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Study on Mechanisms Underlying the Preventive Effects of *Canarium album* L. ethanol Extract on Modulation of Hyperglycemia and Hypercholesterolemia in Diabetic Rats

Yu-Han Kao[1,#], Yu-Te Yeh[1,#], An-Na Chiang[2], Shu-Chen Hsieh[1,*]

[1]Institute of Food Science and Technology, National Taiwan University, Taipei 106, Taiwan
[2]Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei 112, Taiwan
[#] These authors contribute equally.
*Corresponding author: schsieh@ntu.edu.tw

Abstract *Canarium album* L. has attracted attention for its high polyphenol content and various potential pharmacological activities, exhibiting potential to reduce the risk of metabolic disorders. We hypothesized that *Canarium album* L. may regulate glucose and lipid metabolic pathways. To examine the application potential of *Canarium album* L. on metabolism, we designed the present study to investigate the effect of *Canarium album* L. ethanol extract (CO-EtOH) on high-fat diet (HFD) and streptozotocin (STZ)-induced diabetic rats. Our data showed CO-EtOH significantly reduced elevated fasting blood glucose, TC, TNF-α, and bile acid. Administration of CO-EtOH increased hepatic mRNA levels of *GLUT2* and *GK* but decreased those of *PEPCK* and *G6Pase*. Furthermore, CO-EtOH remarkably reduced hepatic levels of cholesterol; lowered the expression of genes involved in cholesterol biosynthesis, transportation, and degradation, including *SREBP-2*, *HMG-CoAR*, *SR-B1*, *CYP7A1*, *BSEP*, *MRP3*, *MRP4*, and *NTCP*; and decreased the expression of *LDLR*, *ABCG8*, *FXR*, and *SHP* that govern cholesterol transportation and bile acid synthesis. The protein expressions of LDLR, ABCA1, FXR, and SHP were also significantly increased upon CO-EtOH treatment. Finally, CO-EtOH could increase glucose uptake in rat skeletal muscle cells in both normal and palmitic acid-challenged conditions. These findings suggest that *Canarium album* L. may have great potential for treating hyperglycemia and hypercholesterolemia.

Keywords: *Canarium album L., type 2 diabetes, high-fat diet, cholesterol, glucose uptake*

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1. Introduction

Type 2 diabetes (T2D) is considered one of the main threats to human health and also ranks as the top 10 causes of death worldwide [1]. International diabetes federation (IDF) defines T2D as a disease characterized by insufficient insulin production or impaired insulin functionality, thus reduces the ability of tissues to utilize glucose and causes hyperglycemia, followed by disturbance of lipid metabolism [2]. Insulin resistance (IR) in the liver leads to elevated expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), which are key enzymes for gluconeogenesis that increase hepatic glucose production [3]. IR also decreases glucokinase (GK) and glycogen synthase (GS), two critical enzymes for glycogen synthesis [4]. The scenario of hepatic IR tightly correlates with fasting hyperglycemia in T2D individuals [5].

T2D is associated with abnormal lipid metabolism [6,7]. Reduced hepatic cholesterol absorption and increased cholesterol synthesis have been demonstrated in T2D patients [8]. Sterol regulatory element binding protein 2 (SREBP-2) is a key transcription factor that regulates cholesterol synthesis, uptake, and efflux [9,10]. The genes involved in cholesterol uptake and efflux, such as low-density lipoprotein receptor (LDLR), ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 are highly associated with T2D [11,12,13,14]. The inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR), a downstream target gene of SREBP-2 and the rate-limiting enzyme for cholesterol synthesis [15,16], are used extensively to treat patients with hypercholesterolemia [17,18].

Excess cholesterol is usually eliminated from the body via liver in the form of bile acids [19,20]. Bile acid synthesis plays a critical role in the maintenance of mammalian cholesterol homeostasis. CYP7A1 gene encodes cholesterol 7α-hydroxylase, which catalyzes the initial step for converting cholesterol to bile acid [21].

Hepatic farnesoid X receptor (FXR) functions as a biological regulator of bile acid synthesis through its transcriptional induction of the inhibitory nuclear receptor small heterodimer partner (SHP) [22]. FXR/SHP pathway is a negative regulator of CYP7A1 gene [23] and regulates bile acid uptake, excretion and transport such as Na^+-dependent taurocholate transporter (NTCP), bile salt exporting pump (BSEP), resistance-associated protein (MRP) 2, ABCG5, ABCG8, MRP3, and MRP4 [24,25]. Insulin-sensitizing drugs have become the major choice to improve T2D; however, their adverse effects have limited the clinical usage [26]. The previous study suggests that alternative methods are effective in controlling T2D, particularly regarding the use of medicinal plants [27]. Overall, there is a huge demand for new medicines, which exhibit their own benefits and safety profiles. Chinese olive fruit also named *Canarium album* L. fruit, is widely cultivated in Taiwan and other Asian regions, and has long been used as a Traditional Chinese Medicine (TCM). The functions of *Canarium album* L. have been explored, including hepatoprotection [28], ant-inflammation [29], inhibition of malignant tumor growth [30], suppression of advanced glycation end products-induced oxidative stress [31], and restraint of excessive lipid accumulation [32]. These properties of *Canarium album* L. fruit can be attributed to its high content of polyphenols and flavonoids [33,34]. In spite of the pharmacological potentials, *Canarium album* L. fruit is still very much under studied. There is a lack of detailed study of effectiveness in the antidiabetic activity of the *Canarium album* L. fruit. In the present study, CO-EtOH, which was the extract with solvent that is commonly applied in food industry, was supplemented to Sprague-Dawley rats fed with HFD combined with STZ to investigate its dual effect in the prevention of both dyslipidemia and hyperglycemia, along with the corresponding molecular mechanism.

2. Materials and Methods

2.1. Plant Material and Preparation of the Plant Extract

Canarium album L. fruits were obtained from Baoshan Township, Hsinchu County, Taiwan. The *Canarium album* L. fruit was dried and extracted with water followed by filtration. The filtrate was concentrated to powder by vacuum drying and then exhaustively extracted with 95% ethanol (v/v) (1000 mL) overnight at room temperature. After being filtered through NO.1 filter paper, the extract was vacuum evaporated with the yield of 23% and dissolved in water, stored at -20°C for 24 hours, and followed by freeze drying. The same batch of *Canarium album* L. fruit extract was used throughout this study. Composition of CO-EtOH was determined using high-performance liquid chromatography (HPLC) analysis with a Photodiode Array Detector (PDA, Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was achieved on a 250 × 3 mm i.d. column containing C18 amide 5 μm, with a 5 mm × 3 mm i.d. guard column (Varian, Darmstadt, Germany). Gradient elution was performed with 0.5% (v/v) formic acid (solvent A) and methanol (solvent B) at a constant flow rate of 0.7 mL min^{-1}. The linear gradient profile was as followings: 90% A and 10% B at the start, 38% A and 62% B at 25 min, and 5% A and 95% B at 60 min. UV-Vis absorption spectra were recorded on-line at 200 and 600 nm during HPLC analysis.

2.2. Experimental Animals

Six-week-old male Sprague-Dawley adult rats with body weight 130 ± 10 g were purchased from BioLASCO Co., Ltd. (Taipei, Taiwan.). The experimental protocol was approved by the Institutional Animal Care and Use Committee of the National Taiwan University, Taipei, Taiwan, Republic of China. These rats were fed ordinary laboratory chow diet and acclimated to the animal room conditioned at 23 ± 2°C, relative humidity of 55 ± 5% and a 12-hour light/dark cycle. After adaptation for one week, rats were provided with either normal chow diet (AIN-93 diet with 10% energy content by fat) or high-fat diet (HFD, AIN-93 diet with 60% energy content by fat). In week 2, HFD-fed rats were intragastrically treated with CO-EtOH (dissolved in 0.5% carboxymethyl cellulose (CMC), 760 mg/kg body weight); control animals were also gavaged with 0.5% CMC and this treatment continued daily for 8 weeks. During the 8-week experimental period, rats were provided free access to food and water. Animals were sacrificed by CO_2 anesthesia, and serum was collected for biochemical analysis. The livers were dissected and stored at –80°C for further experiments.

2.3. Experimental Design

Rats were divided into three groups with 10 animals in each group and housed under controlled environmental conditions. *The treatment groups were* as follows: Normal control group (NC group): healthy rats, Diabetic control group (DC group): HFD+STZ induced diabetic rats, DC+ CO-EtOH group: HFD+STZ induced diabetic rats and intragastric administration of CO-EtOH.

2.4. Induction of Diabetes

To establish a T2D animal model, we followed the modified method described by Srinivasan et al. [35] to feed the rats with HFD during the entire experimental period. In week 7, experimental rats excluding control group were given a single intraperitoneal injection of STZ (35 mg/kg) (Sigma-Aldrich Inc., St. Louis, MO, USA), which can partly degrade the β-pancreatic cells to induce symptoms of T2D at late stage. The blood glucose test was performed by clipping the tip of the tail and the rats in DC group have been confirmed to exhibit blood glucose higher than 300 mg/dL [36].

2.5. Cell Culture

L6 myoblast cells were obtained from America Type Culture Collection (ATCC, ATCC Number CRL-1458) and grown in DMEM containing 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) (Sigma-Aldrich, St Louis, MO, USA) at 37°C in a humidified atmosphere with 5% CO_2. After the cells had reached 70–80% confluence, myoblast

differentiation was induced by growing the cells in DMEM containing 2% horse serum for 7 days. The differentiation of L6 myotube from myoblast was confirmed by morphology and protein expression of MyoD. Before each assay, cells were serum-starved for 12 hours in DMEM containing 0.2% bovine serum albumin (BSA).

2.6. Glucose Uptake

L6 myotube cells were washed with Krebs-Ringer phosphate buffer (KRP buffer: 25 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM KH_2P_4, 2.5 mM $MgSO_4$, 5 mM $NaHCO_3$, and 0.1% BSA, pH 7.4) and incubated in KRP buffer containing 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) for 15 minutes. The reaction was terminated by addition of ice-cold PBS. The cellular uptake of 2-NBDG was measured using a fluorometer at excitation and emission wavelengths of 485 and 535 nm, respectively.

2.7. Quantitative Real-time RT-PCR (RT-qPCR) Analysis

Total RNA was extracted using TRIZOL reagent (Invitrogen Corp., Carlsbad, CA, USA), following the manufacturer's instructions. Purified RNA (2 μg) was treated with RNase-free DNase I (Invitrogen Corp., Carlsbad, CA, USA), and then reverse-transcribed with oligo (dT) primer using the SuperScript First-Strand Synthesis System (Invitrogen) to generate cDNA. The RT-qPCR reaction mixture was carried out on a StepOnePlus™ Real-Time PCR Systems (Thermo Scientific, Waltham, MA, USA). The primer sequence of each gene is shown in Table 1. The relative copy number was calculated using the threshold crossing point (Ct) as calculated by $2^{-\Delta\Delta Ct}$ [37] and the relative expression were normalized to the amount of 18S rRNA.

2.8. Western Blot Analysis

Liver tissue was lysed in lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and phosphatase inhibitors (Sigma-Aldrich, Inc., St. Louis, MO, USA). Equal amount of protein samples (30μg) were subjected to SDS-PAGE and transferred to nitrocellulose membranes after gel electrophoresis. (Millipore, Bedford, MA, USA). The blots were blocked with 5% (w/v) skim milk and probed with primary antibodies against FXR, SHP (Abcam, Cambridge, MA, USA), CYP7A1, LXRα/β (Santa Cruz, CA, USA), LDLR, and ABCA1 (Gene Tex, Irvine, CA, USA) separately, followed by goat anti-rabbit or mouse IgG horseradish peroxidase (HP)-conjugated secondary antibodies. The protein bands were visualized using enhanced chemiluminescence reagents (ECL, PerkinElmer, Boston, MA, USA). Densitometry of bands on films was assessed with Epichemi-3 software (UVP BioImaging System, Upland, CA) and normalized to β-actin levels.

2.9. Biochemical Analysis

Fasting blood samples were collected from the tail vein of the rats after starvation for 12 hours. Fasting blood glucose (FBG) levels were determined by the glucose analyzer (Eumed Biotechnology Co., Ltd., Hsinchu, Taiwan). At the end of the experiment, serum samples were collected by cardiac puncture and centrifuged at 1500 xg for 10 minutes. Total cholesterol (TC), triglyceride (TG), and HDL-cholesterol (HDL-C) levels were measured using an automatic SPOTCHEM EZ SP-4430 blood chemistry analyzer (ARKRAY, Inc., Kyoto, Japan) with different strips. Insulin levels were determined using commercial kits (Mercodia AB, Uppsala, Sweden). Bile acid and TNF-α levels were determined using kits according to the manufacturer's instructions (Cayman, Ann Arbor, MI, USA).

2.10. Quantification of Hepatic Cholesterol Contents

Lipids were extracted from homogenized liver tissues using a mixture of 8:4:3 chloroform/methanol/0.9% NaCl (v/v) to a final dilution of 20-fold the original volume of the tissue sample. The organic layer was then separated, evaporated, and reconstituted in chloroform. The value of TC was measured using a colorimetric assay kit (Sigma-Aldrich, Inc., St. Louis, MO, USA) and normalized against the weight of the extracted liver.

Table 1. Primers used sequences for RT-qPCR

Genes	Forward and Reverse Primers (5' to 3')
ABCA1	FP:GTACCCAGCGTCCTTTGTGT
	RP:CCCAAGAGAGTGGAGAGACG
ABCG1	FP:CTGCAAGAGAGGGATGAAGG
	RP:ACAGGAGGGTTGTTGACCAG
ABCG5	FP:TGGAAGGGGAAGTGTTTGTG
	RP:GAACACCAACTCTCCGTAAG
ABCG8	FP:GAACACCAACTCTCCGTAAG
	RP:GTCAAGTCCACGTAGAAGTC
BSEP	FP:ATGTTGGAACGGAGGAACTG
	RP:CCTTCTCGACCCGATATTCA
CYP7A1	FP:CACCATTCCTGCAACCTTTT
	RP:GTACCGGCAGGTCATTCAGT
FXR	FP:TCAGCCAACATTCCCATC
	RP:CCTGTGACAAAGAAGCCG
G6pase	FP: AACGTCTGTCTGTCCCGGATCTAC
	RP: ACCTCTGGAGGCTGGCATTG
GK	FP:TGGATGAAAGCTCAGCGAA
	RP:ACACGAAACGGGTCTCAAAA
GLUT2	FP: TAGTCAGATTGCTGGCCTCAGCTT
	RP: TTGCCCTGACTTCCTCTTCCAACT
HMG-CoAR	FP: CCCAGCCTACAAACTGGAAA
	RP: CCATTGGCACCTGGTACTCT
LDLR	FP:CAGCTCTGTGTGAACCTGGA
	RP:TTCTTCAGGTTGGGGATCAG
PEPCK	FP:AAAGCATTCAACGCCAGGTTC
	RP:CACCACATAGGGCGAGTCTGTC
SHP	FP:CTTCCTCAGGAACCT
	RP:CCCAGTGAGCCTCCT
SR-B1	FP:TGCCCCAGGTTCTTCACTAC
	RP:CCCTACAGCTTGGCTTCTTG
SREBP-2	FP:AGACTTGGTCATGGGGACAG
	RP:GGGGAGACATCAGAAGGACA
MRP3	FP:ACACCGAGCCAGCCATATAC
	RP:ACATTGGCTCCGATAGCAAC
MRP4	FP: GCCCTTACCCAGCTGCTGA
	RP: CAGAATCCAGAGAGCCTCTTTTACA
r18S	FP:GATCCATTGGAGGGCAAGTCT
	RP:AACTGCAGCAACTTTAATATACGCTATT

2.11. Statistical Analysis.

GraphPad Prism Version 6.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for data analysis. Data were presented as mean ± SEM. Statistical differences among experimental groups were determined using one-way ANOVA and the LSD multiple range test. *, # indicates the values with statistically significant difference, $p < 0.05$.

3. Results

3.1. High Performance Liquid Chromatography (HPLC) Analysis of CO-EtOH

The major compounds of CO - EtOH were analyzed by HPLC. As shown in Figure 1, two major peaks indicated gallic acid and ellagic acid, respectively, as compared to standard references of gallic acid appeared at 12.72 and ellagic acid at 46.21 min. Moreover, the contents (gallic acid and ellagic acid) of CO - EtOH were 10.2 and 15.0 mg/g extract, respectively.

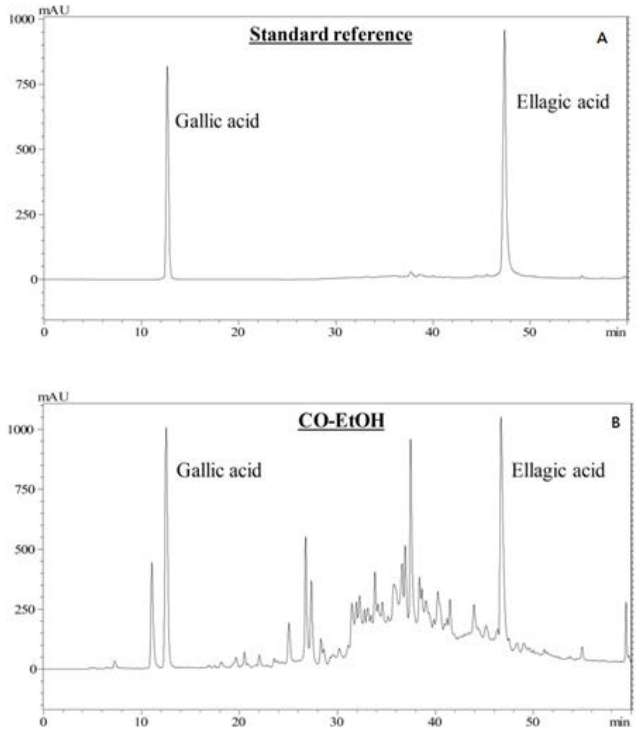

Figure 1. HPLC pattern of CO-EtOH and polyphenol standards. (A) Standard reference: Gallic acid (12.72 min) and ellagic acid (46.21 min). (B) HPLC chromatogram of the ethanol extract of *Canarium album* L. fruits (CO-EtOH)

3.2. Effects of CO-EtOH on Body Weight and Blood/Serum Biochemical Parameters in Diabetic Rats

All animals in this study remained healthy, showing no pathological signs or abnormalities, with similar daily food intake (data not shown) during the experimental period. As shown in Figure 2A, HFD administration for 6 weeks significantly increased body weight of the rats compared with normal control (NC) group. Furthermore, DC + CO-EtOH group exhibited significantly decreased body weight gain compared to DC group before STZ injection in week 7 (Figure 2B). After STZ injection, both DC and DC + CO-EtOH groups decreased body weight, however, the body weight in three groups was comparable. These results indicate that DC + CO-EtOH offers a function in weight control but does not further deteriorate the symptom of weight loss during late stage T2D. In addition, reduced epididymal adipose tissue weight compared with DC was observed upon CO-EtOH treatment (Figure 2C). Fasting blood glucose levels were measured every other week, and a significant increase in blood glucose levels was observed in all diabetic groups compared with NC group (Figure 2D). Administration of CO-EtOH significantly rescued HFD-induced abnormal fasting glucose levels from week 3 to the end of the experimental period ($p < 0.05$). The concentrations of serum TC, TNF-α, and total bile acid were increased significantly by 49%, 46% and 83% in the DC rats (Figure 2E-G) compared with NC rats, and these biochemical parameters were suppressed to near the values of NC group after CO-EtOH administration. However, other serum biochemical parameters such as TG, FFA, and insulin had not been effectively reversed by CO-EtOH (Figure 2H-J).

3.3. Effects of CO-EtOH on Hepatic Glucose Metabolism-related Genes in Diabetic Rats and Glucose Uptake in Differentiated L6 myocyte

To address the mechanism of anti-hyperglycemic effect of CO-EtOH, we examined mRNA expression of *GLUT2*, which is responsible for glucose uptake in the liver. As shown in Figure 3A, there is no significant difference between DC group and NC group. However, CO-EtOH caused a two-fold higher *GLUT2* mRNA level compared with DC group. We further examined the genes involved in liver glucose metabolism among groups and found that DC rats exhibited decreased mRNA expression of *GK*, the key enzyme of glycolysis, to half of the amount as in NC group (Figure 3B). In contrast, the genes involved in gluconeogenesis, such as *PEPCK* and *G6Pase*, exhibited a significant increase in DC by 3.2-fold and 3.6-fold, respectively, compared with NC group (Figure 3C). As expected, administration of CO-EtOH reversed these effects remarkably. Collectively, our finding indicates that CO-EtOH might regulate glucose metabolism to protect HFD and STZ-induced rats from hyperglycemia. We further examined whether CO-EtOH targets muscle cells to decrease FBG induced by HFD and STZ. As revealed in Figure 3D, we used L6 myotube as a cell model and verified the model with insulin to stimulate glucose uptake as measured by 2-NBDG uptake. We further incubated the myotube with 0.75 mM palmitic acid for 14 hours to induce insulin resistance which led to significantly decreased glucose uptake, and found that CO-EtOH treatment for another 30 minutes after palmitic acid induction could stimulate 2-NBDG uptake by 1.8-fold ($p < 0.01$ vs. palmitic acid).

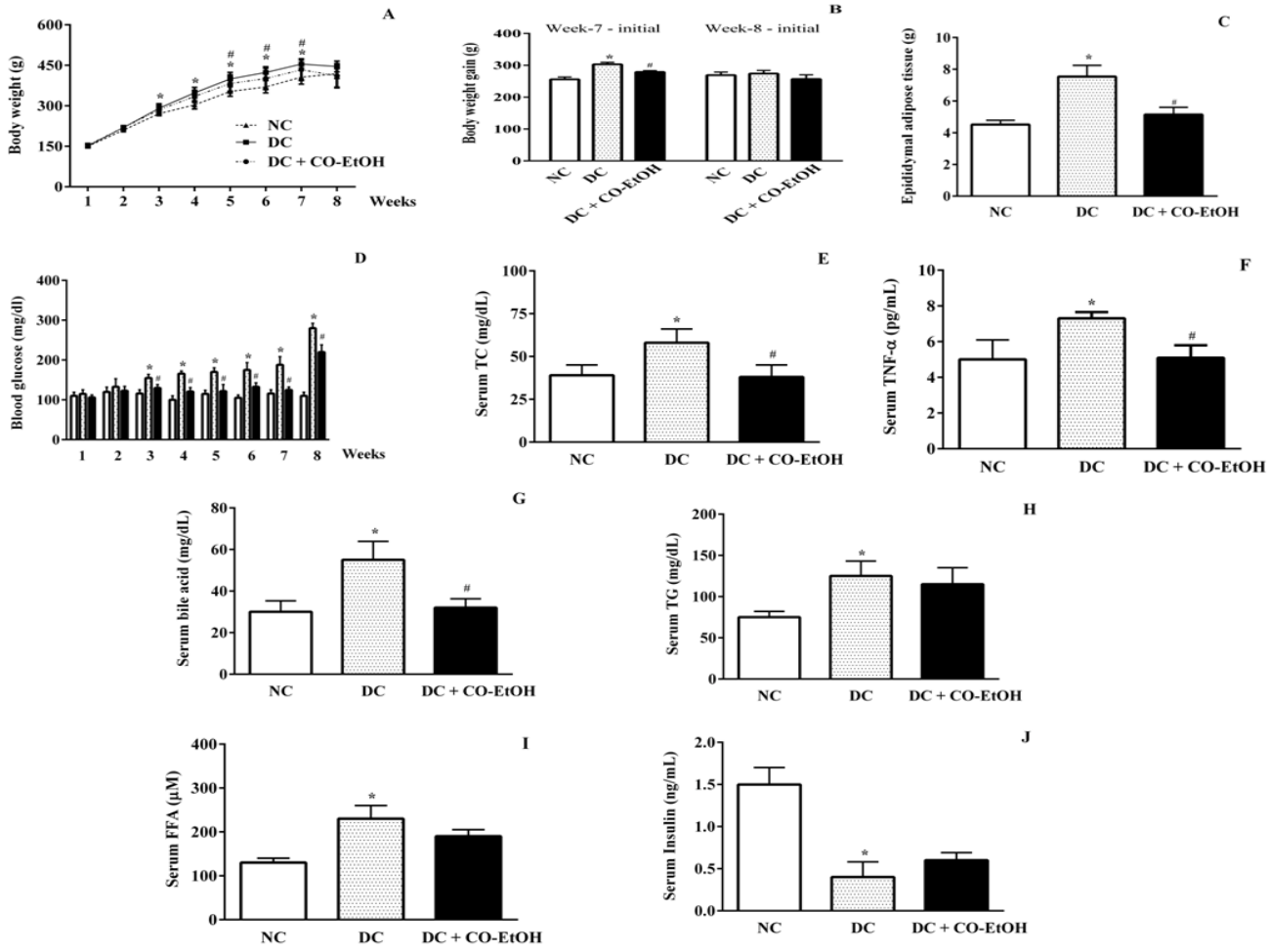

Figure 2. Effect of CO-EtOH on body weight and blood/serum biochemical parameters. Sprague-Dawley rats were orally administered CO-EtOH (760 mg/kg body weight) or CMC daily. (A) body weight (B) body weight gain (C) epididymal adipose tissue weight (D) fasting blood glucose (E) serum TC (F) serum TNF-α (G) serum bile acid (H) serum TG (I) serum FFA and (J) serum insulin. Values are expressed as mean ± SEM (n = 8-10). Statistical significance was analyzed by ANOVA and then by LSD multiple range test. Note: $p < 0.05$ was considered statistically significant. * indicates statistical difference between DC group and NC group. #indicates statistical difference between DC + CO-EtOH group and DC group

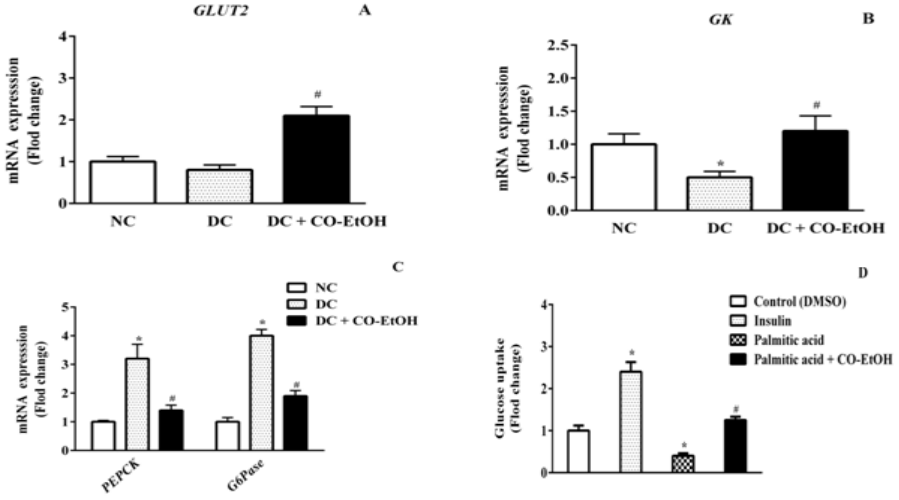

Figure 3. Effect of CO-EtOH on hepatic glucose metabolism-related genes and glucose uptake in L6 myotubes. Sprague-Dawley rats were orally administered CO-EtOH (760 mg/kg body weight) or CMC daily. The expression of *GLUT2* (A), *GK* (B) *PEPCK* and *G6Pase* (C) was evaluated by RT-qPCR. Values are expressed as mean ± SEM (n = 8-10). (D) L6 myotubes were incubated with palmitic acid for 14 hours to induce insulin resistance, followed by 1 mg/mL CO-EtOH for 0.5 hour. Thirty-minute treatment of 100 nM insulin was used as a positive control. Statistical significance was analyzed by ANOVA and then by LSD multiple range test. Note: $p < 0.05$ was considered statistically significant. In animal model: *indicates statistical difference between DC group and NC group. #indicates statistical difference between DC + CO-EtOH group and DC group. In cellular model: *indicates statistical difference between insulin or palmitic acid group and control group. #indicates statistical difference between palmitic acid + CO-EtOH group and palmitic acid group

3.4. Effects of CO-EtOH on hepatic cholesterol levels, cholesterol synthesis transport related genes and proteins in diabetic rats

Hepatic cholesterol levels were remarkably increased in the DC group by 45.8% compared with the NC group. However, treatment with CO-EtOH remarkably reduced hepatic cholesterol content (Figure 4A). We thus investigated the molecular mechanism involved in CO-EtOH-mediated amelioration of the increase in hepatic cholesterol level. As shown in Figure 3B, CO-EtOH apparently suppressed the mRNA levels of *SREBP-2* and *HMG-CoAR*, indicating that CO-EtOH inhibited endogenous cholesterol synthesis. We also found that LDLR, a receptor for delivering cholesterol from liver to peripheral tissues, was decreased in DC group and rescued by CO-EtOH in both mRNA and protein levels (Figure 4C-D); whereas the mRNA level of HDL receptor *SR-B1* significantly increased in DC group and was reversed to near the level in NC group by CO-EtOH treatment (Figure 4D). As for cholesterol efflux, we measured the gene expression of *ABCA1* and *ABCG1*. RT-qPCR results show no differences among three experimental groups (Figure 4E); however, ABCA1 protein was significantly decreased in DC group compared with NC group, and the level was increased by CO-EtOH supplement (Figure 4F).

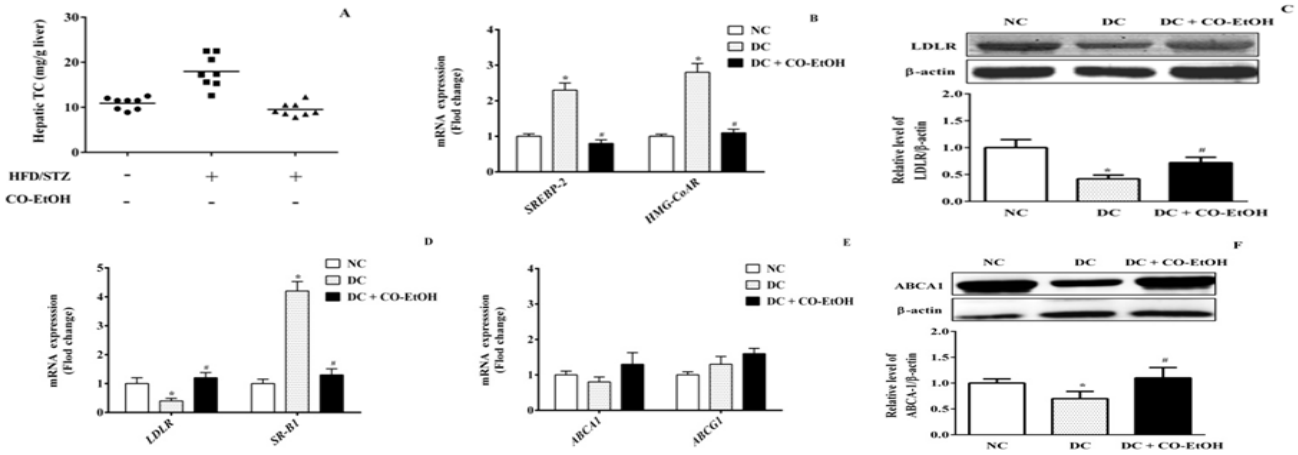

Figure 4. Effect of CO-EtOH on hepatic cholesterol levels, cholesterol synthesis and transportion-related genes and protein expression. Sprague-Dawley rats were orally administered CO-EtOH (760 mg/kg body weight) or CMC daily. (A) The content of hepatic cholesterol was analyzed using enzymatic assay. The expression of *SREBP-2* and *HMG-CoAR* (B) was evaluated by RT-qPCR. The expression of LDLR (C) was detected by western blot. The expression of *LDLR* and *SR-B1 (*D), *ABCA1* and *ABCG1* (E) were evaluated by RT-qPCR. The expression of ABCA1 (F) was detected by western blot. Values are expressed as mean ± SEM (n = 8-10). Statistical significance was analyzed by ANOVA and then by LSD multiple range test. Note: $p < 0.05$ was considered statistically significant. *indicates statistical difference between DC group and NC group. #indicates statistical difference between DC + CO-EtOH group and DC group

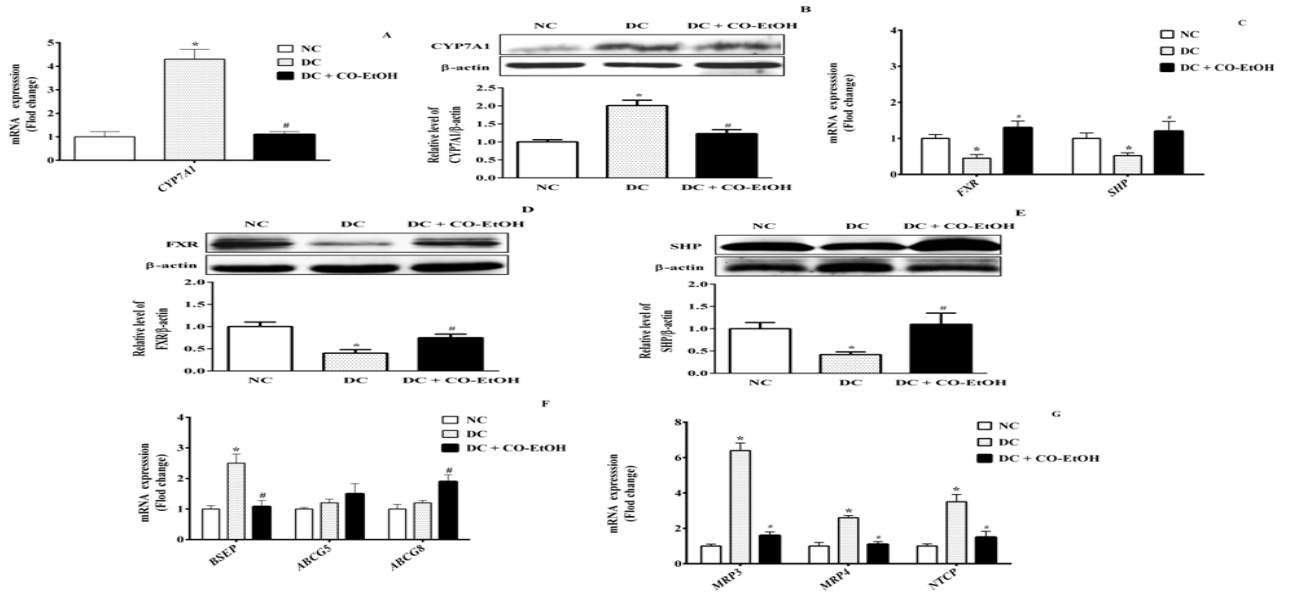

Figure 5. Effect of CO-EtOH on hepatic cholesterol degradation-related genes and proteins. Sprague-Dawley rats were orally administered CO-EtOH (760 mg/kg body weight) or CMC daily. The expression of CYP7A1 (A) was evaluated by RT-qPCR. The expression of CYP7A1 (B) was detected by western blot. The expression of FXR and SHP (C) was evaluated by RT-qPCR. The expression of FXR (D), and SHP (E) were detected by western blot. The expression of BESP, ABCG5, ABCG8 (F), MRP3, MRP4, and NCTP (G) were evaluated by RT-qPCR. Values are expressed as mean ± SEM (n = 8-10). Statistical significance was analyzed by ANOVA and then by LSD multiple range test. Note: $p < 0.05$ was considered statistically significant. * indicates statistical difference between DC group and NC group. #indicates statistical difference between DC + CO-EtOH group and DC group

3.5. Effects of CO-EtOH on Hepatic Cholesterol Degradation-related Genes and Proteins in Diabetic Rats

The decrease in hepatic cholesterol might be due to cholesterol degradation, including bile acid synthesis and secretion [38]. We thus assessed CYP7A1, the enzyme in the first and rate-limiting step for converting cholesterol into 7α hydroxycholesterol. Figure 5A and Figure 5B show that mRNA and protein levels of CYP7A1 were significantly increased in the DC rats compared to NC rats, while CO-EtOH reversed the expression of CYP7A1 in both mRNA and protein levels. We further traced the upstream of CYP7A1 and observed that FXR and SHP mRNA levels, which compared to DC group, were significantly decreased 2.1-fold and 1.8-fold, respectively. After CO-EtOH treatment, SHP mRNA level was not only reversed but was higher than that in NC group (Figure 5C). Similarly, the protein expression of FXR and SHP is consistent with their mRNA expression pattern (Figure 5D-E), indicating the role of CO-EtOH in the regulation of bile acid synthesis. We next examined the fate of bile acid. For that, we investigated the gene expression of BSEP, which is an efflux transporter that plays an important role in the disposition of bile salts from the liver into the bile canaliculus for export into the gut. We found CO-EtOH treatment significantly decreased the HFD-induced BSEP mRNA levels (Figure 5F). The pathways for transporting hepatic cholesterol to bile canaliculus were also assessed. As displayed in Figure 5F, mRNA expression of ABCG5 and ABCG8 remained the same in DC group compared with NC group; however, CO-EtOH administration could significantly increase ABCG8 mRNA levels. At last, we measured the genes involved in the transport of bile acids from hepatocytes to systemic circulation and found that the increase of the expression of MRP3 and MRP4 in DC group was rescued by CO-EtOH. Similar expression pattern of NTCP, a transporter for bile acid uptake in the basolateral (sinusoidal) membrane of hepatocytes to reabsorb bile acid from the intestine, was observed among groups (Figure 5G).

4. Discussion

T2D is a disease of chronic metabolic dysregulation. Besides pharmacological therapies for T2D, identification of modifiable lifestyle factors including dietary factors for reducing the incidence of T2D is a vital area of research [39,40]. One dietary factor of interest is the consumption of polyphenol-rich foods, which have been suggested to lower the risk of T2D [41,42,43,44]. We believe Canarium album L. might have potential benefits for diabetes treatment owing to its rich polyphenol content. In order to mimic human T2D, HFD was combined with a single shot of STZ at 35 mg/kg body weight. Previous studies confirmed high doses of STZ (>45 mg/kg body weight) critically damage the fucntion of pancreatic β-cells and consequenly, the secretion of insulin, which is considered to resemble T1D [45,46]. On the contrary, the combination of HFD and low doses of STZ generates characteristics of T2D because HFD induces obesity and insulin resistance, and a low dose of intraperitoneal STZ induces moderate impairment of insulin secretion [35,47]. Furthermore, in HFD/STZ model, the changes in the gene expression are mostly consistent to that reported in human T2D [48]. This study was thus designed to investigate the effects of CO-EtOH on hyperglycemia and hypercholesterolemia using HFD and low dose STZ-induced diabetic rat model.Normally, glucose production and uptake are kept in balance to maintain glucose homeostasis. However, in T2D and IR state, glucose homeostasis is severely disturbed and results in hyperglycemia. Liver tissues are the major sites for glucose production (gluconeogenesis), and glycogen breakdown (glycogenolysis) upon insulin sitimulation [49]. Aberrantly high levels of glucagon persist in patients with diabetes, leading to elevated rates of gluconeogenesis and glycogenolysis but decreased glycogenesis in the liver that contributes to hyperglycemia [50,51,52]. In our studies, we found CO-EtOH treatment significantly decreased fasting blood glucose levels, which were tightly associated with the increase in gene expression of GK and GLUT2 and the decrease in gene expression of PEPCK and G6Pase, indicating CO-EtOH improved hyperglycemia via regulating glucose metabolic genes involved in glycolysis and *gluconeogenesis*. In T2D, hyperglycemia is accompanied with dyslipidemia [53]. Tan et al. and Zhang et al. reported increased serum TC levels in HFD and STZ-induced diabetic rats [54,55]. Some flavonoids possess a rigid structure like (–)-epigallocatechin-3-gallate (EGCG) and curcumin that prevent HMG-CoA from entering the enzyme-active site of HMG-CoAR [56,57,58]. Clinical drugs like statins are also effective in the treatment of hypercholesterolemia by attenuating cholesterol synthesis in the liver via competitive inhibition of HMG-CoAR [59], which is a SREBP-2 target gene [10]. In the present study, serum and hepatic TC levels were increased as a result of HFD and decreased by CO-EtOH treatment. The mechanism behind the modulation possibly results from decreasing gene expression of SREBP-2 and HMG-CoAR that regulate hepatic *de novo* cholesterol biosynthesis.

The conversion of hepatic cholesterol to bile acid is an important pathway for the elimination of cholesterol from the body, which accounts for around 50% of daily cholesterol excretion [60,61]. The classic bile acid biosynthetic pathway is initiated by CYP7A1 [62]. In this study, we found that HFD led to increased serum and hepatic cholesterol, which in turn accelerated bile acid synthesis caused by elevated CYP7A1 expression. However, CO-EtOH decreased cholesterol synthesis and the subsequent expression of CYP7A1, along with reducing the synthesis of bile acid. The conclusion is consistent with the positive association between bile acid synthesis and plasma cholesterol levels [63]. We further mapped the pathways contributing to the effect of CO-EtOH and revealed that CO-EtOH could target CYP7A1 through affecting its upstream conductors, including hepatic FXR and SHP. Due to decreased bile acid synthesis upon CO-EtOH treatment, it is not surprising that hepatic bile acid excretion-related gene such as BSEP was decreased after CO-EtOH treatment. Furthermore, CO-EtOH affected ABCG5 and ABCG8 heterodimers to facilitate the transport of cholesterol into the bile [64], decreasing NTCP to hinder the reabsorption

of bile acid from the intestine, and declined MRP3 and MRP4 to flux bile acid from the liver to circulation. Taken together, CO-EtOH decreases hepatic cholesterol by controlling the whole scenario, including cholesterol synthesis, cholesterol degradation (bile acid synthesis and bile acid secretion), cholesterol efflux and bile acid absorption. We consider that CO-EtOH possibly reduced bile acid reabsorption by the liver and increased bile acid elimination in the feces resulting from decreased NTCP gene level. The mediators for bile acid efflux, such as MRP3 and MRP4, are upregulated during cholestatic conditions to enhance bile acid efflux from hepatocytes [65]. Our study clearly pointed out the significant decrease of MRP3 and MRP4 mediated by CO-EtOH treatment, and that might explain the reduced level of serum bile acid. Interestingly, increasing amount of studies has proposed bile acid to be not only involved in cholesterol metabolism but also in glucose metabolism homeostasis [66]. Subbiah et al. reported that CYP7A1 activity and bile acid are increased in STZ-treated diabetic rats [67] and concluded that insulin inhibits CYP7A1 activity and mRNA expression in rats [68,69,70]. In our data, we found serum insulin levels significantly decreased in DC group, whereas CO-EtOH showed no siginificant effect on serum isulin, suggesting that CO-EtOH might influence factors other than insulin to regulate CYP7A1 and bile acid.

Our previous work demonstrates that ethyl acetate fraction of Chinese olive fruit extract (CO-EtOAc) displays significant beneficial effects on tumor-bearing mice [30], streptozotocin-induced diabetic rats [71], and high-fat diet-induced fatty liver mice [72]. We suggest that the biofunctional activity might result from its major constituents, gallic acid and ellagic acid. To the best of our knowledge, ethyl acetate is forbidden to be used as a solvent to obtain extract for human, because its toxicity when ingested or inhaled would cause damage of tissues encountering it, and upon repeated or prolonged exposure, the damage can even extend to other organs. However, Shi J et al. reported that ethanol extract is also a good solvent extract for obtaining polyphenolic compounds and is safe for human consumption [73]. In order to enrich the content of gallic acid and ellagic acid, we used water and ethanol as our extract method in this study. Using HPLC analysis, we found that gallic acid and ellagic acid were the major ingredients in CO-EtOH similar to CO-EtOAc. Futhermore, the content of gallic acid and ellagic acid was higer in CO-EtOH than in CO-EtOAc. In the study, we prove Chinese olive as a potential materail for controlling T2D. Except the molecular mechnaim that has already been displayed in CO-EtOAc, in this article, we further explored the molecules involved in CO-EtOH mediated modulation of blood glucose, as well as the molecular mechanism regarding the scenario of cholestrol homeostasis regulated by CO-EtOH.

5. Conclusions

In this study, we used the HFD/STZ-induced T2D rat model to confirm the anti-hyperglycemia and anti-hypercholesterolemia properties of CO-EtOH as displayed by decreased fasting plasma glucose level, serum TC, BA and hepatic TC levels. Further data revealed that CO-EtOH decreased cholesterol synthesis and the consequent bile acid synthesis and transport through FXR/SHP pathway (Figure 6). In addition, we employed the myotube cell model to verify the augmenting effect of CO-EtOH on glucose uptake under palmitic acid-induced insulin resistance. These findings provide insights into the therapeutic potential of *Canarium album* L. extract in the management of metabolic disorders in diabetic patients.

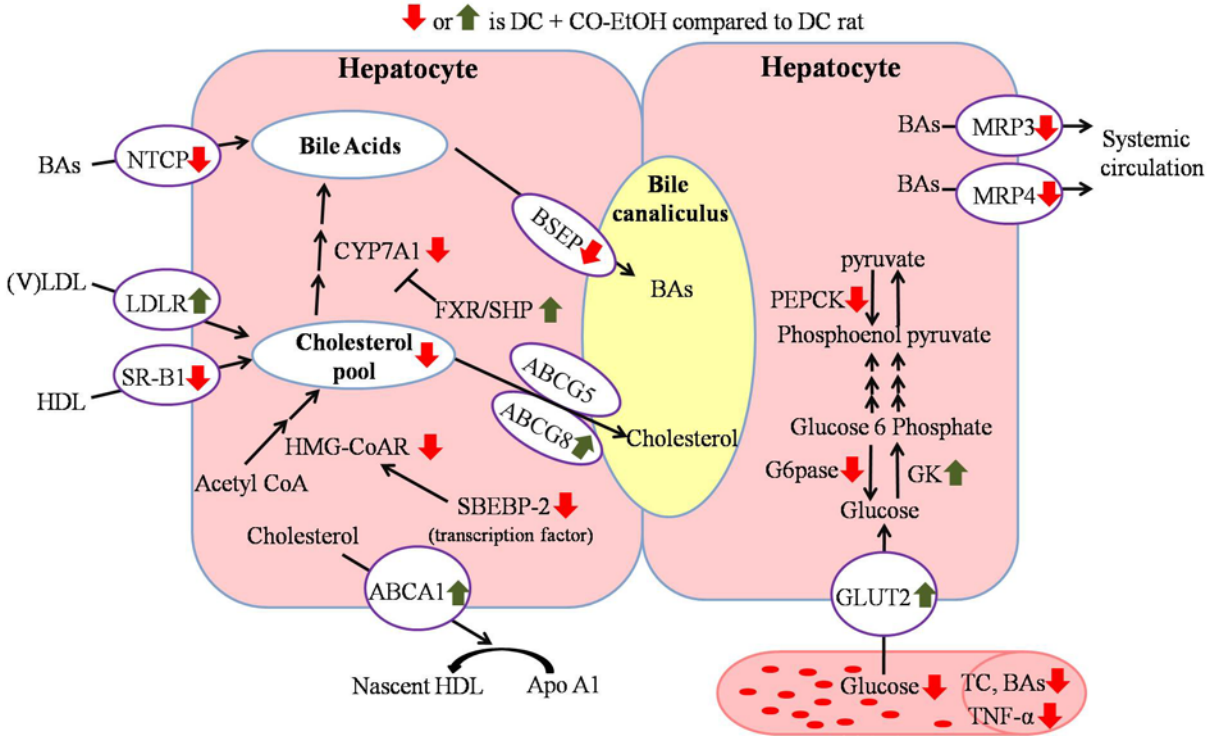

Figure 6. The potential mechanisms of CO-EtOH protect hyperglycemia and hypercholesterolemia in diabetic rats

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Author Contributions

S.C.H. and A.N.C. designed the experiments. Y.H.K. was responsible for Canarium album L. extraction. Y.T.Y., Y.H.K., S.C.H., and A.N.C. performed research, analyzed and wrote the manuscript. S.C.H. had primary responsibility for the final content. All authors have read and approved the final manuscript.

Conflicts of Interest

The authors declare no competing financial interests.

Abbreviations

T2D	type 2 diabetes
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ABCG5	ATP-binding cassette transporter G5
ABCG8	ATP-binding cassette transporter G8
BSEP	bile salt exporting pump
CYP7A1	cholesterol 7a-hydroxylase1
FXR	farnesoid X receptor
GK	glucokinase
FFA	free fatty acid
GLUT4	glucose transporter 4
HMG-CoAR	hydroxyl-3-methylglutaryl coenzyme reductase
LDLR	low density lipoprotein receptor
PEPCK	phosphoenolpyruvate carboxykinase
SHP	small heterodimer partner
SR-B1	scavenger receptor class B type I
SREBP-2	sterol regulatory element-binding protein 2
MRP3	multidrug resistance-associated protein 3
MRP4	multidrug resistance-associated protein 4

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