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Hepatoprotective effect of coffee pulp aqueous extract combined with simvastatin against hepatic steatosis in high-fat diet-induced obese rats

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ABSTRACT

This study investigated the effect of coffee pulp aqueous extract (CPE) on obesity- induced hepatic steatosis in rats and the mechanism involved. Male Wistar rats were fed high-fat diet for 12 weeks and supplemented with 1000 mg/kg BW CPE or 40 mg/kg BW simvastatin or CPE combined with 20 mg/kg BW simvastatin for another 12 weeks. The lipid profiles, presence of insulin resistance, development of hepatic steatosis and related mechanisms were investigated. Results show that CPE, simvastatin and combined treatment improved lipid profiles, insulin resistance, oxidative stress and hepatic steatosis. Correspondingly, CPE induced the lipolytic gene PPARα, while combination treatment additively suppressed the lipogenic genes PPARγ and SREBP1-c. Such effects downregulated the fatty acid transporter FAT/CD36, and activated AMPK, which concomitantly improved obese induced-hepatic steatosis. Collectively, hepato-protective effects of CPE, particularly combined with simvastatin, could broaden the therapeutic options for hyperlipidaemia and NAFLD patients who receive lipid-lowering drugs.

1. Introduction

Obesity is characterized by a chronic imbalance between energy intake and expenditure (Hatzis et al., 2013). A previous study estimated that increased fat- or energy-rich diet consumption causing hyperlipidaemia and obesity will impact nearly 1.12 billion people worldwide in 2030 (Organization, 2017). As a consequence, the risk of diseases such as type 2 diabetes mellitus, cardiovascular disease, hypertension, metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) and cancer has also increased (Barsh, Farooqi, & O'Rahilly, 2000; Luchsinger, 2006). Evidence shows a high prevalence of NAFLD and non-alcoholic steatohepatitis (NASH) in obese patients: up to 91% and 37%, respectively (Sayiner, Koenig, Henry, & Younossi, 2016). Moreover, the 3-year risk of patients with NAFLD or NASH developing hepatocellular carcinoma is up to 12.8% (White, Kanwal, & El-Serag, 2012). Thus, a therapeutic strategy for the prevention of risk factors for obesity and hepatic complications is urgently required.

The liver plays crucial roles in metabolic processes for multi-nutrient metabolism, particularly that of lipids. Previous studies demonstrated that long-chain fatty acids, high-density lipoproteins (HDL), low-density lipoproteins (LDL) and very-low-density lipoproteins (VLDL) can be transported into the liver via fatty acid translocase or

Abbreviations: ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; AST, aspartate aminotransferase; BW, body weight; CAT, catalase; FAT/CD36, fatty acid translocase; CGA, chlorogenic acid; CP, coffee pulp; CPE, coffee pulp aqueous extract; EC, epicatechin; GPx, glutathione peroxidase; HDL, high-density lipoproteins; HFD, high-fat diet; HOMA index, homeostasis assessment; LDL, low-density lipoproteins; LW, liver weight; MDA, malondialhyde level; NAD, nicotinamide adenine dinucleotide; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PPAR, peroxisome proliferator-activated receptor; PVDF, polyvinylidene difluoride; qPCR, quantitative polymerase chain reaction; CuZn-SOD, Cu-Zn superoxide dismutase; SREBP-1, sterol regulatory element-binding protein 1; TBS-T, tris-buffered saline; VLDL, very-low-density lipoproteins; VFW, visceral fat wight

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FAT/CD36 (Febbraio, Hajjar, & Silverstein, 2001; Silverstein & Febbraio, 2009). Recent evidence shows that obesity induces the expression of FAT/CD36 and its transcription factor, sterol regulatory element-binding protein 1c (SREBP-1c), resulting in hepatic lipid accumulation in dairy cows (Prodanovic et al., 2016). Furthermore, NAFLD mice induced by a high-fat diet (HFD) demonstrated upregulation of hepatic FAT/CD36 mRNA and protein expression paralleling a high hepatic triglyceride content (Koonen et al., 2007). Similarly, obesity and type 2 diabetic patients have shown overexpression of subcutaneous fat FAT/CD36 protein (Bonen, Tandon, Glatz, Luiken, & Heigenhauser, 2006). These information indicate that FAT/CD36 is one of the major contributors to the progression of dyslipidaemia, hyperlipidaemia, insulin resistance and hepatic steatosis. Moreover, dephosphorylation of AMP-activated protein kinase (AMPK) and activation of PPARγ were present with HFD-induced hepatic steatosis in mice and obese patients (Kim, Choi, Jang, & Park, 2013; Zhou et al., 2008). A previous study reported that curcumin activates AMPK, a rate-limiting enzyme for accelerating hepatic lipolysis and fatty acid oxidation and decelerating gluconeogenesis in dyslipidaemic rats (Viollet et al., 2009). This effect was able to improve the serum lipid profiles and inhibit lipogenesis-mediated inactivation of fatty acid synthase, hydroxymethylglutaryl-CoA synthase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase and LDL receptor gene expression (Kim et al., 2013). Hence, the changes in expression and/or function of transcription factors for lipid metabolism, AMPK or lipid transporters can cause lipid profile imbalance, insulin resistance or hepatic lipid accumulation.

Coffee pulp (CP) is the first by-product obtained during coffee bean processing and contains several major active compounds, such as proanthocyanidins, anthocyanidins and caffeoylquinic acid and its derivatives (Ramirez-Coronel et al., 2004). More recently, our study found that CP aqueous extract (CPE) enriched in chlorogenic acid (CGA), caffeine, epicatechin (EC), and catechin, respectively (Ontawong et al., 2019). Among these, CGA, a major component found in CP, decreases plasma and hepatic lipid levels and increases HDL in (*fa/fa*) Zucker rats and high-cholesterol diet-fed rats (Rodriguez de Sotillo & Hadley, 2002; Wan et al., 2013). In addition, CGA improves insulin sensitivity and decreases hepatic lipid accumulation in HFD-induced obese mice (Ma, Gao, & Liu, 2015) while EC, a minor component present in CPE, reduces plasma lipid profile and protected hepatic lipid accumulation in hyperlipidemic rats (Cheng et al., 2017). Moreover, EC also decreases blood glucose and lipid peroxidation and elevates glutathione levels and anti-oxidative activities of the enzymes, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in streptozotocin-induced diabetic rats (Quine & Raghu, 2005). Like CGA, caffeine, a constituent found in CP, activates AMPK and SREBP1c and SREBP2, leading to a reduce lipid content in HepG2 cells (Quan, Kim, & Chung, 2013). Here, we examined the beneficial effect of CP aqueous extract (CPE), either alone or combined with simvastatin, against hyperlipidaemia, insulin resistance, obesity and hepatic steatosis in HFD-induced obese rats, as well as the mechanism involved.

2. Materials and methods

2.1. Chemicals

Polyclonal rabbit anti-FAT/CD36 and Na^+-K^+ATPase were obtained from Novus Biologicals, CO, USA. Monoclonal mouse anti-β-actin was purchased from Abcam (Cambridge, MA, USA). Polyclonal AMP-activated protein kinase (AMPK), phosphorylated AMPK, simvastatin and complete protease inhibitor cocktail were purchased from Merck (Darmstadt, Germany). CelLytic™ MT cell lysis reagent was obtained from Sigma-Aldrich (St. Louise, MO, USA). All other chemicals used were of high purity.

2.2. CPE extract preparation, purification and qualification

Dried CP was kindly provided by Hillkoff (Chiang Mai, Thailand). CPE was previously identified for species and a voucher specimen (number 003806) has been deposited at the herbarium of Faculty of Science, Naresuan University, Phitsanulok, Thailand. One hundred grams of dried CP was weighed and blended thoroughly followed by hot water infusion for 10 min. The liquid extract was then filtered through filter paper (Whatman, Kent, UK) three times. The filtrate was evaporated by using a lyophilizer (Labconco, Kansas City, MO, USA). The total phenolic content of CPE was quantified to reach a minimum of 23.33 ± 3.16 mg GAE/g extract before use in this study. The major active components of CPE were identified using HPLC as we recently described (Ontawong et al., 2019), and the amounts of CGA, caffeine, catechin and epicatechin were reported to be 12.04 ± 0.38, 3.55 ± 0.66, 0.26 ± 0.01 and 0.18 ± 0.04 mg/g of CPE, respectively.

2.3. Animals

Male Wistar rats weighing 170–190 g were obtained from National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. Animal facilities and protocols were approved by the Laboratory Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (Protocol number: 02/2560). All rats were housed in a room maintained at 25 ± 1 °C on a 12:12 h dark–light cycle and acclimated for 1 week with free access to chow and water. Thirty-six rats were randomly distributed into six groups: normal diet (ND), ND supplemented with CPE at 1000 mg/kg BW (ND + CPE), HFD-induced obesity (HF), HF supplemented with CPE at 1000 mg/kg BW (HF + CPE), HF treated with simvastatin at 40 mg/kg BW (HF + Sim40) and HF supplemented with CPE at 1000 mg/kg BW combined with a half dose of simvastatin at 20 mg/kg BW (HF + CPE + Sim20). To make provision for reducing daily dose of simvastatin and drug toxicity while lipid-lowering effect was achievable, the combination treatment of 20 mg/kg BW simvastatin with CPE at 1000 mg/kg BW was selected in order to determine the efficacy of the combination treatment compared with single treatment. Similarly, a previous study reported that 20 mg/kg BW of simvastatin had an additive effect with nutraceutical product on lipid-lowering action without any adverse effects. In contrast, simvastatin at 40 mg/kg BW alone showed a few adverse effects e.g. myalgia and increase in creatine phosphokinase (Lasker & Chowdhury, 2012). Moreover, CGA at 10 mg/kg BW decreased total cholesterol and LDL, increased HDL and decreased the hepatic lipid content in hypercholesterolemic rats (Wan et al., 2013); therefore, 1000 mg/kg BW CPE containing approximately 12 mg/kg BW CGA and simvastatin was administered daily by oral gavage starting at week 13 and continued for the next 12 weeks until sacrifice. In order to avoid CPE-simvastatin interaction, rats from combination group was orally gavage fed twice daily; CPE in the morning and simvastatin in the evening, while others received distilled water as suggested in a study by Wallace and colleagues (Wallace et al., 2003).

2.3.1. Determination of plasma metabolic parameters

Quantitative total plasma glucose, triglycerides, total cholesterol and HDL were determined using enzymatic assay kits obtained from Biotech (Biotechnical Co., Ltd, Bangkok, Thailand). LDL levels were subsequently calculated from the formula: LDL (mg/dL) = total cholesterol − HDL − (triglyceride/5). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were quantitatively analysed using a colorimetric assay kit at the Small Animal Teaching Hospital, Faculty of Veterinary Medicine, Chiang Mai University. Plasma insulin concentrations were determined using an ELISA assay kit (Merck,

Darmstadt, Germany). Homeostasis assessment (HOMA index) was calculated to estimate insulin resistance from the following formula:

$$\frac{\text{Fasting plasma insulin } (\mu\text{U/mL}) \times \text{Fasting plasma glucose (mmol/L)}}{22.5}$$

2.3.2. Determination of hepatic lipid peroxidation

Hepatic malondialdehyde (MDA) levels, which represent oxidative stress-induced hepatic lipid peroxidation, were analysed according to the manufacturer's protocol (Cayman Chemical, Michigan, MI, USA). In brief, liver tissues were cut and suspended in CelLytic™ MT cell lysis reagent containing protease inhibitors (Roche, Applied Science, IN, USA) according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA). The tissues were then homogenized and centrifuged at 1600*g* for 10 min. The supernatant was subsequently collected for MDA measurement. Each sample was expressed as the total MDA level and normalized to the total protein concentration (nmol/mg protein) using the Bradford assay (Bio-Rad, Hercules, CA, USA).

2.3.3. Determination of liver and faecal lipid contents

Liver tissues and dried faeces were ground, and lipid contents were extracted by chloroform and isopropanol at a ratio of 7:11 as modified from Folch, Lees, and Sloane Stanley (1957). Triglycerides and total cholesterol were subsequently determined using an enzymatic colorimetric assay as mentioned above.

2.3.4. Determination of mRNA expression of lipid transporters and lipogenic, lipolytic and anti-oxidative genes using quantitative polymerase chain reaction (qPCR) technique

Total RNA was purified from freshly isolated rat liver tissues using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. First strand cDNA was subsequently obtained using the iScript cDNA synthesis kit (Macrogen, Seoul, Korea), and qPCR was performed using SYBR green real-time PCR master mix (Bioline, London, UK) on an ABI 7500 (Life Technologies, Grand Island, NY, USA). Forward and reverse primers were referenced or designed and purchased from Macrogen (Seoul, Korea) as shown in Table 1 (Chen & Cheng, 2006; Ghanbari-Niaki, Gholizadeh, Ghanbari-Abarghooi, Roudbari, & Chaichi, 2014; Houssier et al., 2008; Kim et al., 2004; Li et al., 2015; Limaye, Raghuram, & Sivakami, 2003; Spangenburg, Brown, Johnson, & Moore, 2009; Tan, Wang, Lv, & Li, 2008; Yu et al., 2003).

2.3.5. Determination of protein expression of lipid transporters and related proteins using western blotting analysis

Liver tissues were cut and suspended in CelLytic™ MT cell lysis reagent containing protease inhibitors as mentioned above. The sample was disrupted by homogenization, centrifuged at 5000*g* for 10 min at 4 °C and designated as whole cell lysate. Supernatants from homogenized samples were subsequently re-centrifuged at 100,000*g* for 2 h, and the supernatant from this step was designated as the cytoplasmic fraction while the pellet was re-suspended using the same lysis buffer and used as the membrane fraction. The protein concentration in each sample was determined using the Bradford assay, and the samples were stored at −80 °C prior to use. For electrophoresis, samples were resolved in 4X Laemmli buffer, electrophoresed in 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, West Milwaukee, WI, USA). Non-specific binding was then eliminated by blocking with 5% (w/v) non-fat dry milk in 0.05% Tween-20 in Tris-buffered saline (TBS-T) for 1 h, and the PVDF membrane was incubated with either polyclonal anti-rabbit or anti-mouse antibody (see details in Figure legend) overnight. The membrane was washed with TBS-T and incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit or anti-mouse IgG (Merck, Darmstadt, Germany) for 1 h. The target protein was detected using Super Signal West Pico chemiluminescent substrate (GE Healthcare, West Milwaukee, WI, USA) and quantitatively analysed by using the Image J program from Research Services Branch (RSB) of the National Institute of Health/National Institute of Mental Health (NIH/NIMH) (Bethesda, MD, USA).

2.3.6. Determination of liver histology and lipid accumulation

Liver tissue was excised and fixed in 4% paraformaldehyde solution for 24 h. The sample was infiltrated with xylene, embedded in paraffin and cut into 5-μm thick sections. Liver samples were subsequently stained with haematoxylin and eosin (H&E) to evaluate liver morphology. A microscopic examination was performed, and photographs were taken under a regular light microscope. For lipid accumulation, liver cryosectioning was performed by embedding in optimal cutting temperature compound and cutting into 10-μm thick sections. The section was dehydrated in 70% alcohol, stained with 2% Oil Red O in acetone for 20 min and subsequently submersed in 70% alcohol to remove excess dye. Slides were counterstained with haematoxylin and washed with water. Neutral lipid accumulation was determined using bright-field microscopic evaluation.

2.4. Statistical analysis

Data were expressed as mean ± S.E.M. Statistical differences were assessed using one-way ANOVA followed by the Tukey's post hoc test using SPSS version 23 (IBM Corp., NY, USA). Differences were

Table 1
Primer sequences and expected amplicon sizes for gene amplification.

cDNA	Genbank Acc. no.	Forward primer	Reverse primer	Amplicon size (bp)
FAT/CD36	NM031561.2	5′-GACAATCAAAAGGGAAGTTG-3′	5′-CCTCTCTGTTTAACCTTGAT-3′	159
NPC1L1	NM001002025.1	5′-CCACGAGAGGTCCACATTGG-3′	5′-GAAGAAGCAGATGGCCTCAGA-3′	87
LDLR	NM001195800.1	5′-CAGCTCTGTGTGAACCT-3′	5′-TTCTTCAGGTTGGGGATCA-3′	188
ABCG5	NM022436.2	5′-GGGAAGTGTTTGTGAACGGC-3′	5′-GTGTATCTCAGCGTCTCCCG-3′	121
ABCG8	NM130414.2	5′-CGTCAGATTTCCAATGACTTCCG-3′	5′-TCCGTCCTCCAGTTCATAGTACA-3′	243
ABCA1	NM178095.2	5′-ACGAGATTGATGACCGCCTC-3′	5′-AGCATCCACCCCACTCTCTTC-3′	110
PPARα	NM013196.1	5′-AATCCACGAAGCCTACCTGA-3′	5′-GTCTTCTCAGCCATGCACAA-3′	132
PPARγ	NM013124.3	5′-CCCTGGCAAAGCATTTGTAT-3′	5′-GGTGATTTGTCTGTTGTCTTTCC-3′	100
SREBP-1c	NM001276708.1	5′-GGAGCCATGGATTGCACATT-3′	5′-GCTTCCAGAGAGGAGCCCAG-3′	185
Cu-Zn SOD	X05634	5′-GCAGAAGGCAAGCGGTGAAC-3′	5′-TAGCAGGACAGCAGATGAGT-3′	387
GPx	NM030826	5′-CTCTCCGCGGTGGCACAGT-3′	5′-CCACCACCGGGTCGGACATAC-3′	297
CAT	NM012520.2	5′-CCTCCTCGTTCAAGATGTGGTTTTC −3′	5′-CGTGGGTGACCTCAAAGTATCCAAA −3′	122
Actin	NM031144	5′-CCTAAGGCCAACCGTGAAAA −3′	5′-GGAGCGCGTAACCCTCATAG-3′	181

ABCA1, ATP binding cassette subfamily A member 1; ABCG5, ATP binding cassette subfamily G member 5; ABCG8, ATP binding cassette subfamily G member 8; CAT, Catalase; Cu-Zn SOD, copper zinc superoxide dismutase; FAT/CD36, Fatty acid translocase; GPx, glutathione peroxidase; LDLR, Low density lipoprotein; PPARα, Peroxisome proliferator activated receptor alpha; PPARγ, Peroxisome proliferator activated receptor gamma; SREBP-1c, Sterol regulatory element-binding protein 1c.

Table 2
Effects of coffee pulp aqueous extract (CPE) on body weight and tissue weight in high-fat diet-induced obese rats.

Organ	ND	ND + CPE	HF	HF + CPE	HF + Sim40	HF + CPE + Sim20
Food intake	27.47 ± 0.49	27.66 ± 0.49	29.14 ± 0.41	27.36 ± 0.41	28.66 ± 0.51	27.29 ± 0.29
Calories intake (kcal/day)	110.44 ± 1.98	111.19 ± 1.98	155.89 ± 2.20*	146.39 ± 2.17*#	153.36 ± 2.71*	146.00 ± 1.55*#
Water intake (mL/day)	22.40 ± 1.25	20.40 ± 0.54	19.85 ± 0.44	20.42 ± 0.53	21.67 ± 1.15	20.28 ± 0.60
BW (g)	578.57 ± 19.80	538.57 ± 20.29	745.00 ± 47.15*	694.29 ± 40.39	715.83 ± 46.89	620.00 ± 20.74
BW gain (g)	374.29 ± 22.17	325.71 ± 21.36	568.33 ± 36.21*	480.71 ± 28.93	555.00 ± 51.10*	459.17 ± 20.51
LW (g)	15.35 ± 0.77	15.50 ± 0.86	27.61 ± 2.48*	25.05 ± 2.48*	29.37 ± 1.59*	26.05 ± 1.44*
Liver index**	2.63 ± 0.09	2.87 ± 0.09	3.85 ± 0.37*	3.61 ± 0.28	4.11 ± 0.03*	4.20 ± 0.10*
VFW (g)	42.07 ± 5.92	34.85 ± 4.23	82.05 ± 8.03*	58.90 ± 8.98	76.14 ± 7.98*	53.11 ± 8.11

ND, normal diet; ND + CPE, ND supplemented with CPE at 1000 mg/kg BW; HF high-fat diet-induced obese; HF + CPE, HF supplemented with CPE at 1000 mg/kg BW; HF + Sim40, HF treated with simvastatin at 40 mg/kg BW; HF + CPE + Sim20, HF treated with CPE at 1000 mg/kg BW combined with simvastatin at 20 mg/kg BW. Values shown are mean ± SEM (n = 6), *$p < 0.05$ represents the significant difference compared with normal rats, #$p < 0.05$ represents the significant difference compared with HF group. BW, Body weight; LW, Liver weight; VFW, Visceral fat weight; **Liver index was calculated by liver weight divided by body weight and multiply by 100.

considered to be significant when $p < 0.05$.

3. Results

3.1. Effects of CPE on general characteristics of high-fat diet-induced obese rats

As shown in Table 2, there was no difference in any parameters between ND and ND + CPE. However, HFD-fed rats had a significant increase in caloric intake, body weight (BW), BW gain, liver weight (LW), liver index and visceral fat weight (VFW) compared with normal diet-fed rats, indicating the development of obesity. Supplementation of CPE at 1000 mg/kg BW (HF + CPE) and the combination group (HF + CPE + Sim20) significantly reduced caloric intake when compared with HF group. Furthermore, these two experimental groups normalised the BW, BW gain and VFW similarly to ND group while rats treated with simvastatin alone (HF + Sim40) normalised the BW. These results suggest that CPE supplementation alone and combination treatment of CPE with a half dose of simvastatin additively controlled caloric intake which may help to control BW and visceral fat mass in HFD-induced obese rats. However, the liver index of rats in the HF + Sim40 and combination treatment groups remained higher than that in HF group.

In addition, the plasma total cholesterol, LDL, HOMA index and liver enzymes (AST and ALT), were increased in HFD-fed rats compared with the ND group (Table 3). In contrast, CPE supplementation was able to improve these lipid parameters, insulin resistance and liver function when compared with HF group. In particular, the combination treatment was able to reduce total cholesterol, triglyceride, LDL and HOMA index. CPE was, to highlight, more effective at reducing the liver enzyme AST and ALT than simvastatin and combination treatment, suggesting that CPE had a lipid-lowering effect and improved insulin resistance without hepatic toxicity.

3.2. CPE and combination treatment of CPE and simvastatin improve morphological features of hepatic steatosis in high-fat diet-induced obese rats

To determine the effect of CPE on liver morphology in high-fat diet-induced obese rats, liver tissue stained with H&E was semi-quantitatively analysed. As shown in Fig. 1A and B, liver morphology in ND and ND + CPE rats were not different, whereas numerous fat vacuoles in microvesicular steatosis pattern were shown in HF group, as indicated by the black bold arrow, when compared with ND (Fig. 1C). Rats supplemented with either CPE or simvastatin alone demonstrated smaller fat vacuoles in liver tissues than that HF group (Fig. 1D and E). On the other hand, treatment of obese rats with CPE combined with simvastatin at 20 mg/kg BW markedly reduced the size and the number of lipid vacuoles as observed by macrovesicular steatosis (black arrow) and seemed likely to restore liver morphology when compared with HFD-fed rats (Fig. 1F), indicating that single treatment and combination treatment of CPE with a half dose of simvastatin may improve hepatic steatosis in obese rats. Consistent with H&E staining, ND rat liver showed few fat vacuoles, similarly to the ND + CPE group, using Oil red O staining (Fig. 2A and B). In contrast, hepatic steatosis presented as markedly condensed, red-stained vacuoles in HF rat liver tissues (Fig. 2C), whereas the HF + CPE and HF + Sim40 treated groups had a lower level of red-stained vacuoles than the HF group (Fig. 2D and E). Furthermore, HF + CPE group also showed a marked decrease in fat droplet size, while combination treatment significantly reduced both fat droplet size and levels of lipid accumulation (Fig. 2D and F). These data indicate that CPE had the potential to decrease hepatic steatosis in the obese condition.

Table 3
Effects of coffee pulp aqueous extract (CPE) on plasma metabolic parameters in high-fat diet-induced obese rats.

Plasma parameter	ND	ND + CPE	HF	HF + CPE	HF + Sim40	HF + CPE + Sim20
Plasma glucose (mg/dL)	134.83 ± 6.38	141.60 ± 18.66	151.78 ± 6.15	137.56 ± 3.50	123.26 ± 5.19	131.56 ± 20.44
Plasma cholesterol (mg/dL)	64.46 ± 5.74	66.98 ± 6.25	121.80 ± 6.44*	73.17 ± 4.30#	74.18 ± 10.32#	72.20 ± 2.62#
Plasma triglyceride (mg/dL)	65.05 ± 13.15	61.32 ± 6.12	93.24 ± 9.55	66.56 ± 9.67	55.81 ± 6.17	52.23 ± 5.00#
Plasma HDL (mg/dL)	44.89 ± 2.96	41.42 ± 2.63	40.91 ± 3.99	40.73 ± 2.94	27.76 ± 2.96	32.91 ± 3.30
Plasma LDL (mg/dL)	8.02 ± 2.13	13.29 ± 5.11	62.42 ± 8.46*	19.13 ± 4.28#	35.25 ± 9.17	28.85 ± 5.63#
Plasma insulin (ng/mL)	3.15 ± 0.73	2.97 ± 0.81	5.76 ± 0.79	1.92 ± 0.88#	3.50 ± 0.93	2.33 ± 0.47
HOMA index	22.52 ± 2.98	22.11 ± 5.71	62.36 ± 10.64*	18.19 ± 7.94#	25.09 ± 8.62#	20.27 ± 4.97#
Aspartate aminotransferase (U/L)	139.86 ± 26.31	172.00 ± 8.43	244.50 ± 34.68*	146.60 ± 11.94#	198.83 ± 22.73	192.33 ± 29.62
Alanine aminotransferase (U/L)	61.43 ± 11.46	67.80 ± 5.07	163.83 ± 53.30*	68.80 ± 3.77#	96.17 ± 15.05	107.67 ± 4.86

ND, normal diet; ND + CPE, ND supplemented with CPE at 1000 mg/kg BW; HF high-fat diet-induced obese; HF + CPE, HF supplemented with CPE at 1000 mg/kg BW; HF + Sim40, HF treated with simvastatin at 40 mg/kg BW; HF + CPE + Sim20, HF treated with CPE at 1000 mg/kg BW combined with simvastatin at 20 mg/kg BW. Values shown are mean ± SEM (n = 6), *$p < 0.05$ represents the significant difference compared with normal rats, #$p < 0.05$ represents the significant difference compared with HF group. HDL, High density lipoprotein; HOMA, Homeostatic Model Assessment; LDL, Low density lipoprotein.

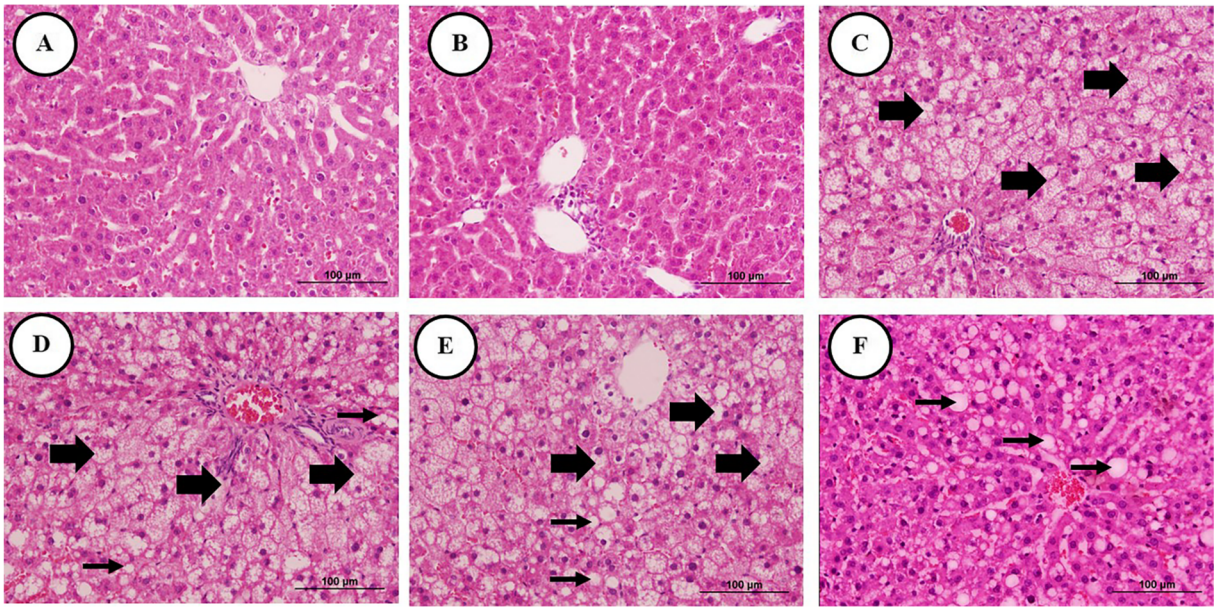

Fig. 1. Micrographs of conventional haematoxylin and eosin (H&E) staining of rat liver. (A) Normal diet (ND), (B) Normal diet treated with coffee pulp aqueous extract (CPE) (ND + CPE), (C) High-fat diet (HF), (E) HF treated with CPE (HF + CPE), (D) HF treated with simvastatin (HF + Sim40), (F) HF treated with CPE and simvastatin (HF + CPE + Sim20). Black bold arrow indicates microvesicular steatosis. Black arrow indicates macrovesicular steatosis. The data were performed at least three times from separate sets of animals (original magnification 100×).

3.3. CPE and combination treatment of CPE and simvastatin improve hepatic steatosis in high-fat diet-induced obese rats

Corresponding to histopathological assessments, hepatic triglyceride and total cholesterol contents in HF group dramatically increased, similarly to faecal triglycerides and cholesterol contents, when compared with either ND or ND + CPE (Fig. 3). In contrast, simvastatin and combination treatments significantly reduced hepatic triglyceride and total cholesterol contents whereas CPE alone significantly decreased only cholesterol content (Fig. 3A and B). In addition, a greater reduction in triglyceride content also occurred in combination treatments compared with CPE supplementation alone (Fig. 3A). Furthermore, single treatment with CPE or simvastatin significantly reduced faecal triglyceride secretion, whereas these three treatments elevated cholesterol secretion similarly to HF group (Fig. 3C and D). Such results indicate that combination treatment was greatly able to decrease hepatic steatosis, coincident with the promotion of intestinal faecal cholesterol excretion.

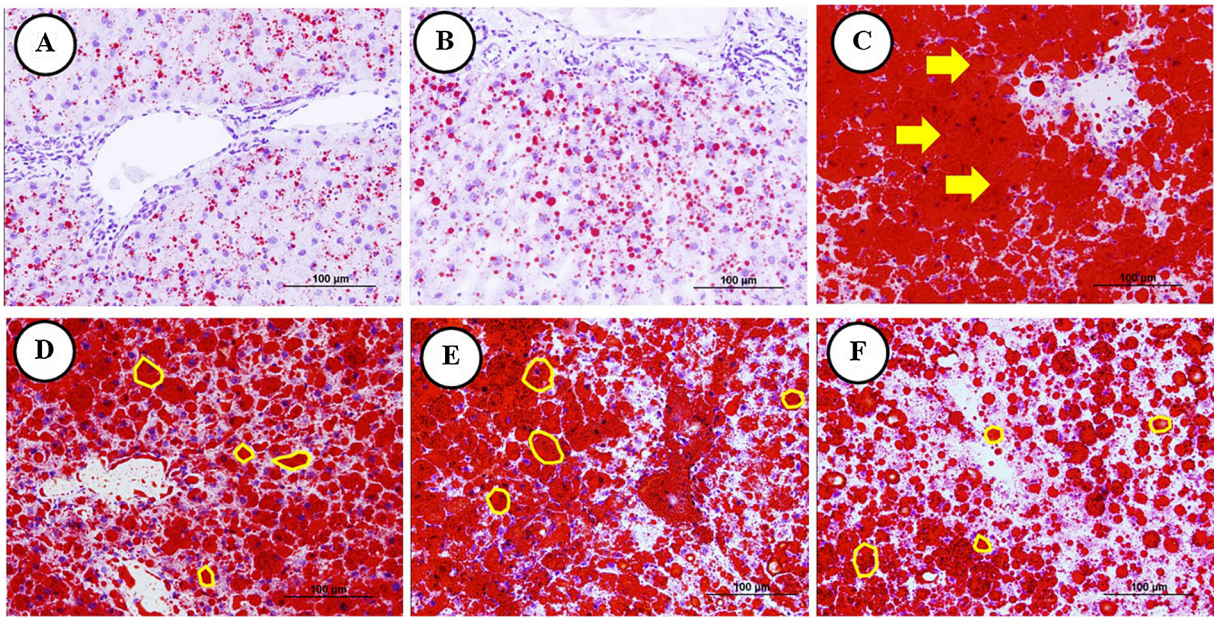

Fig. 2. Micrographs of conventional oil red O staining of rat liver tissues. (A) Normal diet (ND), (B) Normal diet treated with coffee pulp aqueous extract (CPE) (ND + CPE), (C) High-fat diet (HF), (E) HF treated with CPE (HF + CPE), (D) HF treated with simvastatin (HF + Sim40), (F) HF treated with CPE and simvastatin (HF + CPE + Sim20). Red stained colour indicates fat accumulation in liver tissues. Yellow arrow indicates the fat vacuoles in liver tissues. Enclosed line indicates the size of fat vacuoles in liver tissues. The data were performed at least three times from separate sets of animals (original magnification 100×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

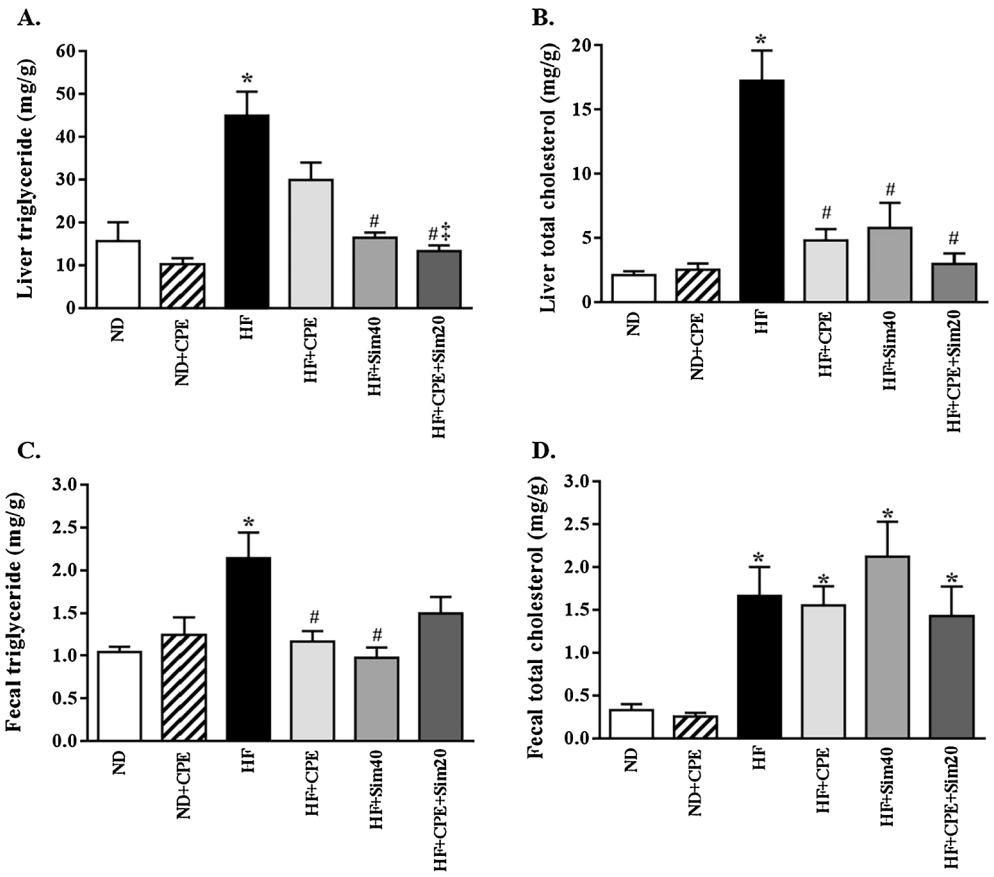

Fig. 3. Effect of coffee pulp aqueous extract (CPE) on hepatic lipids and faecal secretion. The level of (A) hepatic triglycerides, (B) hepatic total cholesterol, (C) faecal triglycerides and (D) faecal cholesterol in Normal diet (ND), Normal diet treated with coffee pulp aqueous extract (CPE) (ND + CPE), High-fat diet (HF), HF treated with CPE (HF + CPE), HF treated with simvastatin (HF + Sim40), HF treated with CPE and simvastatin (HF + CPE + Sim20) rats. Values shown are mean ± S.E.M (n = 6), $^{*}p < 0.05$ represents a significant difference compared with normal rats, $^{\#}p < 0.05$ represents a significant difference compared with HF group, $^{\ddagger}p < 0.05$ represents a significant difference compared with HF + CPE group.

3.4. CPE exerts antioxidant effects

To further clarify the hepatoprotective mechanisms of CPE against hepatic steatosis, the mRNA expression of lipid peroxidation and antioxidant gene markers, including catalase (CAT), glutathione peroxidase (GPx), and Cu-Zn superoxide dismutase (Cu-ZnSOD), was determined. As shown in Fig. 4A, liver tissues from HFD-fed rats demonstrated a significant increase in lipid peroxidation, as indicated by a high level of MDA compared with that of ND and ND + CPE rats. In contrast, total MDA was significantly decreased in the simvastatin and combination groups when compared with the HF group. Moreover, HF, HF + Sim40 and HF + CPE + Sim20 had a low expression of the free radical scavenging transcripts GPx (Fig. 4B). Interestingly, CuZn-SOD gene expression was significantly increased in ND + CPE when compared with ND group, respectively, while a greater reduction in CuZn-SOD mRNA expression was shown in simvastatin and combination treatments compared with CPE supplementation alone. These data indicate that improvement of hepatic steatosis by CPE and simvastatin partially involved its antioxidant capacity.

3.5. CPE improves hepatic steatosis by regulating SREBP-1c/PPARγ and PPARα mRNA expression

To further clarify the mechanisms of the hepatic lipid-lowering effect of CPE, the expression levels of genes regulating hepatic lipid metabolism were determined. Consistent with the hepatic lipid content, in HFD-fed rat liver, the expression of genes involved in lipogenesis, SREBP-1c and PPARγ, was significantly increased, and that of a gene related to fatty acid oxidation, PPARα, was significantly reduced compared with the ND and ND + CPE groups (Fig. 5). In contrast, PPARα was upregulated by CPE supplementation, whereas both PPARα and SREBP-1c were downregulated by combination treatment. Either single or combination treatment also markedly suppressed PPARγ expression. Again, a greater reduction in PPARα mRNA expression was shown in combination treatment compared with CPE supplementation alone. These results confirm that combination treatment improved hepatic steatosis by diminishing lipogenesis resulted in lower fatty acid oxidation.

3.6. Combination treatment of CPE and simvastatin improves hepatic steatosis via FAT/CD36 and AMPK protein expression

As shown in Fig. 6A, FAT/CD36 mRNA was significantly upregulated in the HF group when compared with ND and ND + CPE, whereas expression of the LDLR gene, which encode a cholesterol uptake transporter, was markedly downregulated in the HF condition. However, a decrease in FAT/CD36 mRNA expression was shown in HF + CPE + Sim20. Like mRNA expression, the hepatic membrane protein FAT/CD36 was upregulated in the HF group compared with the ND and ND + CPE groups, with no change in the expression of this protein in the whole cell or cytosolic fractions (Fig. 6B). In contrast, HF + CPE + Sim20 revealed a significantly lower level of membrane FAT/CD36 protein expression than HF alone. Nonetheless, there was no statistically significant difference in whole cell or cytosolic FAT/CD36 protein expression among experimental groups. Likewise, phosphorylation of AMPK, a rate-limiting enzyme for accelerating hepatic lipolysis and fatty acid oxidation and decelerating gluconeogenesis, was reduced in HF group when compared with ND, while it was markedly restored in the combination group compared with the HF group. Neither CPE alone nor simvastatin completely restored phosphorylated AMPK from HFD-induced obese rats. Therefore, combination treatment with CPE and simvastatin had an additive reducing effect on hepatic lipogenesis, primarily through inhibition of FAT/CD36 expression at both transcriptional and translational levels, resulting in modulating AMPK activity and improved hepatic steatosis.

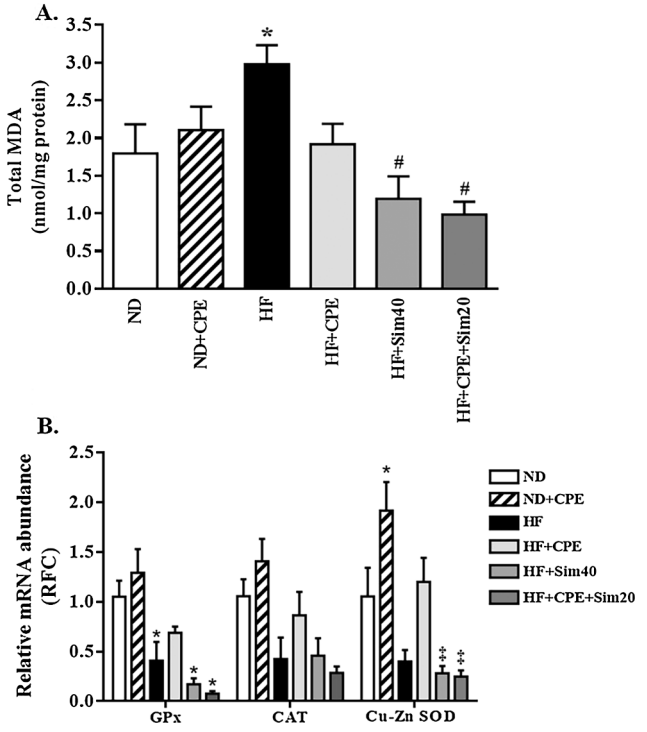

Fig. 4. Effect of coffee pulp aqueous extract (CPE) on MDA levels and antioxidant gene expression. (A) Hepatic malondialdehyde concentration (MDA) (B) hepatic mRNA expression of catalase (CAT), glutathione peroxidase (GPx), and copper-zinc superoxide dismutase (Cu-Zn SOD) from Normal diet (ND), Normal diet treated with coffee pulp aqueous extract (CPE) (ND + CPE), High-fat diet (HF), HF treated with CPE (HF + CPE), HF treated with simvastatin (HF + Sim40), HF treated with CPE and simvastatin (HF + CPE + Sim20) rats. Values shown are mean ± S.E.M (n = 6); $^{*}p < 0.05$ represents a significant difference compared with normal rats, $^{\#}p < 0.05$ represents a significant difference compared with HF group, $^{\ddagger}p < 0.05$ represents a significant difference compared with HF + CPE group.

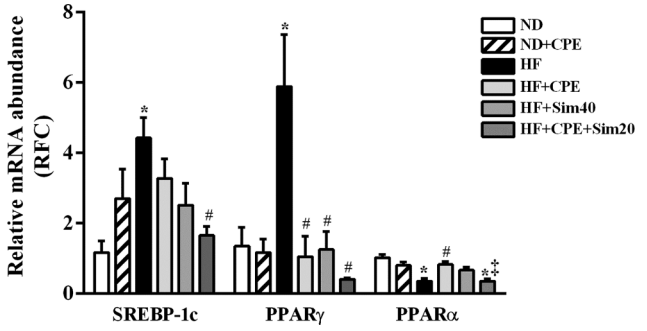

Fig. 5. Effect of coffee pulp aqueous extract (CPE) on hepatic lipogenesis and lipolysis gene markers. Hepatic mRNA expression levels of SREBP-1c, PPARγ, and PPARα from Normal diet (ND), Normal diet treated with coffee pulp aqueous extract (CPE) (ND + CPE), High-fat diet (HF), HF treated with CPE (HF + CPE), HF treated with simvastatin (HF + Sim40), HF treated with CPE and simvastatin (HF + CPE + Sim20) rats. Values shown are mean ± S.E.M (n = 6); $^{*}p < 0.05$ represents a significant difference compared with normal rats, $^{\#}p < 0.05$ represents a significant difference compared with HF group, $^{\ddagger}p < 0.05$ represents a significant difference compared with HF + CPE group.

4. Discussion

It has been described in a review that obesity induces insulin resistance and triggers altered transcription of genes regulating hepatic lipogenesis, lipolysis and fatty acid oxidation, resulting in hepatic steatosis (Fabbrini, Sullivan, & Klein, 2010). Hence, the identification of new strategies to modulate these key regulators in obesity-induced hepatic steatosis might prove useful for clinical management, which could also prevent further development of NAFLD, NASH, cirrhosis and hepatocellular carcinoma. At present, prescription drugs for NAFLD, such as glucagon-like peptide-1 analogues and statin, are available and have been raised as the guidance statements (Chalasani et al., 2018). Nonetheless, adverse side effects, including lactic acidosis, rhabdomyolysis and drug interactions have been reported (Filippatos et al., 2008; Golomb & Evans, 2008). Thus, the lipid-lowering effects of natural medicines have raised attention.

This study demonstrated the lipid-lowering effect of CPE against obesity and hepatic steatosis *in vivo* due to its anti-lipogenic and antioxidant activities. A previous study showed that coffee pulp aqueous extract contains 28.74% gallic acid, which exhibits high antioxidant activity against 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and 1,1-diphenyl-2-picrylhydrazyl radical scavenging activities *in vitro* (Duangjai et al., 2016). In addition, CGA, a major constituent of *Eucommia ulmoides* leaves, Panax ginseng berry and CPE, has shown both direct and indirect beneficial effects on glucose and lipid metabolism (Attele et al., 2002; Hao et al., 2016). For example, CGA reduces plasma lipids, weight gain, hepatic steatosis and insulin resistance in (*fa/fa*) Zucker rats and high-cholesterol diet-induced obese mice (Ma et al., 2015; Rodriguez de Sotillo & Hadley, 2002). CGA also increases superoxide anion scavenging activity and effectively inhibits hepatic lipid peroxidation in rats with paraquat-induced oxidative stress (Tsuchiya, Suzuki, & Igarashi, 1996). Similarly, this study revealed, by normalization of three major antioxidant genes, that CPE has a high antioxidant capacity and showed strong induction of Cu-Zn SOD mRNA synthesis in normal rats.

The present study also showed that CPE up-regulated PPARα lipolysis gene, and down-regulated PPARγ lipogenesis gene. Simvastatin treatment only directly inhibited PPARγ gene expression similarly to previous study (Yang et al., 2016). In addition, the combination of CPE and a half dose of simvastatin had an additive effect on improvement of obesity-induced hepatic steatosis by down-regulation of FAT/CD36, SREBP1-c and activation of AMPK. Recent study demonstrated that cholesterol crystals induced ROS production, resulting in up-regulation of CD36 expression in macrophage cells (Kotla, Singh, & Rao, 2017). On the other hand, CGA, a major constituent found in CPE, suppresses FAT/CD36 mRNA expression, resulting in reduced hepatic FFA accumulation (Huang, Liang, Zhong, He, & Wang, 2015). Similarly, simvastatin downregulates mRNA and protein expression of this transporter in ApoE KO mice (Yin et al., 2017). Since the activation of AMPK has been implicated as the crucial step for enhancing lipid utilization and improving insulin resistance (Srivastava et al., 2012), an increase in AMPK phosphorylation was shown in muscle and heart tissues in FAT/CD36$^{-/-}$ mice. These findings suggest that FAT/CD36 controls AMPK activity to maintain cellular fatty acid homeostasis (Samovski et al., 2015). Consistent with the effect of statins on AMPK activation (Sun et al., 2006), catechin and caffeine, the minor components found in CPE, also stimulate AMPK in 3T3-L1 adipocytes and HepG2 cells, respectively (Murase, Misawa, Haramizu, & Hase, 2009; Quan et al., 2013). Correspondingly, activation of AMPK inhibits nuclear translocation of SREBP-1c which subsequently reduces fatty acid synthesis and hepatic steatosis in mice (Li et al., 2011). Hence, the additive effect of combination of simvastatin and CPE, where the latter exerts antioxidant property, could down-regulate FAT/CD36, SREBP1-c and subsequently increases phosphorylation of AMPK, resulting in improved insulin resistance and reduced hepatic lipid content. Like SREBP-1c, PPARγ has shown to induce lipoprotein lipase and FAT/CD36, which are the causes of obesity and related diseases (Boelsterli & Bedoucha, 2002). A previous study reported that 50% downregulation of PPARγ improves insulin sensitivity in heterozygous PPARγ knockout mice (Miles, Barak, He, Evans, & Olefsky, 2000). Again, CPE and/or simvastatin reduced PPARγ mRNA levels, which could further downregulate FAT/CD36 cell surface expression and attenuate insulin resistance and hepatic

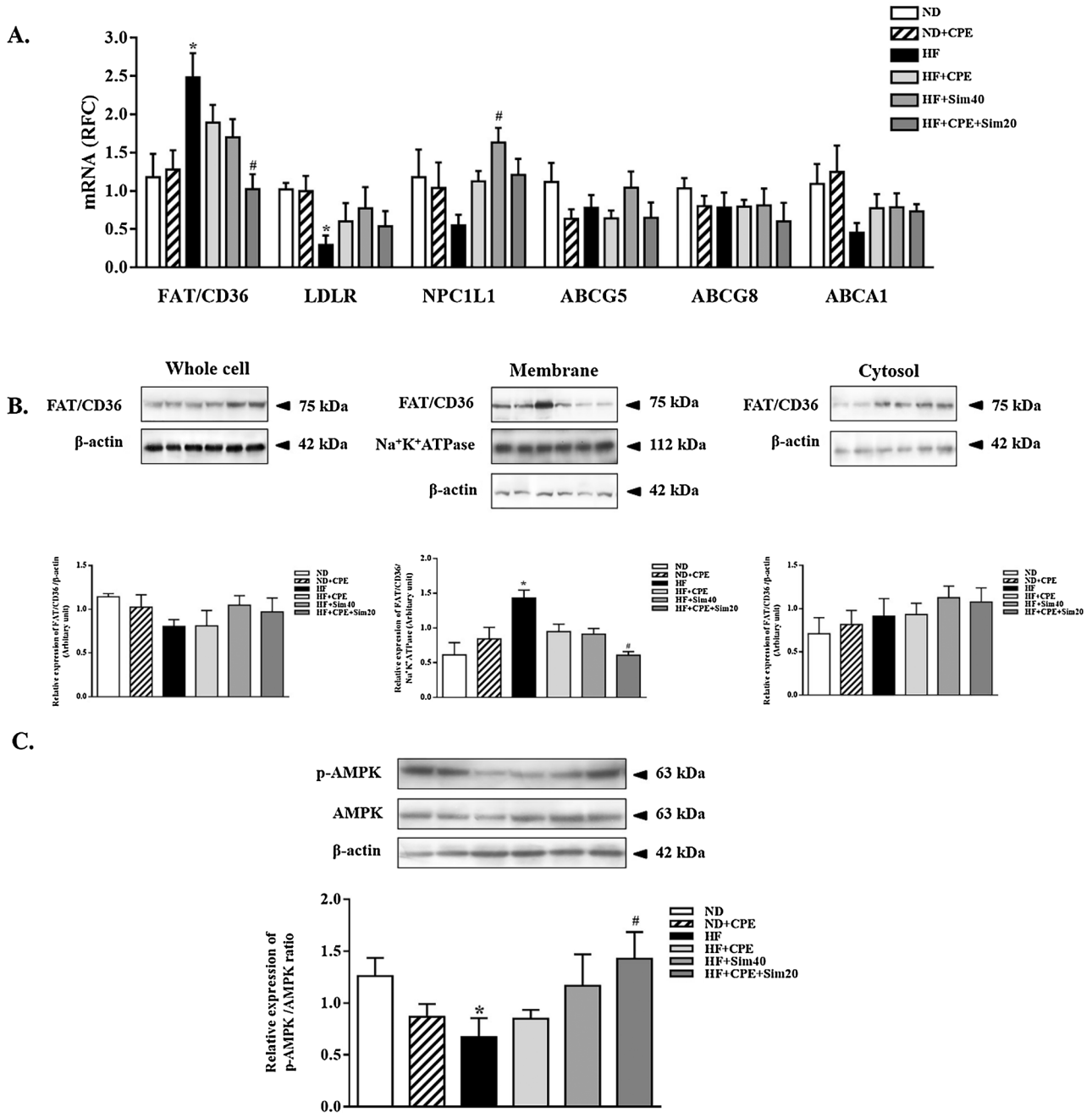

Fig. 6. Effect of coffee pulp aqueous extract (CPE) on lipid transporter expression and AMPK phosphorylation. (A) Hepatic mRNA expression levels of lipid transporters, (B) FAT/CD36 protein expression in whole cell, membrane and cytosolic fractions and (C) Phosphorylated AMPK and total AMPK protein expression in whole cell lysate fraction in Normal diet (ND), Normal diet treated with coffee pulp aqueous extract (CPE) (ND + CPE), High-fat diet (HF), HF treated with CPE (HF + CPE), HF treated with simvastatin (HF + Sim40), HF treated with CPE and simvastatin (HF + CPE + Sim20) rats. A representative blot of FAT/CD36, p-AMPK and AMPK protein expression is shown on the top panel and quantification of relative protein expression in each fraction normalised by their references including Na^+K^+ATPase and β-actin, respectively, is presented on the bottom. Values shown are mean ± S.E.M (n = 3); $^*p < 0.05$ represents a significant difference compared with normal rats, and $^\#p < 0.05$ represents a significant difference compared with HF group.

steatosis.

Recently, ischemic heart disease *patients treated with the combination of* 20 mg of simvastatin and a nutraceutical product composed of 200 mg of bergamot juice dry extract, 120 mg of phytosterols, 80 mg of artichoke leaf extract and 20 mg of vitamin C showed a marked reduction in total cholesterol, LDL cholesterol and triglycerides, without any side effects, similarly to 40 mg simvastatin alone (Campolongo et al., 2016). However, patients treated with 40 mg simvastatin show myalgia, whereas no adverse event occurred in the combination treated group (Campolongo et al., 2016). Moreover, treatment with 250 mg/kg BW procyanidin-rich in grape seed extract and 2% cholestyramine, the bile acid sequestrant, in mice could reduce intestinal and hepatic lipid synthesis and downregulate the expression of genes involved in hepatic cholesterol synthesis and lipogenesis compared with cholestyramine treatment alone (Heidker, Caiozzi, & Ricketts, 2016). Thus, combination therapy with a lipid-lowering drug and CPE might also be an option for minimizing adverse side-effects and reducing the dosage of lipid-lowering therapies.

In conclusion, this study reported for the first time the beneficial effects of CPE combined with simvastatin against hyperlipidaemia,

insulin resistance and hepatic steatosis. Besides the classical action of simvastatin, CPE partly contributed a hepatic lipid-lowering effect by inhibiting free fatty acid absorption mediated by FAT/CD36 and improving insulin resistance via AMPK, SREBP-1c and PPARγ. CPE also has a direct counteractive effect on oxidative stress via modulation of antioxidant genes. This magnificent effect of CPE makes it a new alternative approach as a natural lipid lowering-supplement which could broaden the therapeutic options for hyperlipidaemia and NAFLD patients who receive lipid-lowering drugs.

5. Ethical statement of animal experiment

I testify on behalf of all co-authors that animal facilities and protocols in our article submitted to *Journal of Functional Foods* were approved by the Laboratory Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (Protocol number: 02/2560). All rats were housed in a room maintained at 25 ± 1 °C on a 12:12 h dark–light cycle and acclimated for 1 week with free access to chow and water.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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