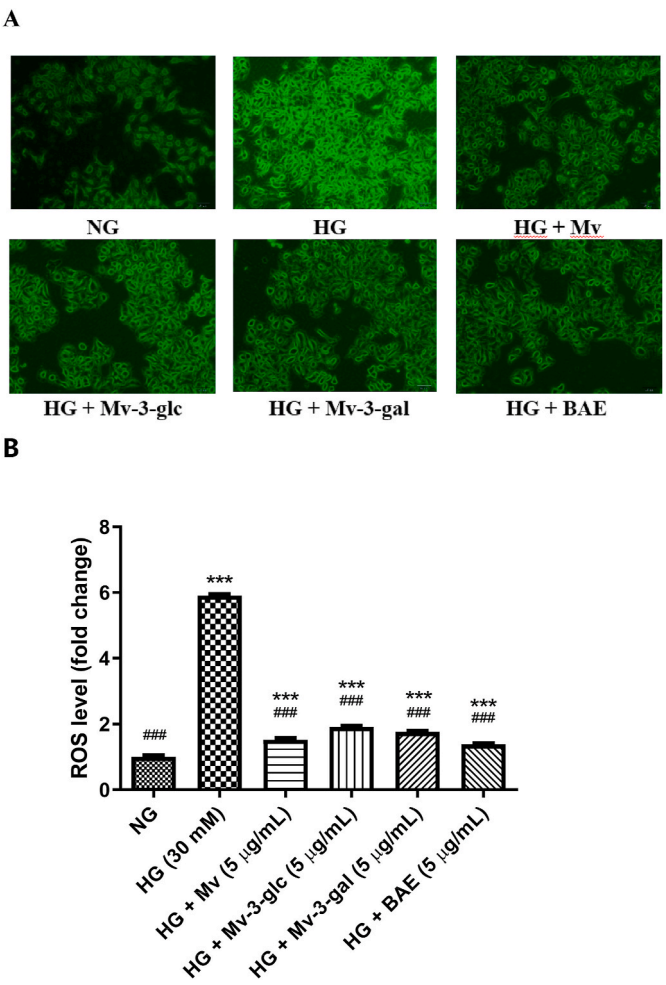

Fig. 2. Antioxidant effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on reactive oxygen species (ROS) level in high glucose-stimulated HepG2 cells. (**A**) The fluorescence intensity (**B**) ROS fold change. A representative set of images from three independent experiments is shown. All images presented are at × 200 magnification. Bars represent mean values ± SD ($n = 3$). *** indicates $P < 0.001$ compared with the control NG; ### indicates $P < 0.001$ compared with the HG model.

had increased and hepatic oxidative stress had occurred. Fluorescence intensity of ROS in HepG2 cells was decreased after pretreatment with 5 μg/mL of Mv, Mv-3-glc, Mv-3-gal, and BAE for 24 h (Fig. 2A). Pretreatment with Mv, Mv-3-glc, Mv-3-gal, and BAE significantly inhibited ROS formations about 89.48%, 81.54%, 84.57%, and 92.38%, respectively (Fig. 2B). Blueberry anthocyanin extract (BAE: 769.83 ± 20.41 A U.) significantly decreased ROS levels more than malvidin and its derivatives. Malvidin (Mv: 849.53 ± 31.43 A U.) significantly exhibited stronger antioxidant effect than its derivatives. Antioxidant effect of malvidin-3-galactoside (Mv-3-gal: 984.66 ± 18.77 A U.) was significantly higher than malvidin-3-glucoside (Mv-3-glc: 1067.83 ± 20.85 A U.).

3.1.3. Effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on glucose metabolism in high glucose-stimulated HepG2 cells and supernatants

Gluconeogenesis is stimulated by the activation of transcription factor FOXO1, in which the interaction with its co-activator, PGC1α, could increase the expression levels of gluconeogenesis (PEPCK and G6Pase) [29]. The hypoglycemic effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on PEPCK and PGC1-α in the high glucose-stimulated HepG2 cells were evaluated using Western Blot (Fig. 3), whereas on FOXO1 and G6Pase in the high glucose-stimulated HepG2 supernatant were evaluated using ELISA (Table 1). PEPCK level in HepG2 cells was increased 1.87-fold in the diabetic group compared to control. Our results which can be seen in Fig. 3A, showed that BAE, malvidin, and its derivatives had similar ability to decrease the up-regulation of PEPCK expression level in high glucose-induced HepG2 cells by 89.02% 87.36%, 92.59%, and 97.84%, respectively. The expression levels of PGC-1α in HepG2 cells increased 1.75-fold in the diabetic (high-glucose) group compared to the control (non-glucose), while Mv, Mv-3-glc, Mv-3-gal, and BAE significantly reversed this change. Pretreatment with BAE showed the strongest capacity to decrease PGC-1α (93.22% decrease) level in high glucose-induced HepG2 cells more so than malvidin (63.49% decrease) and its derivatives mv-3-glc (59.21% reduction) and mv-3-gal (55.57% decrease) which showed similar ability to decrease PGC-1α level in high glucose-induced HepG2 cells (Fig. 3B).

Table 1 shows that with normal cells, FOXO1 expression was higher by 2.12-fold in high-glucose treatment compared with non-glucose treatment. FOXO1 over-activation stimulated by high glucose conditions could induce pro-inflammatory factors in diabetics and promote lipid accumulation [30]. FOXO1 up-regulation during high-glucose conditions were inhibited by pretreatment with Mv, Mv-3-glc, Mv-3-gal, and BAE, thus the inhibitory effect on FOXO1 in high glucose-induced HepG2 cells were 89.73%, 89.94%, 72.71%, and 76.64%, respectively (Table 1). The expression of G6Pase was strongly up-regulated when exposed to high glucose compared with a normal glucose group but these effects were reduced by BAE, malvidin, and its derivatives. These compounds decreased the up-regulation of G6Pase expression level in high glucose-induced HepG2 cells. The inhibitory effect of Mv, Mv-3-glc, Mv-3-gal, and BAE on G6Pase in high glucose-stimulated HepG2 supernatant were about 74.12%, 85.89%, 72.33%, and 77.58%, respectively (Table 1).

ELISA was also used to evaluate the hypoglycemic effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on the phosphorylation of GSK3β at Ser9 inactive form in high glucose-stimulated HepG2 supernatant, whereas Western Blot was used to evaluate the phosphorylation of GS at Ser641 inactive form in high glucose-stimulated HepG2 cells. The expression level of the phosphorylation of GSK3β at Ser9 inactive form was high in unstimulated cells whereas the expression level of the phosphorylation of GS at Ser641 inactive form was low. When exposed to high glucose, the expression level of the phosphorylation of GSK3β at Ser9 inactive form was strongly down-regulated to 1.3-fold (Table 1), and the expression level of the phosphorylation of GS at Ser641 inactive form was strongly up-regulated to 2.6-fold. This experiment showed that BAE, malvidin and its derivatives had the same ability to increase the phosphorylation of glycogenolysis enzyme GSK3β at Ser9 inactive form in high glucose-induced HepG2 cells. Pretreatment with Mv, Mv-3-glc, Mv-3-gal, and BAE significantly increased the phosphorylation of GSK3β at Ser9 inactive form by about 1.54-fold, 1.78-fold, 2.08-fold, and 2.12-fold, respectively (Table 1). The results show a dramatic enhancement of GS activity. Pretreatment of BAE, malvidin, and its derivatives showed the same ability to increase GS synthesis by decreasing the phosphorylation of GS at Ser641 inactive form in high glucose-induced HepG2 cells. Pretreatment with Mv, Mv-3-glc, Mv-3-gal, and BAE significantly decreased p-GS/GS expression levels to 82.94%, 85.99%, 89.83%, and 96.22% in HepG2, respectively (Fig. 3C).

In addition, pretreatment of BAE, malvidin, and its derivatives showed the ability to inhibit the up-regulation of glucose transporter GLUT2 expression level in high glucose-induced HepG2 cells in this experiment. The inhibitory effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on GLUT2 in high glucose-stimulated HepG2 supernatant were 74.59%, 80.38%, 54.68%, and 58.51%, respectively (Table 1).

3.1.4. Effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on lipid metabolism in high glucose-stimulated HepG2 cells and supernatants

The effects of high glucose-induced hepatic fat accumulation on ACC and HMGCR in HepG2 supernatant were evaluated using ELISA (Table 1), whereas the phosphorylation of ACC at Ser79 and SREBP-1c in

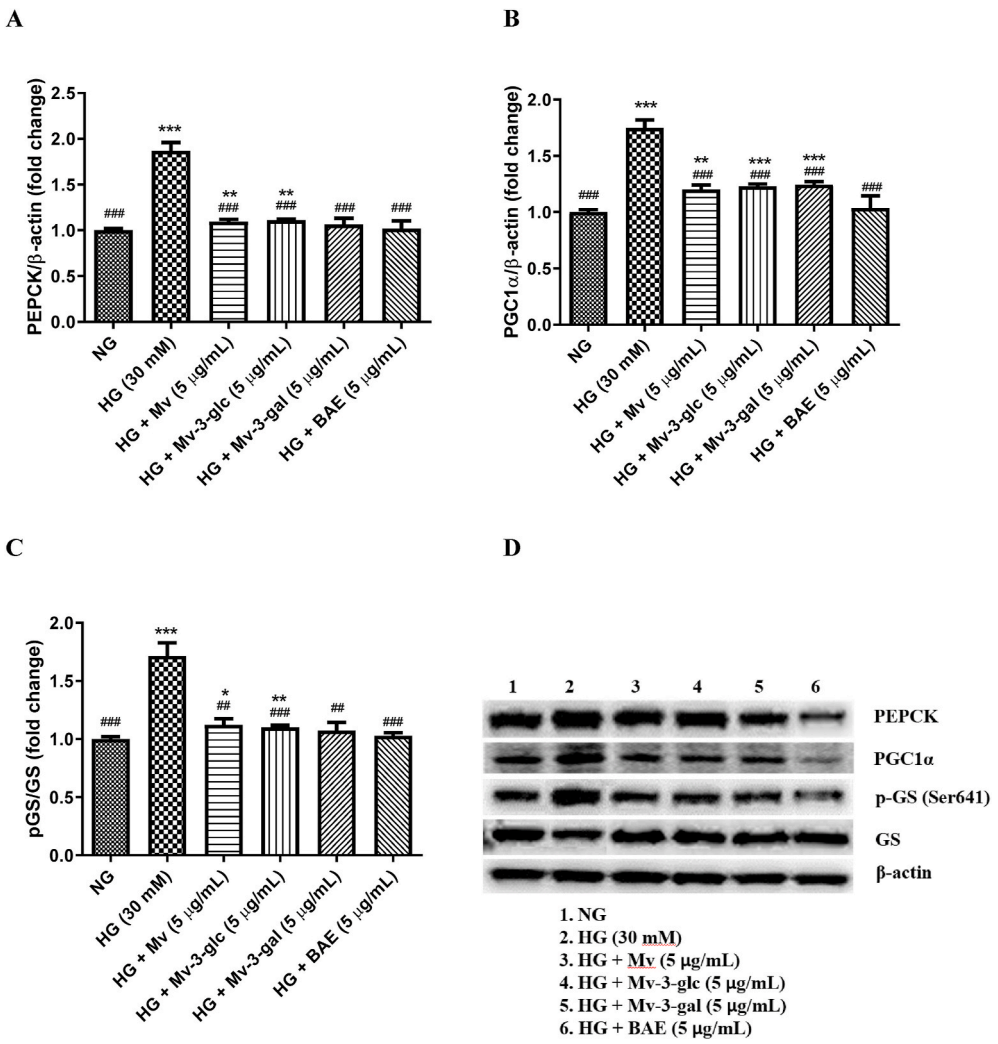

Fig. 3. Inhibitory effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on glucogenesis relative protein levels in high glucose-stimulated HepG2 cells. (**A**) PEPCK, (**B**) PGC1α, (**C**) p-GS/GS fold change, and (**D**) Representative Western blot bands are shown. Bars represent mean values ± SD ($n = 3$). *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared with the control NG; ## and ### indicate $P < 0.01$ and $P < 0.001$ compared with the HG model.

Table 1
Effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on FOXO1, G6Pase, p-GS, p-GSK3β, GLUT2, ACC, HSL, and HMGCR expression level in high glucose-stimulated HepG2 supernatant (n = 3).

Treatment	Protein in HepG2 supernatants							
	Glucogenesis			Glycogenolysis	Glucose Transporter	Lipogenesis		Lipolysis
	FOXO1 (μg/g)	G6Pase (μg/g)	p-GS (μmol/g)	p-GSK3β (μg/g)	GLUT2 (μg/g)	ACC (nmol/g)	HMGCR (μg/g)	HSL (μg/g)
NG (Control)	2.12 ± 0.18 a	3.14 ± 0.23 a	3.37 ± 0.23 a	20.18 ± 0.26 b	1052.18 ± 73.44 a	5.23 ± 0.27 a	2.74 ± 0.10 a	23.05 ± 1.44 b
HG	4.50 ± 0.30 b	6.82 ± 0.78 b	8.82 ± 0.42 e	14.86 ± 1.50 a	2458.75 ± 173.58 c	9.19 ± 0.63 c	6.83 ± 0.44 c	13.46 ± 2.68 a
HG + Mv	2.36 ± 0.08 a	4.09 ± 0.40 a	6.11 ± 0.06 d	23.06 ± 1.05 b,c	1409.52 ± 131.77 a,b	6.50 ± 0.50 a,b	4.02 ± 0.36 b	19.63 ± 2.52 a,b
HG + Mv-3-glc	2.36 ± 0.36 a	3.66 ± 0.46 a	5.63 ± 0.15 c,d	24.33 ± 2.22 b,c	1328.09 ± 292.55 a,b	6.39 ± 0.58 a,b	4.04 ± 0.46 b	20.29 ± 3.05 a,b
HG + Mv-3-gal	2.77 ± 0.31 a	4.16 ± 0.34 a	5.31 ± 0.21 c	25.94 ± 2.33 c	1689.62 ± 133.81 b	6.73 ± 0.49 b	3.66 ± 0.57 a,b	19.45 ± 2.13 a,b
HG + BAE	2.68 ± 0.18 a	3.96 ± 0.21 a	4.37 ± 0.23 b	26.15 ± 1.54 c	1635.80 ± 186.72 b	6.48 ± 0.45 a,b	3.85 ± 0.45 a,b	24.38 ± 0.84 b

Different letters in the same column indicate significant differences ($P < 0.05$).

HepG2 cells were evaluated using Western Blot (Fig. 4). Pretreatment of high glucose induced HepG2 cells with Mv, Mv-3-glc, Mv-3-gal, and BAE inhibited ACC activity by significantly increasing the phosphorylation of ACC at Ser79 inactive form by 1.46-fold, 1.87-fold, 2.18-fold, and 1.97-fold, respectively (Fig. 4B). High-glucose treatment significantly enhanced the expression level of ACC co-activator, SREBP-1c, in HepG2 cells. BAE had the strongest ability to inhibit ACC activity by decreasing the up-regulation of SREBP-1c expression level, nearly the same levels as the control. Malvidin-3-galactoside also significantly decreased the up-regulation of SREBP-1c expression level to nearly the same levels as control. Malvidin (78.59%) and malvidin-3-glucoside (77.53%) also showed the ability to decrease SREBP-1c expression level in high glucose-induced HepG2 cells but not to the same extent as malvidin-3-galactoside (92.72%) or BAE (95.58%) (Fig. 4A). High-glucose treatment significantly enhanced ACC and HMGCR expressions in HepG2 supernatant. Pretreatment with BAE, malvidin, and its derivatives resulted in inhibited cholesterol synthesis to similar extents by inhibiting the up regulation of ACC and HMGCR expression levels.

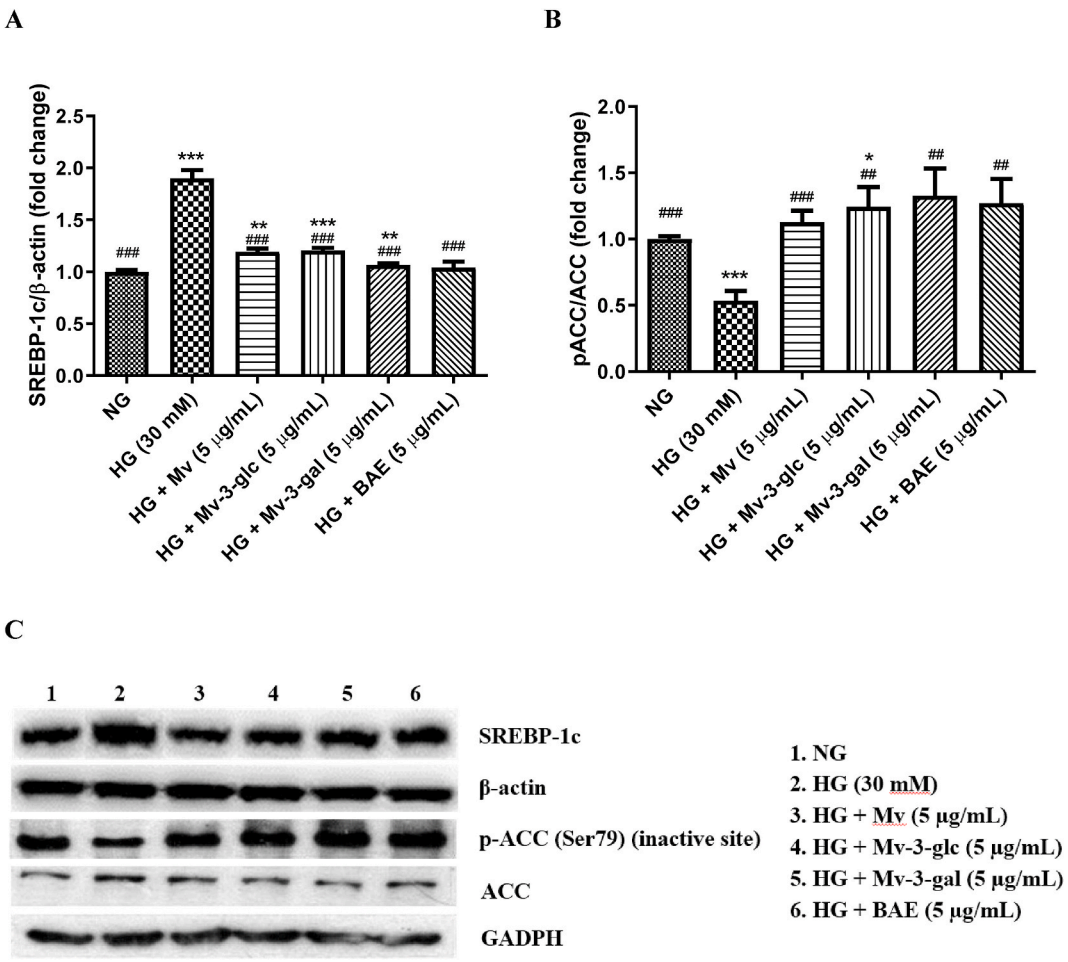

Fig. 4. Regulatory effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on lipogenesis relative protein levels in high glucose-stimulated HepG2 cells. (**A**) SREBP-1c, (**B**) p-ACC/ACC fold change, and (**C**) Representative Western blot bands are shown. Bars represent mean values ± SD ($n = 3$). *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared with the control NG; ## and ### indicate $P < 0.01$ and $P < 0.001$ compared with the HG model.

Pretreatment with Mv, Mv-3-glc, Mv-3-gal, and BAE decreased ACC expression levels by 67.93%, 70.71%, 62.12%, and 68.43%, respectively, and decreased HMGCR expression levels by 68.72%, 68.18%, 77.55%, and 72.94%, respectively (Table 1).

On the other hand, pretreatment with BAE showed the strongest ability to inhibit the downregulation of lipolysis enzyme HSL expression levels in HepG2 supernatant caused by high-glucose treatment, followed by malvidin-3-glucoside, malvidin, and malvidin-3-galactoside. Pretreatment with Mv, Mv-3-glc, Mv-3-gal, and BAE increased HSL expression levels by 89.69%, 89.59%, 76.07%, and 68.68%, respectively (Table 1).

3.1.5. Effects of Mv, Mv-3-glc, Mv-3-gal, and BAE on AMPK activation in high glucose-stimulated HepG2 cells

AMPK is a major regulator of the liver and whole-body glucolipid homeostasis [31]. AMPK is activated via increasing the phosphorylation of Thr172 [32]. The phosphorylation of AMPK at Thr172 active form in HepG2 cells was significantly decreased in the high-glucose compared to the non-glucose group. BAE showed the strongest ability to activate AMPK in high-glucose-induced HepG2 cells compared to malvidin and its derivatives. Pretreatment with Mv, Mv-3-glc, Mv-3-gal, and BAE significantly increased p-AMPK/AMPK expression levels by about 1.32-fold, 1.26-fold, 1.39-fold, and 1.91-fold, respectively (Fig. 5A and B) which indicated they have similar abilities to activate AMPK in high glucose-induced HepG2 cells.

3.2. In vivo studies

3.2.1. Effect of BAE on body and tissue weight in induced diabetic mice

Oral administration of blueberry anthocyanin for five weeks to C57BL/6J mice did have a significant ($P < 0.0001$) effect on the body weight, since the control had a significant high body weight compared to the diabetic model group, while BAE treatments alleviated the body weight decrease of diabetic mice (Fig. 6A). In blueberry anthocyanin extracts – low concentration (BAE-L) and blueberry anthocyanin extracts – high concentration (BAE-H) treatments, daily administration of BAE had no significant differences on body weight indicating its dose-independent manner ($P > 0.05$). Furthermore, Table 2 shows the weight of selected internal mice organs. Results showed that weights were slightly similar to the control group. However, compared with control group, the liver, spleen and thymus weights were significantly different ($P < 0.05$) in BAE-L. Moreover, there was not statistically significance in the liver length and body width among treatments ($P > 0.05$).

3.2.2. Effect of BAE on blood glucose and urine glucose levels in induced diabetic mice

There was a significant ($P < 0.0001$) elevation in fasting blood glucose in model mice as compared to control group, implying that the diabetic model of mice was successful. However, supplementation of BAE to model mice, BAE-L and BAE-H for 5 weeks resulted in significant recovery of fasting blood glucose levels (Fig. 6B) and ameliorated glucose tolerance (Fig. 6C) in diabetic mice. The control group

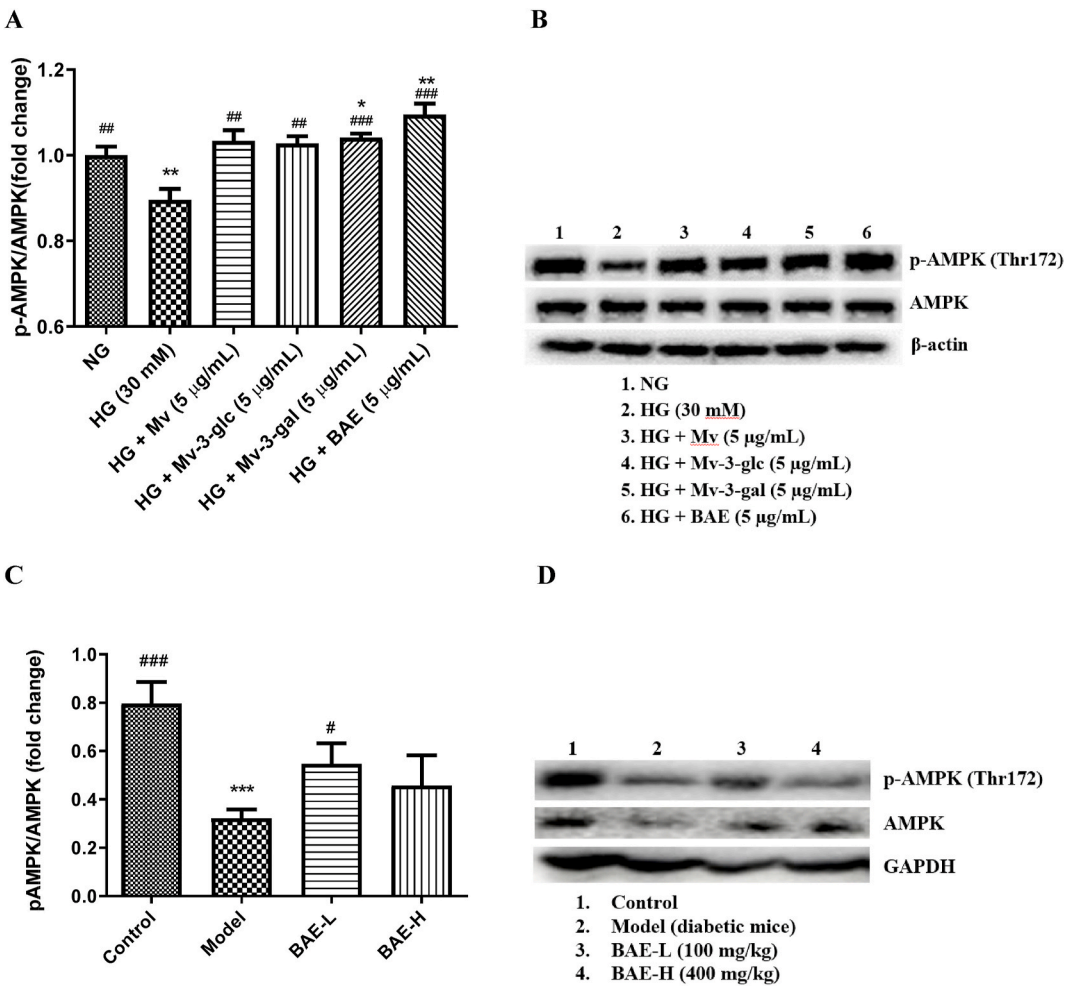

Fig. 5. Activation effects of BAE on glucolipid metabolism center AMPK signaling pathway *in vitro* and *in vivo*. (**A**) p-AMPK/AMPK fold change by Mv, Mv-3-glc, Mv-3-gal, and BAE in high glucose-stimulated HepG2 cells and (**B**) their representative Western blot bands are shown. (**C**) p-AMPK/AMPK fold change by BAE-L and BAE-H in induced diabetic mice and (**D**) their representative Western blot bands are shown. Bars represent mean values ± SD ($n = 3$ for cells in repeated experiment, n = 4–6 for mice). *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared with the NG group/the control; #, ##, and ### indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared with the model HG group/the induced diabetic mice.

maintained a constant glucose level during that period. Compared with the control, BAE-L group notably reduced the blood glucose level in diabetic mice from week 2. Similarly, high-dose BAE obviously reduced the blood glucose levels from week 2 with continued gradual reduction until week 4. BAE at two doses (100 mg/kg and 400 mg/kg per day) significantly decreased blood glucose levels in diabetic mice at 30, 60 and 120 min after glucose load during glucose tolerance test. These results indicate that dietary inclusion of BAE for 5 weeks can effectively regulate glucose metabolism in diabetic mice.

The diabetic model of mice drank more water and produced more urine than the control group. The supplementation of BAE to model group could lessen the symptoms of polydipsia and polyuria in the diabetic mice. Moreover, BAE-L and BAE-H treatments significantly decreased urine glucose levels ($P < 0.01$ and $P < 0.001$, respectively, Fig. 7D). The levels of excreted urine showed significant increases in the model group compared to control, indicating that the former group of mice suffered from diabetes.

3.2.3. *Effect of BAE on triglyceride, total cholesterol, and insulin levels in serum of induced diabetic mice*

Administration of BAE (100 mg/kg and 400 mg/kg per day) significantly affected the lipid levels in induced diabetic mice. Total cholesterol and triglycerides were significantly elevated ($P < 0.01$) in model mice in comparison to the control (Fig. 7A and B). BAE-L treatment only induced a slight decrease of blood lipid indexes but there was no significant difference with the model diabetic mice ($P > 0.05$). Treatment BAE-H resulted in a significant diminution of total cholesterol and triglycerides ($P < 0.01$) and the levels of these parameters were similar to the control ($P > 0.05$). On the other hand, Fig. 7C shows the insulin levels in mice serum after five weeks of treatment. Both BAE-L and BAE-H significantly decreased ($P < 0.001$) the insulin levels in serum of induced diabetic mice. These results indicate that inclusion of dietary BAE for 5 weeks can effectively ameliorate insulin resistance in diabetic mice.

3.2.4. *Effect of BAE on liver antioxidant and glucose transporter levels in induced diabetic mice*

In this study, the effect of BAE on antioxidant enzyme activity of SOD and GSH-PX levels in diabetic mice was evaluated in order to elucidate whether the hypolipidemic and hypoglycemic effects of BAE were associated with the protection of the antioxidant defense systems. As shown in Fig. 8A and B, the model group had a significant decrease in activity of SOD and GSH-PX compared with control group ($P < 0.05$). After BAE treatment, the activity of SOD was significantly increased ($P < 0.01$) in BAE-L and BAE-H when compared with the model group. Moreover, the GSH-PX activity of BAE-H group (400 mg/kg BAE) was significantly higher than the model one ($P < 0.05$). GLUT2 is the main glucose transporter in the plasma membranes of hepatocytes, therefore,

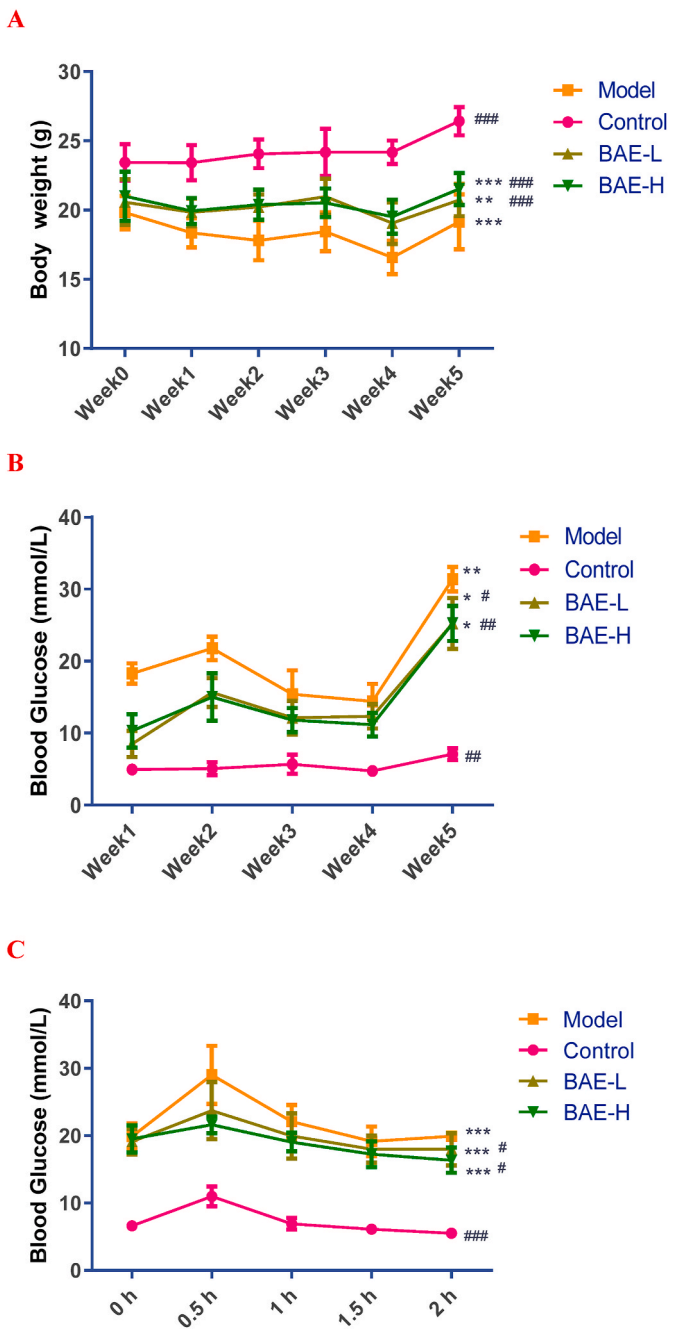

Fig. 6. Effects of BAE on body weight and blood glucose levels in induced diabetic mice. (**A**) Body weight and (**B**) Blood glucose change of mice from the control, model, BAE-L, and BAE-H groups during gavage for five weeks, and (**C**) Glucose tolerance of mice from the control, model, BAE-L, and BAE-H groups after gavage for five weeks. Bars represent mean values ± SD ($n = 6$). *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared with the NG group/the control; #, ##, and ### indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared with the model HG group/the induced diabetic mice.

the effects of BAE on glucose GLUT2 content in induced diabetic mice were studied (Fig. 8C). GLUT2 levels in mice liver were not statistically affected by BAE treatments but were significantly higher in diabetic mice treated with BAE, compared to the model group.

3.2.5. *Effects of BAE on AMPK activation in induced diabetic mice*

AMPK signaling pathway is one of the principal factors for cellular energy homeostasis, which can be recognized as the crucial target in the prevention and treatment of obesity and diabetes. The effects of BAE on the AMPK signaling pathway in induced diabetic mice are shown in Fig. 5C and D. The level of AMPK significantly decreased ($P < 0.001$) in model group in comparison with the control. Groups with BAE showed the strongest ability to activate AMPK compared to the control. Moreover BAE-L and BAE-H showed similar ability to activate AMPK in samples from diabetic mice, and also significantly increased p-AMPK/AMPK expression levels by about 0.54-fold and 0.45-fold, respectively (Fig. 5C).

Table 2
Tissue weight of liver, kidney, spleen, and thymus as well as liver length and body width of mice from the control, model, BAE-L, and BAE-H groups after gavage for five weeks ($n = 6$).

Tissue	Liver weight (g)	Kidney weight (g)	Spleen weight (g)	Thymus weight (g)	Liver length (cm)	Body width (cm)
Control	1.32 ± 0.11 b	0.35 ± 0.02 a,b	0.12 ± 0.02 b	0.035 ± 0.002 c	1.95 ± 0.15 a	3.49 ± 0.13 a
Model	1.14 ± 0.15 a	0.31 ± 0.04 a	0.07 ± 0.01 a	0.014 ± 0.002 a	2.09 ± 0.25 a	3.32 ± 0.37 a
BAE-L	1.18 ± 0.08 a	0.35 ± 0.02 b	0.09 ± 0.02 a	0.015 ± 0.004 a,b	1.82 ± 0.29 a	3.54 ± 0.25 a
BAE-H	1.30 ± 0.07 b	0.32 ± 0.02 a,b	0.15 ± 0.03 b	0.021 ± 0.006 b	1.95 ± 0.36 a	3.44 ± 0.19 a

Different letters in the same column indicate significant differences ($P < 0.05$).

4. Discussion

Diabetes is a chronic metabolic disorder affecting a great proportion of the population worldwide. Blueberries are rich in anthocyanins which have been studied for their numerous beneficial effects on human health. Anthocyanins are able to ameliorate dysfunction in lipid and glucose metabolism, which are fundamental risk factors for obesity and diabetes. The objectives of the present study were to investigate the hypoglycemic and hypolipidemic effects of a blueberry extract rich in anthocyanins on HepG2 cells and induced diabetic mice, as well as to explore the underlying mechanisms.

BAE intake *in vivo* for 5 weeks significantly attenuated the hyperglycemia and hyperlipidemia syndromes in diabetic mice. In this study, we found that administration of BAE, both 100 mg/kg and 400 mg/kg per day could reduce the body weight loss. However, BAE treatments significantly decreased serum triglycerides and cholesterol in diabetic mice. The presence of hypocholesterolemic compounds that can act as inhibitors of some enzymes such as hydroxyl methyl glutaryl CoA reductase, which participates in the synthesis of cholesterol or reduces the absorption of cholesterol in the intestine, could be a factor that is attributable to the cholesterol lowering property of BAE [33]. Furthermore, BAE resulted in a significantly reduced peak of glucose within 2 h, which is supported by other authors [34–36] that reported the potential antidiabetic effect of anthocyanins.

Excessive oxidative stress in body cells plays an important role in the development of diabetes, and the persistent hyperglycemia of diabetes could also lead to increased oxidative stress in the body [37], therefore, the inhibition or reduction of oxidative stress could be an additional therapy for preventing or delaying the incidence of diabetes. It has been reported that anthocyanins could potentially exert antioxidant capacity under high oxidative stress conditions such as obesity and hypercholesteremia [38]. Our results showed a significantly increased activity of SOD levels in BAE-H treatment. These data suggested that BAE could suppress oxidant stress by improving the activity of SOD, which might be attributed to the hyperglycemia and hyperlipidemia alleviation effect of BAE. Similar findings of positive effects of BAE on antioxidant defensiveness were also established in the liver and hippocampus tissues, which was revealed by the strengthened antioxidant enzyme activities including SOD [39]. Anthocyanins could ameliorate dysfunction in lipid and glucose metabolism, which are important risk factors for

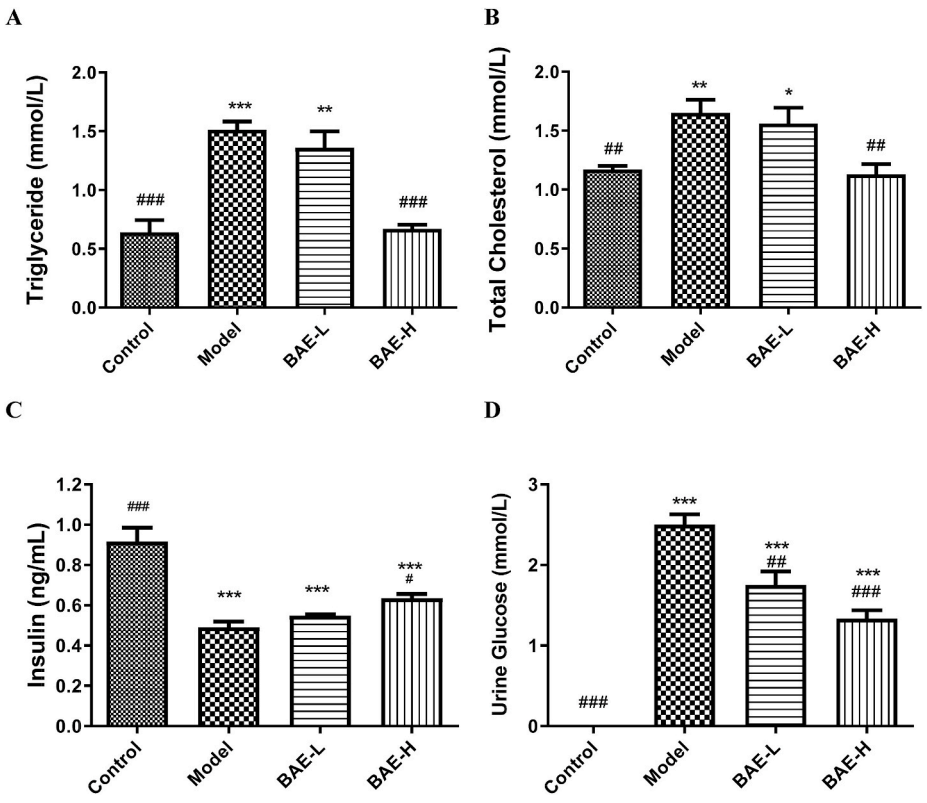

Fig. 7. Effects of BAE on serum biochemical indexes and urine glucose level in induced diabetic mice. (**A**) Triglyceride, (**B**) Total cholesterol, and (**C**) Insulin content in the serum of mice from the control, model, BAE-L, and BAE-H groups after five weeks, and (**D**) Urine glucose level of mice from the control, model, BAE-L, and BAE-H groups after five weeks. Bars represent mean values ± SD ($n = 6$). *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared with the NG group/the control; #, ##, and ### indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared with the model HG group/the induced diabetic mice.

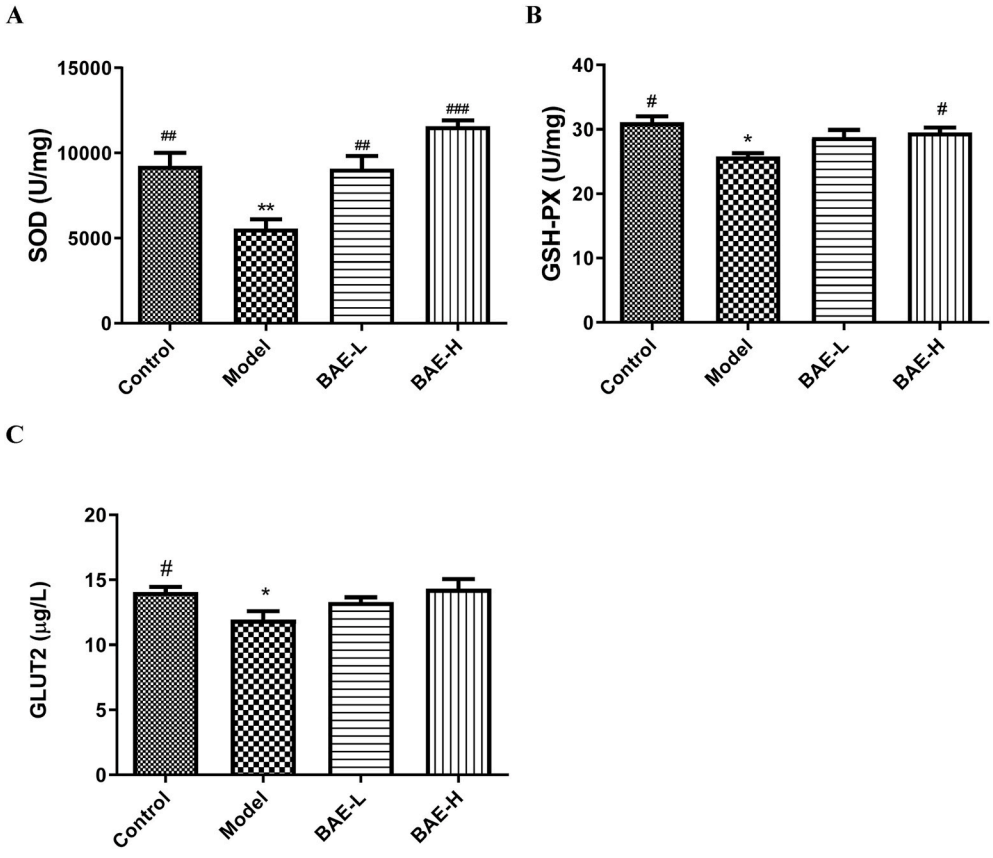

Fig. 8. Effects of BAE on liver antioxidant and glucose transporter levels in induced diabetic mice. (**A**) SOD activity, (**B**) GSH-PX activity, and (**C**) GLUT2 content in the liver of mice from the control, model, BAE-L, and BAE-H groups after five weeks. Bars represent mean values ± SD ($n = 4$–6). * and ** indicate $P < 0.05$ and $P < 0.01$ compared with the NG group/the control; #, ##, and ### indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared with the model HG group/the induced diabetic mice.

diabetes and obesity. One of the crucial factors for cellular energy homeostasis is AMPK signaling pathway, which can be recognized as the key target in the prevention/treatment of diabetes and obesity [40].

Pancreatic β-cells in the liver are affected by glycoxidative stress because of the low quantities of ROS detoxifying enzymes there [41]. Hepatic glycoxidative stress is an essential contributor to diabetic progression as it decreases the insulin stimulation of the insulin signaling proteins and causes insulin resistance [41]. The increase in ROS after 24 h of high glucose stimulation showed that high glucose disrupted the hepatic cellular homeostasis and led to apoptosis. The decrease in cell viability after 24 h of high glucose stimulation resulted in insulin resistance and caused dysfunction in glucose consumption and uptake. Pretreatment with BAE, malvidin, and its derivatives decreased ROS generation and ameliorated cell viability in this study. BAE showed the strongest antioxidant ability to keep β-cells from hepatoxicity triggered by hepatic glycoxidative stress, followed by malvidin, malvidin-3-galactoside, and malvidin-3-glucoside. The hydroxylation and methoxylation patterns of malvidin-3-glucoside and malvidin-3-galactoside on 3,5-dimethoxy substituents in the B-ring conferred these antioxidant properties [42]. Pretreatment with BAE resulted in the greatest ability to protect hepatic β-cells from hepatotoxicity triggered by oxidative stress. However, malvidin also showed the capacity to inhibit ROS generation. Therefore, blueberry anthocyanin extract has the potential to be developed as anti-diabetic nutraceutical to inhibit insulin resistance induced by hepatic oxidative stress in diabetes prevention.

Fig. 9 shows proposed mechanisms for improving hyperglycemia and hyperlipidemia through AMPK activation using BAE. AMPK is a molecule that has actions in the liver, skeletal muscle tissue, adipose tissue and hypothalamus. Typically activated by nutrient deficiencies, it restores the energy balance in two main ways through a complex system of downstream activated molecules [43]. First, AMPK stimulates glucose uptake and lipid oxidation to increase production of ATP, and second, it turns off energy consumption processes. AMPK is involved in pathways that regulate lipid and glucose metabolism. Upregulation of AMPK through a reduction in reactive oxygen species by BAE could lead to positive effects in reducing hyperglycemia and hyperlipidemia in diabetics. As AMPK activity increases, it inhibits multiple factors involved in glucose synthesis including PGC1α, FOXO1, PEPCK, G6Pase and GS which in turn downregulates glucose synthesis [43,44]. According to this study, it inhibits the activity of GLUT2 which decreases glucose transport. It also increases the activity of GSK3β which is involved in glycogenolysis, thereby increasing glycogenolytic activity of the cell. All these lead to a reduction in blood glucose. AMPK also inhibits factors which play a role in lipid synthesis including HMGCR which is involved in sterol and isoprenoid synthesis, SREBP-1 which promotes fatty acid synthesis and ACC which is active in lipogenesis and in addition, AMPK promotes the activity of HSL which is involved in lipolysis [43,45]. These activities can then lead to a reduction in blood lipids. BAE, malvidin, and its derivatives showed their strong capacity to enhance AMPK activation via phosphorylation at Thr172 active form in the insulin-independent pathway. Therefore, BAE, malvidin, and its derivatives can be considered as a potential nutraceutical to improve insulin sensitivity in hepatic cells and increase glucose homeostasis.

Gluconeogenesis catalyze the final release of glucose into the blood circulation, in which its over-expression in the liver would lead to insulin resistance and the rise of hepatic glucose production [46]. Insulin directly inhibits gluconeogenesis and its activators, thus consequently reducing the levels of circulating blood glucose [29]. Our results showed that BAE, malvidin, and its derivatives decrease the formation of hepatic glucose by suppressing gluconeogenesis (PEPCK and G6Pase) and its co-activators (PGC-1α and FOXO1) in the insulin-independent pathway. Therefore, BAE, malvidin, and its derivatives could improve insulin sensitivity and respond to the post-prandial increases in blood glucose, and thus inhibiting gluconeogenesis and its activators.

The superfluous glucose which is not utilized as an instantaneous fuel for energy is initially stored as glycogen [47]. Glycogen synthase (GS) forms glycogen from glucose, but glycogen synthase kinase 3β (GSK3β) inhibits GS activity by converting glycogen to glucose. Insulin inhibits glycogenolysis by inactivating GSK3β, consequently promoting

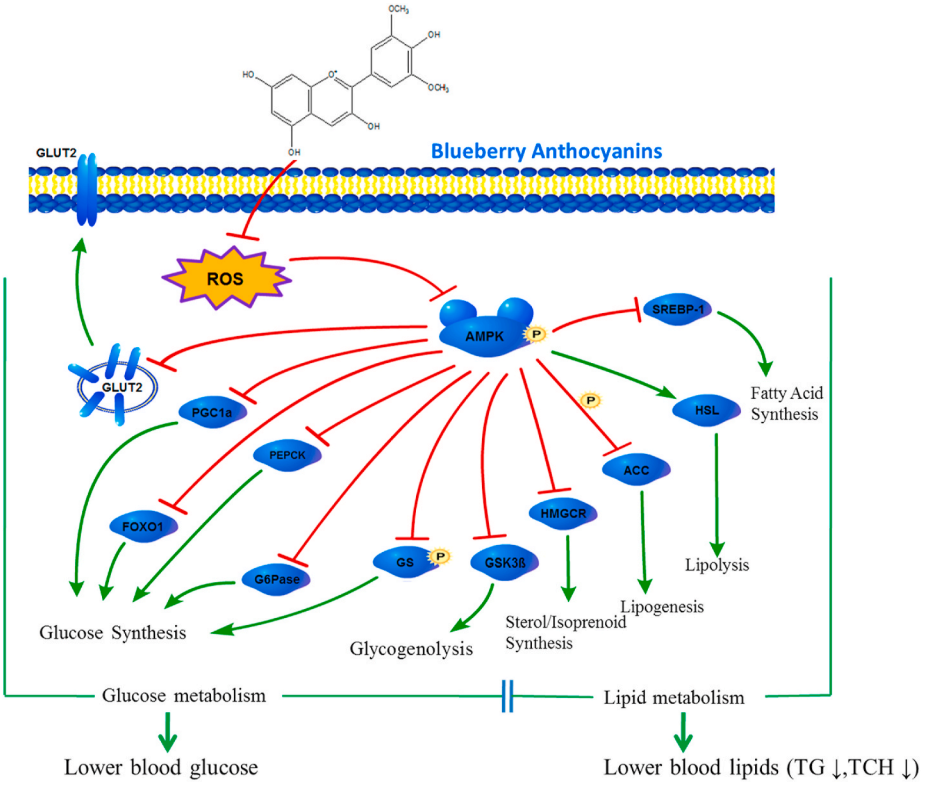

Fig. 9. Schematic of TLR4 signaling cascades. Blueberry anthocyanins (Mv, Mv-3-glc, Mv-3-gal, and BAE) regulated glucose and lipid metabolism by activation AMPK signal pathway. Activation of AMPK signal transduction through ROS inhibition leads to lower blood glucose and blood lipids (TG and TCH) through reducing glucogenesis (GS, G6Pase, PEPCK, PGC1α, and FOXO1), glucose transporter (GLUT2), and lipogenesis (ACC, HMGCR, and SREBP-1), as well as enhancing glycogenolysis (GSK3β) and lipolysis (HSL).

glycogenesis by enhancing GS activity in hepatocytes [48]. In this study, BAE, malvidin, and its derivatives could improve insulin sensitivity by acting as an effective GSK3β inhibitor and GS stimulator in the insulin-independent pathway.

GLUT2 is a bidirectional transporter that takes up glucose during the absorptive (glycolysis) phase and discharges it into the bloodstream during gluconeogenesis and glycogenolysis [49]. When the extracellular glucose concentration increases, more glucose enters pancreatic β-cell via the low-affinity glucose transporter GLUT2 [50]. GLUT2 over-expression in the liver impairs glucose-stimulated insulin secretion and increase the risk of fasting hyperglycemia and T2DM [4]. Malvidin combined with sugars in blueberry extract prevented the sudden rise of glucose by decreasing GLUT2 expression level. Flavonoid containing sugar could reduce glucose transport and absorption by decreasing the absorption rate, whereas anthocyanin itself is not carried away by GLUT2 [51]. The former would be beneficial in reducing the sudden increase of glucose. Anthocyanin and GLUT2 coalition occurred via hydrophilic moieties, i.e. the 3-glucosyl moiety and the B ring for the monoglucoside, the 5-glucosyl moiety and the A ring for the diglucoside, and the A or B ring for the aglycones [52]. In this study, BAE, malvidin, and its derivatives reduced GLUT2 over-expression levels in hepatic cells, thus keeping the balance of glucose-stimulated insulin secretion in pancreatic β-cells and also reducing glucose transport and absorption into blood circulation.

The over-expression of lipogenesis, that is, ACC, SREBP-1c, HMGCR, and HSL in obese patients has been strongly associated with diabetes and fatty liver diseases [53]. Pretreatments with BAE, malvidin, and its derivatives could have inactivated ACC by causing an increase in the phosphorylation of ACC at Ser79 inactive form. ACC inactivation could stimulate long-chain fatty acids to enter the mitochondria for oxidation, thus increasing fatty acid uptake [54]. These pretreatments also inhibited fatty acids synthesis by decreasing the expression of ACC co-activator, SREBP-1c.

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) plays an important role in cholesterol synthesis [55]. Hormone-sensitive lipase (HSL) mediates the release of free fatty acids into vasculature [56]. BAE, malvidin, and its derivatives effectively decreased cholesterol synthesis and free fatty acid circulation into vasculature by significantly decreasing HMGCR and HSL expression levels in hepatocytes. It was also reported that a high dose of BAE could suppress the accumulation of serum ceramides, diacylglycerols, triacylglycerol and cholesterol, and thus prevent lipid metabolic dysfunction [57]. Therefore, BAE plays a role as a hypolipidemic nutraceutical that effectively reduces the fatty acid and cholesterol synthesis, and increases fatty acid oxidation, thus consequently promoting lipid homeostasis.

From our findings, BAE, malvidin, and its derivatives have the potential hypoglycemic and hypolipidemic effects of treating diabetes via AMPK activation that increase insulin sensitivity by inhibiting gluconeogenesis and lipogenesis in human hepatocarcinoma cells, and by decreasing glucose transport into the bloodstream.

5. Conclusion

The hypoglycemic and hypolipidemic effects of anthocyanin extract from rabbiteye blueberry cultivar *in vitro* and *in vivo* have been investigated in this study. The results demonstrated that hepatic oxidative stress was significantly increased by high glucose, which increased ROS by up to 6-fold and decreased cell viability. Pretreatment with Mv, Mv-3-glc, Mv-3-gal and blueberry anthocyanin extract (BAE) significantly reduced this damage by lowering the ROS generation and increasing the cell viability. These findings show that BAE, malvidin, and its derivatives have a powerful antioxidant capacity to protect hepatic cells from oxidative deterioration by decreasing the formation of ROS and increasing cell viability. Therefore, these pretreatments could effectively decrease fatty acid and cholesterol synthesis and reduce free fatty acid circulation in blood vessels. BAE could significantly ameliorate hyperglycemia and hyperlipidemia in induced diabetic mice via a significant reduction in body weight loss, glucose and lipid levels. The antioxidant activity of SOD and AMPK activation were notably improved by BAE. BAE, malvidin, and its derivatives proved to be efficacious anti-diabetic nutraceuticals with hypoglycemic and hypolipidemic activities. They effectively activated AMPK which, in turn, improved insulin sensitivity and protected the body from the over-expression of gluconeogenesis and lipogenesis; therefore, they could effectively maintain glucolipid homeostasis and prevent diabetes. These findings suggest that BAE could exhibit great anti-diabetic effects *in vitro* and *in vivo*, and anthocyanins being one of the major constituents of the blueberry extract, play an important role in exerting hypoglycemic and hypolipidemic activities.

Declaration of competing interest

There are no conflicts to declare.

Acknowledgments

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