Prev. Nutr. Food Sci. 2022;27(4):384-398
https://doi.org/10.3746/pnf.2022.27.4.384
ISSN 2287-8602

Pluchea indica Leaf Extract Alleviates Dyslipidemia and Hepatic Steatosis by Modifying the Expression of Lipid Metabolism-Related Genes in Rats Fed a High Fat-High Fructose Diet

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ABSTRACT: This study evaluated the effect of *Pluchea indica* leaf extract (PIE) on dyslipidemia and lipid accumulation in the liver, emphasizing its molecular mechanisms in regulating lipid metabolism in rats fed a high fat-high fructose diet (HFFD). Male rats were fed HFFD (40% lard and 20% fructose) for ten weeks. They were then divided into four groups receiving distilled water, PIE (100 or 300 mg/kg/d), and pioglitazone (10 mg/kg/d) for a further six weeks, during which the HFFD was continued. After the experiment, fasting blood glucose (FBG), oral glucose tolerance (OGT), serum insulin and leptin levels, lipid profiles, and hepatic triglyceride content were measured. Histological examination and expression levels of lipid metabolism-related genes in the liver were measured. HFFD-fed rats indicated a significantly increased FBG, serum leptin, and homeostasis model assessment of insulin resistance (HOMA-IR) scores with impaired OGT and dyslipidemia compared to rats fed a normal diet. PIE significantly reduced FBG, serum leptin, and HOMA-IR scores and improved OGT. Additionally, PIE significantly improved dyslipidemia and decreased serum-free fatty acids and liver triglyceride content. Hepatic histological examination showed a marked reduction lipid accumulation in relation to HFFD controls. Interestingly, PIE significantly downregulated the expression of lipid synthesis-related genes and upregulated the expression of fatty-acid oxidation-related genes. In conclusion, PIE alleviates dyslipidemia and hepatic steatosis in HFFD rats plausibly by increasing insulin resistance and modifying the gene expression associated with lipid metabolism. PIE may be used as preventive nutrition for dyslipidemia and hepatic steatosis.

Keywords: dyslipidemias, hepatic steatosis, non-alcoholic fatty liver disease, *Pluchea indica* (L.)

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by excessive fat accumulation in hepatocytes in people who drink little or no alcohol. NAFLD ranges in severity from non-alcoholic fatty liver, or simple hepatic steatosis, to non-alcoholic steatohepatitis (NASH), cirrhosis, and ultimately hepatocellular carcinoma (Glen et al., 2016; Pydyn et al., 2020). NAFLD is associated with various kinds of metabolic disorders, such as insulin resistance, dyslipidemia, and obesity (Byrne and Targher, 2015), and is considered the hepatic manifestation of the metabolic syndrome (Marchesini et al., 2001). Metabolic syndrome is a cluster of conditions characterized by abdominal obesity, insulin resistance, hypertension, and dyslipidemia (Grundy et al., 2004). NAFLD is becoming the most common liver illness worldwide, with prevalence ranging between countries from 13.8～31.8% and a global prevalence about 25% (Younossi et al., 2016). In one study, NAFLD's prevalence was 19.3% and 60.5% in non-obese and obese persons, respectively (Wei et al., 2015). Hence, obesity is a significant risk factor for NAFLD development. Great effort is being put into developing new therapeutic agents for obesity and NAFLD (Sheng et al., 2019).

The mechanism of NAFLD pathogenesis remains unclear. However, a multiple-hit hypothesis has now been recognized, substituting plan outdated two-hit hypothesis for the progression of NAFLD. The multiple-hit hypothesis proposes that several factors may act in parallel, pro-

Received 26 May 2022; Revised 23 August 2022; Accepted 26 September 2022; Published online 31 December 2022

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viding more pathways toward NAFLD (Arroyave-Ospina et al., 2021). Insulin resistance is a key factor in developing hepatic steatosis and increased hepatic *de novo* lipogenesis. Impaired inhibition of lipolysis of adipose tissue causes an increased influx of fatty acids to the liver (Buzzetti et al., 2016), so preventing or treating insulin resistance could alleviate NAFLD. Hepatic *de novo* lipogenesis (DNL), the synthesis of fatty acids from a non-lipid source, including glucose and fructose, plays a role in NAFLD development also (Paglialunga and Dehn, 2016). Sterol regulatory element binding protein 1c (SREBP1c), a transcription factor, regulates genes needed for *DNL* in the liver, including *fatty-acid synthase* (*FAS*) and *glycerol-3-phosphate acyltransferase* (*GPAT*). Expression of SREBP1c, induced by insulin, causes increased lipogenesis through carbohydrate conversion into fatty acids (Ferré and Foufelle, 2010). Thus, high blood levels of insulin, in cases of insulin resistance, may cause hepatic steatosis because of SREBP1c activation (Xu et al., 2013). SREBP1c expression is also increased by chronic fructose intake, independently of insulin (Dekker et al., 2010; Li et al., 2016). During lipid catabolism, a transcription factor peroxisome proliferator-activated receptor α (PPARα) regulates the expression of genes involved in the oxidation of fatty acids, including peroxisomal *acyl-CoA oxidase 1* (*ACOX1*) and *carnitine palmitoyl transferase 1* (*CPT1*). In persons with a chronic high-fat diet, a reduction in the expression of PPARα may result in hyperlipidemia and accumulation of fat in the liver (Ipsen et al., 2018; Stec et al., 2019).

It is well known that high consumption of a diet rich in fat and sugar is associated with increased metabolic disturbances, such as obesity, insulin resistance, dyslipidemia, and type 2 diabetes mellitus (T2DM) (Wong et al., 2016). Dietary fat seems to primarily induce NAFLD development in rats with direct impacts on hepatic fat accumulation. In contrast, dietary fructose primarily induces insulin resistance of adipose tissue leading to the release of free fatty acids (FFAs) into the circulation, which is subsequently absorbed and accumulated by the liver. Thus, a combination of fat and fructose could accelerate NAFLD development (Jensen et al., 2018). Presently, there is no approved specific therapy for NAFLD apart from lifestyle changes, such as a controlled diet and regular exercise (Smeuninx et al., 2020). However, there is increasing evidence to suggest the efficacy of medicinal plants or their bioactive compounds in treating NAFLD (Bagherniya et al., 2018; Yan et al., 2020).

Pluchea indica (L.) Less., belonging to Asteraceae, is commonly known as Indian camphorweed, Indian fleabane, and Indian pluchea. In Thai, it is called "*Khlu*". It grows in mangrove forests and other saline habitats in many countries in Asia, including Thailand (Nopparat et al., 2019). In Thailand, *P. indica* leaves are used as the primary ingredient in several local dishes, such as yum (sour and spicy salad) and kang ped (spicy coconut milk soup), and herbal tea (Suriyaphan, 2014).

Leaves of this plant possess different biological activities, including antioxidant, anti-inflammatory, and anticancer activities (Cho et al., 2012; Buapool et al., 2013; Widyawati et al., 2014; Srimoon and Ngiewthaisong, 2015). Many studies have also shown that *P. indica* leaf extract has anti-diabetic effects. Methanolic extract of *P. indica* leaves decreased blood glucose levels in normal and streptozotocin-induced diabetic rats (Pramanik et al., 2006). Recently, a study reported the anti-diabetic activity of ethanolic extract of *P. indica* leaves through the protection of β-cells by inhibiting apoptosis and enhancing cell proliferation in streptozotocin-induced diabetic mice (Nopparat et al., 2019). Similarly, an *in vitro* study reported that *P. indica* tea could lower lipid levels by inhibiting adipogenesis in 3T3-L1 cells and pancreatic lipase activity (Sirichaiwetchakoon et al., 2018). Moreover, *P. indica* tea has a hypolipidemic effect in mice fed with a high-fat diet (Sirichaiwetchakoon et al., 2020). These studies showed the hypoglycemic and hypolipidemic activities of *P. indica* leaf extract. However, there is a poor understanding of the underlying mechanisms through which such extracts act on dyslipidemia and NAFLD in mammals with an induced insulin-resistant condition and fed a high fat-high fructose diet (HFFD). It is hypothesized that 50% ethanolic extract of *P. indica* leaves may alleviate dyslipidemia and NAFLD by improving insulin resistance and regulation of lipid metabolism in the liver. To test this hypothesis, the effects of the ethanolic extract of *P. indica* leaves on dyslipidemia and NAFLD were examined with a focus on the insulin-resistant conditions and its molecular mechanisms in regulating lipid metabolism in rats fed on a HFFD.

MATERIALS AND METHODS

Chemicals

Pioglitazone HCl was collected from Berlin Pharmaceutical Industry Co. Ltd. (Bangkok, Thailand). Thiopental sodium was from Unique Pharmaceutical Laboratories (A Div. of J.B. Chemicals & Pharmaceuticals Ltd., Ankleshwar, India).

Preparation of the 50% ethanolic extract of *P. indica* leaves (PIE)

Leaves of *P. indica* were obtained from Muang District, Samut Songkhram Province, Thailand, and were verified by Associate Professor Dr. Prathan Luecha. A voucher specimen (no. PSKKU-PL-019) of *P. indica* was deposited at the Department of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen Univer-

sity, Khon Kaen, Thailand. The fine powder of the dried leaves (200 g) was extracted thrice with 1 L of 50% ethanol for 24 h at room temperature. The pooled extract was concentrated using a rotary evaporator and freeze-dried using a lyophilizer. The powdered crude extract (PIE) was stored in dark, tightly sealed bottles and stored at 4°C until used. This procedure yielded about 30.22% of the dry weight.

Preparation of HFFD

A normal rat diet (CP Mice Feed, Samut Prakan Province, Thailand) comprising 24% protein, 4.5% fat, 47% carbohydrate, and 5% fiber. HFFD was prepared by mixing 400 g fat (lard), 200 g fructose, and 400 g powdered normal rat diet (Ponglong et al., 2019). The HFFD contained about 5.61 kcal/g, while the normal diet (ND) contained 3.04 kcal/g.

Determination of phenolic compounds in PIE

Some of the phenolic compounds in PIE were quantitated through a rapid high-performance liquid-chromatography analysis using an assay based on a reversed-phase column and an ultraviolet-visual diode array detector (high-performance liquid chromatography-diode-array detector) as described by Peñarrieta et al. (2007). Standard phenolic compounds comprised tannic acid, rutin, quercetin, gallic acid, isoquercetin, catechin, apigenin, eriodictyol, kaempferol, and hydroquinone. The measurements were performed using the external service provided by Central Laboratory Co., Ltd. (Chiang Mai, Thailand).

Animals and study designs

Male Sprague-Dawley rats (160～180 g) were collected from Nomura Siam International Co., Ltd. (Bangkok, Thailand). The animals were housed in a temperature-controlled room (25±2°C with a 12-h dark-light cycle) at the Northeast Laboratory Animal Center, Khon Kaen University. The rats were allowed to acclimatize to the animal house for a week before conducting the experiments. All procedures were conducted according to the guidelines for the care and use of experimental animals and were approved by the Animal Ethics Committee of Khon Kaen University (no. IACUC-KKU-82/2562).

Initially, rats were divided into two major groups, ND, and HFFD groups. The ND group (n=6) was fed an ND and provided regular drinking water throughout the experiment.

The HFFD group was fed HFFD and given 10% fructose in drinking water for ten weeks. After ten weeks of this regime, the animals in the HFFD group were divided into four subgroups (n=6 each) as follows:

Group 1: given distilled water at 1 mL/kg body weight (BW)/d (HFFD control)

Groups 2 and 3: given PIE 100 or 300 mg/kg BW/d, respectively (HFFD+PIE)

Group 4: given pioglitazone (PIO, a positive control) 10 mg/kg BW/d (HFFD+PIO)

All the treatments were administered orally daily for a further six weeks, and the animals were fed HFFD and 10% fructose in drinking water throughout the treatment period. The doses of PIE were adjusted based on previous work (Nopparat et al., 2019).

At the end of the experiment, the animals were fasted overnight, and an oral glucose tolerance test (OGTT) was performed. Blood was also obtained from the lateral tail vein to determine lipid profiles, serum insulin, and leptin levels. The animals were subsequently anesthetized by intraperitoneal injection of thiopental sodium 80 mg/kg. Then blood samples were collected from the abdominal vein to measure liver enzymes. The liver and the visceral fat pad were rapidly excised to examine the expression of lipid metabolism-related genes and histological changes, as described in detail below.

Determination of BW and food intake

The rats were weighed weekly throughout the experiment. Food intake was monitored daily. Energy intake was calculated daily by multiplying food intake (g) by the caloric content of the diet. The percentage of BW gain (%BWG) was calculated using the following equations:

$$\%BWG = \frac{\text{Final BW} - \text{Initial BW}}{\text{Initial BW}} \times 100$$

Serum and tissue preparations

The blood was centrifuged at 3,849 *g* for 20 min for serum separation. The serum was stored at −80°C for biochemical analysis. The liver and epididymal fat pad were washed, blotted dry, and weighed. A part of the epididymal fat pad and liver were fixed in 10% neutral buffered formalin for histological examination, and the residues were stored at −80°C for examination of gene expression. Relative organ weight was expressed as a ratio between organ and BW (g/100 g BW).

OGTT

The rats were fasted overnight (10～12 h), and blood samples were drawn from the lateral tail vein to determine the fasting blood glucose (FBG) using an Accu-Chek Performa glucometer (Roche Diagnostics, Mannheim, Germany). Then, glucose was administered orally to the animals (2 g/kg). The blood-glucose concentrations were determined at 30, 90, and 120 min after glucose loading. An area under the curve (AUC) of blood glucose (0～120 min) was calculated using a trapezoidal technique.

Measurement of serum insulin and leptin levels

Serum insulin and leptin levels were measured using commercial enzyme-linked immunosorbent assay kits (EMD Millipore, Billerica, MA, USA). The index of the homeostasis model assessment of insulin resistance (HOMA-IR) (Matthews et al., 1985), an indicator of insulin sensitivity, was calculated as follows:

$$\text{HOMA-IR} = \frac{\text{Fasting glucose (mmol/L)} \times \text{Fasting insulin (}\mu\text{IU/mL)}}{22.5}$$

Measurement of serum lipid profiles, liver triglycerides (TGs), and liver enzymes

The serum TGs, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were determined using commercial kits (Wako Pure Chemical Industries Ltd., Osaka, Japan). Serum-FFAs were also determined using a commercial kit (Abcam, Cambridge, MA, USA). Very-low-density lipoprotein cholesterol (VLDL-C) was calculated from the serum TG using the Friedewald's formula (Friedewald et al., 1972):

$$\text{VLDL-C} = \text{TG}/5$$

To predict the risk of atherosclerosis and cardiovascular disease, the atherogenic index of plasma (AIP) was determined using the following equations (Onat et al., 2010):

$$\text{AIP} = \log_{10}(\text{TG/HDL-C})$$

Hepatic TG levels were determined as previously described (Ponglong et al., 2019). Briefly, liver tissue was homogenized and extracted using isopropanol. After centrifugation, TG concentrations in the supernatant were measured using commercial kits (Wako Pure Chemical Industries Ltd.). Aspartate transaminase (AST) and alanine transaminase (ALT) were measured by the Clinical Chemistry Laboratory Unit of the Faculty of Associated Medical Sciences, Khon Kaen University.

Histological analysis

The liver tissue and epididymal fat pad were fixed in 10% neutral buffered formalin. The processed tissue sections (5-μm thickness) were stained using hematoxylin and eosin (H&E). Histological changes were investigated under a light microscope (Carl Zeiss Microscopy, Jena, Germany) in which histological images were randomly captured using an AxioCam IC microscope camera and ZEN 2 software (Blue edition, Carl Zeiss Microscopy). The size of adipocytes was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and expressed as μm^2. The average size of adipocytes of each animal was calculated from ten microscope fields.

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

Relative expression of lipogenesis-related genes: *SREBP1c*, *FAS*, and *GPAT*, and fatty-acid oxidation-related genes: *PPARα*, *CPT1*, and *ACOX1*, were evaluated in the livers of the rats.

Total RNA was isolated from the liver tissue using TRIzol reagent (Invitrogen, San Diego, CA, USA) as described previously (Pai et al., 2019). Complementary DNA (cDNA) was synthesized from the isolated total RNA using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The RT-qPCR was conducted using CAPITAL QPCR Green Master Mix (Biotechrabbit, Berlin, Germany) on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Warrington, UK). RT-qPCR cycling conditions were preincubation 95°C for 3 min, 40 cycles of amplification 95°C for 15 s, and 60°C for 31 s, followed by the melting-curve stage: 95°C for 3 s, 72°C for five minutes, and 97°C for 15 s, and cooling at 40°C for 10 min. Relative mRNA transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and expressed as the x-fold gene expression change compared with the control group. β-Actin was used as an internal control to normalize the relative quantification levels. The sequences of the primers used for the real-time PCR reaction are shown in Table 1.

Statistical analysis

Data are shown as means±standard error of the mean. The groups of data were compared using a one-way analysis of variance followed by the Tukey's posthoc test (Systat Software Inc., San Jose, CA, USA). Any *P*-value <0.05 was considered statistically significant.

RESULTS

Phenolic compounds in PIE

Tannic acid, rutin, quercetin, gallic acid, isoquercetin, and catechin were the major phenolic compounds found in the PIE (Table 2).

Effects of PIE on BW gain and food and energy intake

To investigate the effect of PIE on BW gain, food, and energy intake in rats fed HFFD, the BW and food intake were recorded daily and weekly, respectively. At week 16, rats in the HFFD control group had significantly greater BWG than that in the ND control group ($P<0.05$; Fig. 1A). Increases in BW of HFFD rats treated using PIE (100 or 300 mg/kg) were significantly lower than those of the HFFD control group. However, PIO treatment in-

Table 1. Primer sequences used in this study

Gene	Primer sequences (3′→5′)	Product size (bp)	GenBank accession no.
SREBP1c		64	NM_001276707.1
Forward	CCGAGGTGTGCGAAATGG		
Reverse	TTGATGAGCTGAAGCATGTCTTC		
FAS		196	NM_017332.1
Forward	TCGACCTGCTGACGTCTATG		
Reverse	TCTTCCCAGGACAAACCAAC		
GPAT		145	NM_017274.1
Forward	CCACATCAAGGATACAGCTCAT		
Reverse	CATTCGTGTGTTTACATCGGC		
PPARα		140	NM_013196.1
Forward	TAATTTGCTGTGGAGATCGGC		
Reverse	TTGAAGGAGTTTTGGGAAGAGAA		
CPT1		128	NM_031559.2
Forward	CAGCTCGCACATTACAAGGA		
Reverse	TGCACAAAGTTGCAGGACTC		
ACOX1		75	NM_017340.2
Forward	CTGATGAAATACGCCCAGGT		
Reverse	GGTCCCATACGTCAGCTTGT		
β-Actin		150	NM_031144.3
Forward	GGAGATTACTGCCCTGGCTCCTA		
Reverse	ACTCATCGTACTCCTGCTTGCTG		

SREBP1c, sterol regulatory element binding protein 1c; FAS, fatty-acid synthase; GPAT, glycerol-3-phosphate acyltransferase; PPARα, peroxisome proliferator-activated receptor α; CPT1, carnitine palmitoyl transferase 1; ACOX1, acyl-CoA oxidase 1.

Table 2. Phenolic content of *Pluchea indica* leaf ethanolic extract

Phenolic compounds	μg/g dry extract
Tannic acid	881.57
Rutin	401.32
Quercetin	300.61
Gallic acid	272.14
Isoquercetin	259.47
Catechin	178.11
Apigenin	40.63
Eriodictyol	<10
Kaempferol	Undetectable
Hydroquinone	Undetectable

duced an increase in the BW of HFFD rats relative to that of HFFD controls.

The food intake of the HFFD groups was significantly lower, whereas the energy intake was significantly greater than in the ND group ($P<0.05$; Fig. 1B and 1C). Interestingly, the PIE (100 or 300 mg/kg) and PIO-treated groups demonstrated a significant reduction in the average daily food and energy intake relative to the HFFD control group ($P<0.05$). These results show that PIE might prevent HFFD-induced obesity.

Effects of PIE on FBG, OGT, serum insulin, and serum leptin levels

To investigate the PIE effect on HFFD-induced insulin resistance in rats, FBG, AUC of blood glucose of OGTT, serum insulin, HOMA-IR score, and serum leptin levels were measured. At the end of the experiment, the HFFD control group had significantly higher levels of FBG, the AUC of blood glucose, serum insulin, and HOMA-IR score compared with the ND group ($P<0.05$; Fig. 2A～2D, respectively). All these parameters showed that HFFD rats were in an insulin-resistant condition.

PIE (100 or 300 mg/kg) and PIO treatments significantly decreased the levels of all these indicators of insulin resistance: FBG, the AUC of blood glucose, serum insulin, and HOMA-IR score compared with the HFFD control group ($P<0.05$; Fig. 2A～2D, respectively).

Additionally, serum leptin levels, which are associated with obesity and insulin resistance, were also higher in the HFFD group than that in the ND group ($P<0.05$; Fig. 2E). As well, PIE (100 or 300 mg/kg) and PIO treatments significantly lowered the high serum leptin levels compared with the HFFD control group ($P<0.05$; Fig. 2E).

Effects of PIE on serum lipid profiles

To investigate the effect of PIE on dyslipidemia induced by HFFD, alterations in serum lipid profiles, including TG, VLDL-C, LDL-C, HDL-C, and FFA, were investigated. As indicated in Fig. 3A～3E, respectively, there were significant increases in the serum TG, VLDL-C, LDL-C, and FFA, and a significant reduction in HDL-C in the HFFD control group compared with the ND group ($P<0.05$). Treatment with PIE (100 or 300 mg/kg/d) significantly reduced the serum TG, VLDL-C, LDL-C, and FFA levels and significantly increased the HDL-C levels compared with the HFFD control group ($P<0.05$). In the PIO-

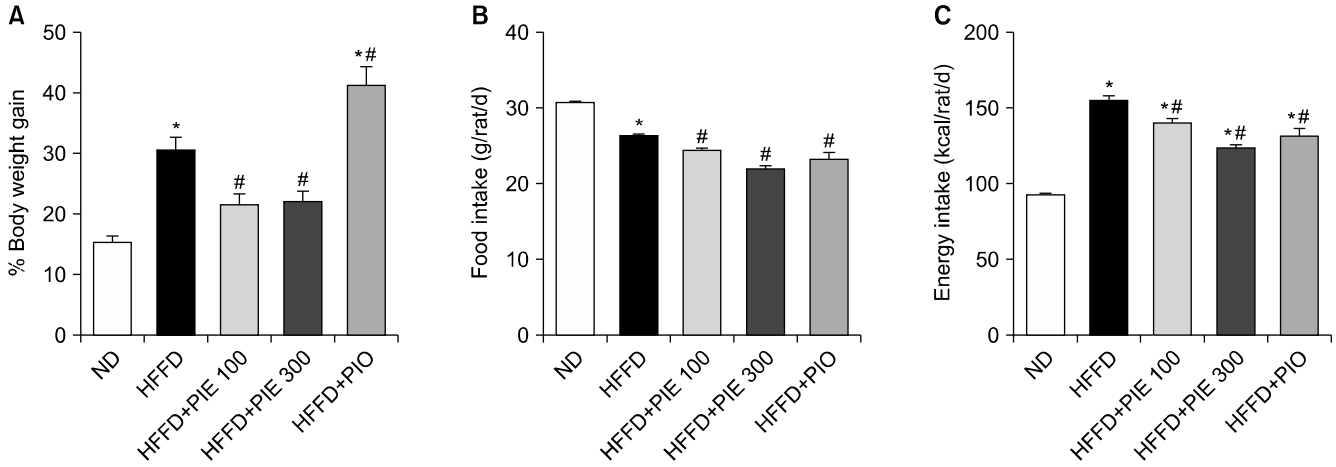

Fig. 1. Effects of *Pluchea indica* leaf extract (PIE) on body weight gain at the end of treatment (A), average daily food intake (B), and average daily energy intake (C) in high fat-high fructose diet (HFFD) rats after treatment for six weeks. Data are presented as mean±SEM (n=6 per group). $^{*}P<0.05$ when compared with the normal diet (ND) group and $^{\#}P<0.05$ when compared with the HFFD group. HFFD + PIE 100 or 300, HFFD rats + PIE 100 or 300 mg/kg/d; HFFD + PIO, HFFD rats+pioglitazone 10 mg/kg/d.

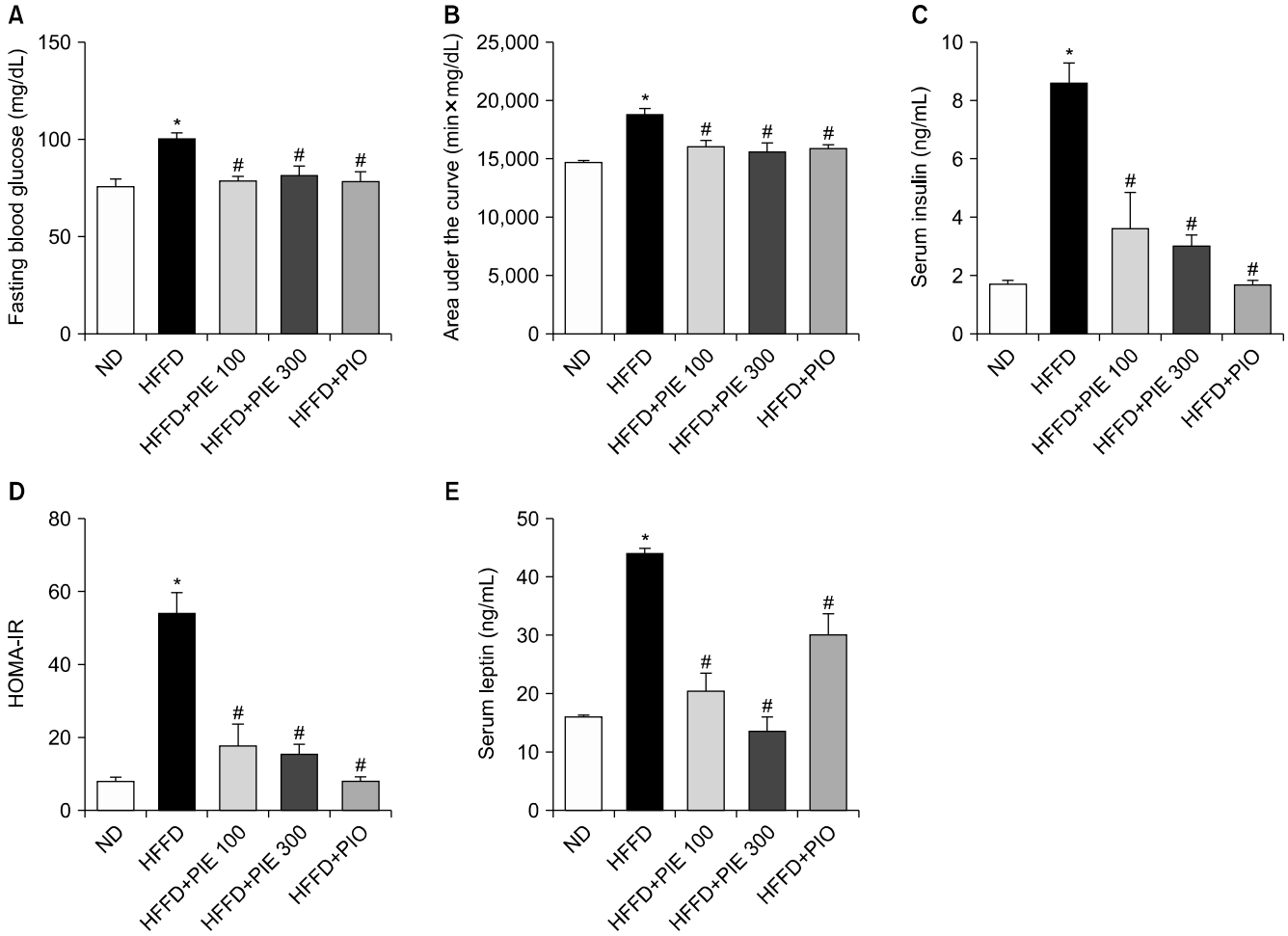

Fig. 2. Effects of *Pluchea indica* leaf extract (PIE) on fasting blood glucose (A), area under the curve of blood glucose (B), serum insulin (C), homeostasis model assessment of insulin resistance (HOMA-IR) index (D), and serum leptin (E) in high fat-high fructose diet (HFFD) rats. Data are presented as mean±SEM (n=6 per group). $^{*}P<0.05$ when compared with the normal diet (ND) group and $^{\#}P<0.05$ when compared with the HFFD group. HFFD + PIE 100 or 300, HFFD rats + PIE 100 or 300 mg/kg/d; HFFD + PIO, HFFD rats + pioglitazone 10 mg/kg/d.

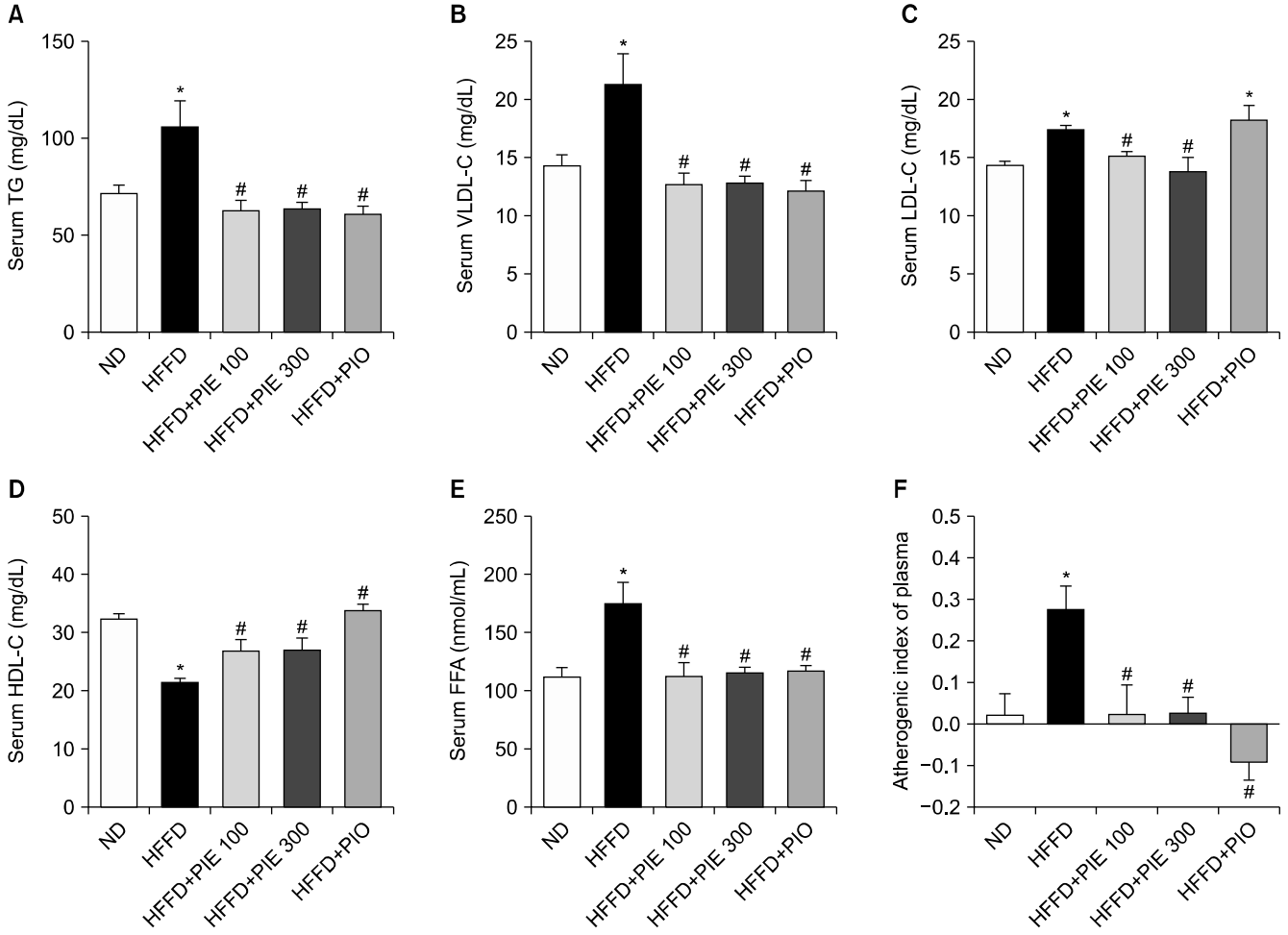

Fig. 3. Effects of *Pluchea indica* leaf extract (PIE) on the levels of serum TGs (A), VLDL-C (B), LDL-C (C), HDL-C (D), FFA (E), and atherogenic index of plasma (F) in high fat-high fructose diet (HFFD) rats. Data are presented as mean±SEM (n=6 per group). *P<0.05 when compared with the normal diet (ND) group and $^{\#}P$<0.05 when compared with the HFFD group. TG, triglyceride; VLDL-C, very-low-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FFA, free fatty acid; HFFD + PIE 100 or 300, HFFD rats + PIE 100 or 300 mg/kg/d; HFFD + PIO, HFFD rats + pioglitazone 10 mg/kg/d.

treated group, serum TG, VLDL-C, and FFA, but not LDL-C, were significantly reduced, and HDL-C was increased considerably relative to the HFFD control group (P<0.05). The AIP, the logarithm to the base 10 of the ratio of TG/HDL-C, was significantly greater in the HFFD control group (P<0.05) while it was comparable to normal levels in the PIE (100 or 300 mg/kg) and PIO-treated groups (P<0.05; Fig. 3F). Altogether, treatment with PIE improved lipid profiles.

Effects of PIE on fat tissue weight and adipocyte hypertrophy

Increased intra-abdominal lipid accumulation is a feature of obesity. Thus, the epididymal fat pad from each rat was weighed and stained using H&E to measure the size of fat cells. The relative epididymal fat tissue weight and the average size of adipocytes in the HFFD control group were significantly greater than those in the ND group (P<0.05; Fig. 4). In HFFD rats treated using PIE (100 or 300 mg/kg) and PIO, the relative epididymal fat tissue weight and the average size of adipocytes were smaller than those of the HFFD control group (P<0.05). These results indicated that PIE treatment might decrease lipid deposition in adipocytes.

Effects of PIE on liver weight and fat accumulation in the liver

To investigate the effect of PIE on HFFD-induced hepatic steatosis, liver tissues were examined using H&E staining, and hepatic TGs were measured. The relative liver weight and hepatic TG content were significantly greater in the HFFD control group compared with ND rats (P< 0.05; Fig. 5A and 5B). Interestingly, treatment using PIE (100 or 300 mg/kg) or PIO significantly reduced the relative liver weight and hepatic TG content compared with the HFFD control group (P<0.05; Fig. 5A and 5B).

Histological examination of the liver tissue indicated a marked accumulation of fat in the liver cells of the HFFD group relative to the ND group (Fig. 5C). Remarkably, the liver tissue of the PIE (100 or 300 mg/kg) and PIO-

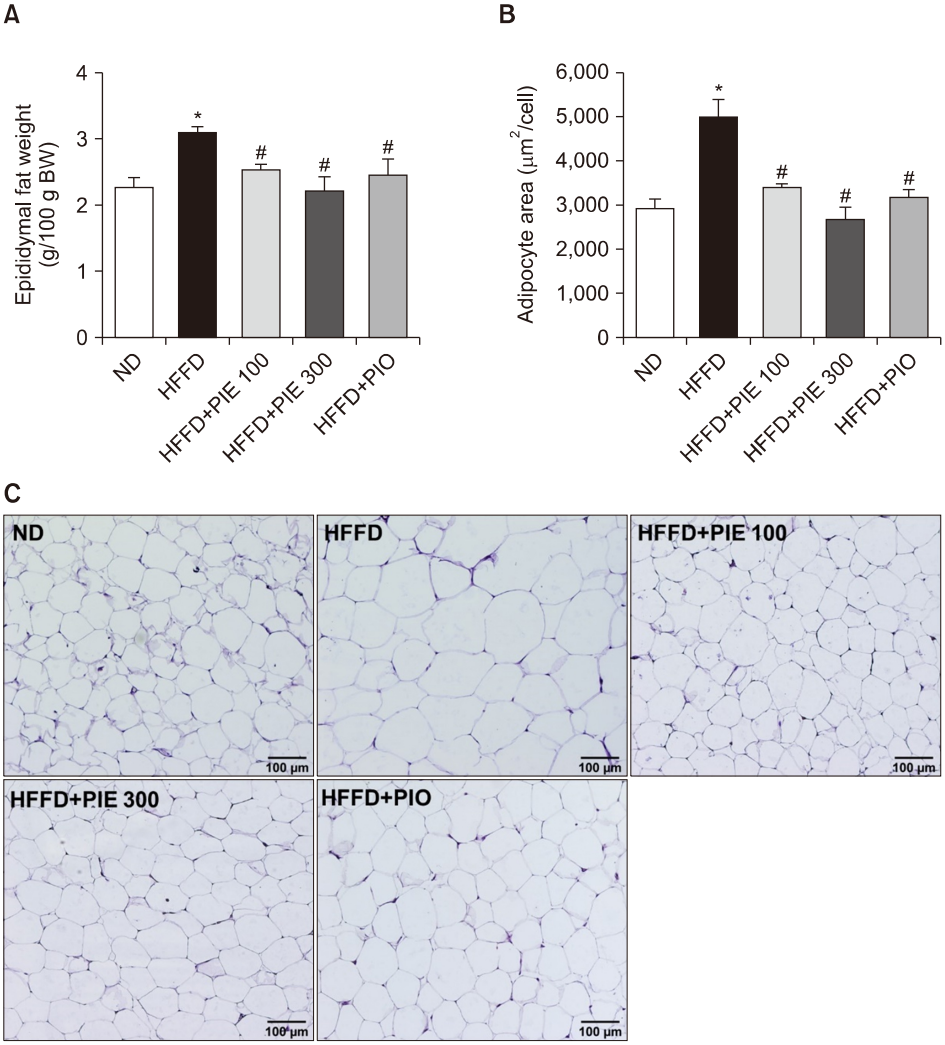

Fig. 4. Effect of *Pluchea indica* leaf extract (PIE) on relative weight of epididymal fat tissue (A), the average size of adipocytes (B), and histological pictures of adipocytes (C) in high fat-high fructose diet (HFFD) rats. Epididymal adipose tissue was stained using H&E (magnification: 100×). Data are presented as mean± SEM (n=6 per group). $^{*}P<0.05$ when compared with the normal diet (ND) group and $^{\#}P<0.05$ when compared with the HFFD group. BW, body weight; HFFD + PIE 100 or 300, HFFD rats + PIE 100 or 300 mg/kg/d; HFFD + PIO, HFFD rats + pioglitazone 10 mg/kg/d.

treated groups showed a clear decrease in fat accumulation (Fig. 5C), which was consistent with the levels of liver TG content.

Fat accumulation in the liver or NAFLD may cause some degree of liver damage. The serum levels of the hepatic enzymes AST and ALT were measured to examine whether feeding rats with HFFD would cause liver damage and whether any damage might be alleviated by PIE treatment. As shown in Fig. 5D and 5E, these levels were similar in the HFFD control and ND groups. PIE treatment did both affect the AST and ALT levels. However, a significant reduction in serum ALT level was observed in the PIO-treated group compared with the HFFD control group ($P<0.05$). These results showed that, in this study, feeding rats with HFFD for 16 weeks inducted hepatic steatosis with no hepatic injury.

Effects of PIE on the expression of genes associated with regulations of hepatic lipogenesis and fatty-acid oxidation

To investigate whether the PIE effects on serum lipid profiles and hepatic lipid accumulation were associated with a decrease in lipid synthesis and promotion of fatty-acid oxidation, the expression levels of lipogenesis-associated genes and fatty-acid oxidation-associated genes were assessed using RT-qPCR.

In the HFFD control group, the expression levels of genes regulating lipid synthesis, *SREBP1c*, *FAS*, and *GPAT* were significantly greater than that in the ND group. Still, the expression of all of these genes was significantly downregulated by PIE or PIO treatment ($P<0.05$; Fig. 6A～6C, respectively). Moreover, the expression levels of the key genes of fatty-acid oxidation, *PPARα*, *CPT1*, and *ACOX1*, were significantly reduced in the HFFD control group compared with the ND group ($P<0.05$; Fig. 6D～6F, respectively). The reduction in expression levels of *PPARα* and *ACOX1* was also reversed by the PIE (100 or 300 mg/kg for *PPARα* and 100 mg/kg for *ACOX1*) or PIO treatment compared with the HFFD group ($P<0.05$). However, there was no significant change in the expression level of the *CPT1* gene in the PIE- or PIO-treated groups. These results indicated that the hepatic steatosis alleviating effect of PIE may be associated with a reduction in hepatic lipid synthesis and an increase in fatty-acid β-oxidation.

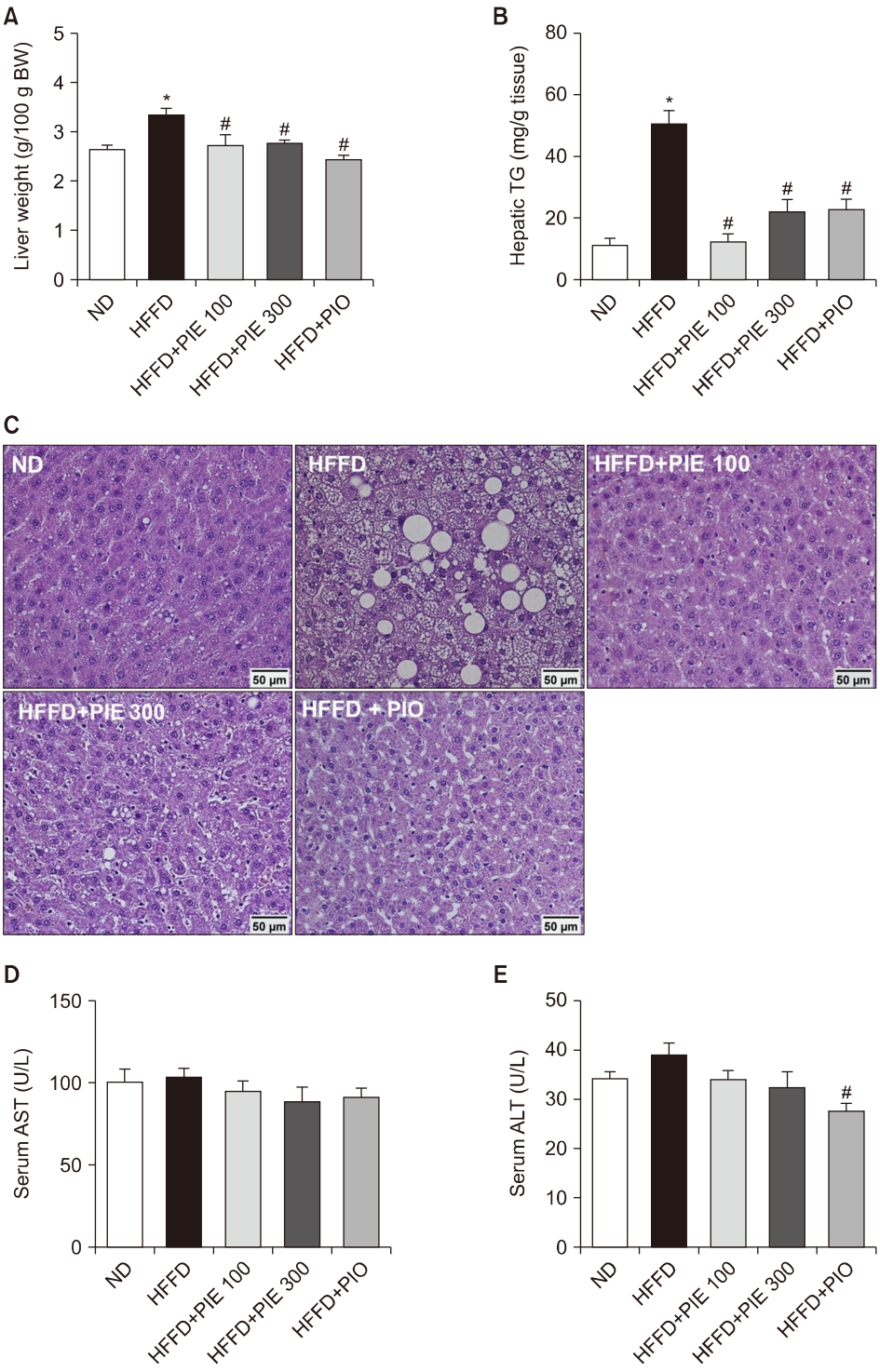

Fig. 5. Effects of *Pluchea indica* leaf extract (PIE) on liver weight (A), hepatic TGs (B), liver histological pictures (C), serum AST (D), and serum ALT (E) in high fat-high fructose diet (HFFD) rats. Liver tissue was stained using H&E (magnification: 200×). Data are presented as mean± SEM (n=6 per group). $^{*}P<0.05$ when compared with the normal diet (ND) group and $^{\#}P<0.05$ when compared with the HFFD group. BW, body weight; TG, triglyceride; AST, aspartate transaminase; ALT, alanine transaminase; HFFD + PIE 100 or 300, HFFD rats + PIE 100 or 300 mg/kg/d; HFFD + PIO, HFFD rats + pioglitazone 10 mg/kg/d.

DISCUSSION

In this study, the effects of PIE on HFFD-induced dyslipidemia and NAFLD in rats were evaluated. The results highlight that PIE is an alternative agent that can be used to alleviate dyslipidemia and NAFLD. Our findings showed that treatment using PIE for six weeks caused a significant reduction in BW gain and improvement in insulin resistance and dyslipidemia. Particularly, PIE decreased hepatic steatosis and adipocyte hypertrophy induced by HFFD. The beneficial effects of PIE on dyslipidemia and NAFLD could be the result of the inhibition of hepatic lipogenesis and the promotion of fatty-acid oxidation activities.

The chronic consumption of diets rich in saturated fats and processed sugars, particularly high fructose, is strongly associated with systemic insulin resistance, obesity, dyslipidemia, and T2DM (Calvo-Ochoa et al., 2014). High fat diet results in high levels of circulating lipids and the formation of lipid intermediates like diacylglyc-

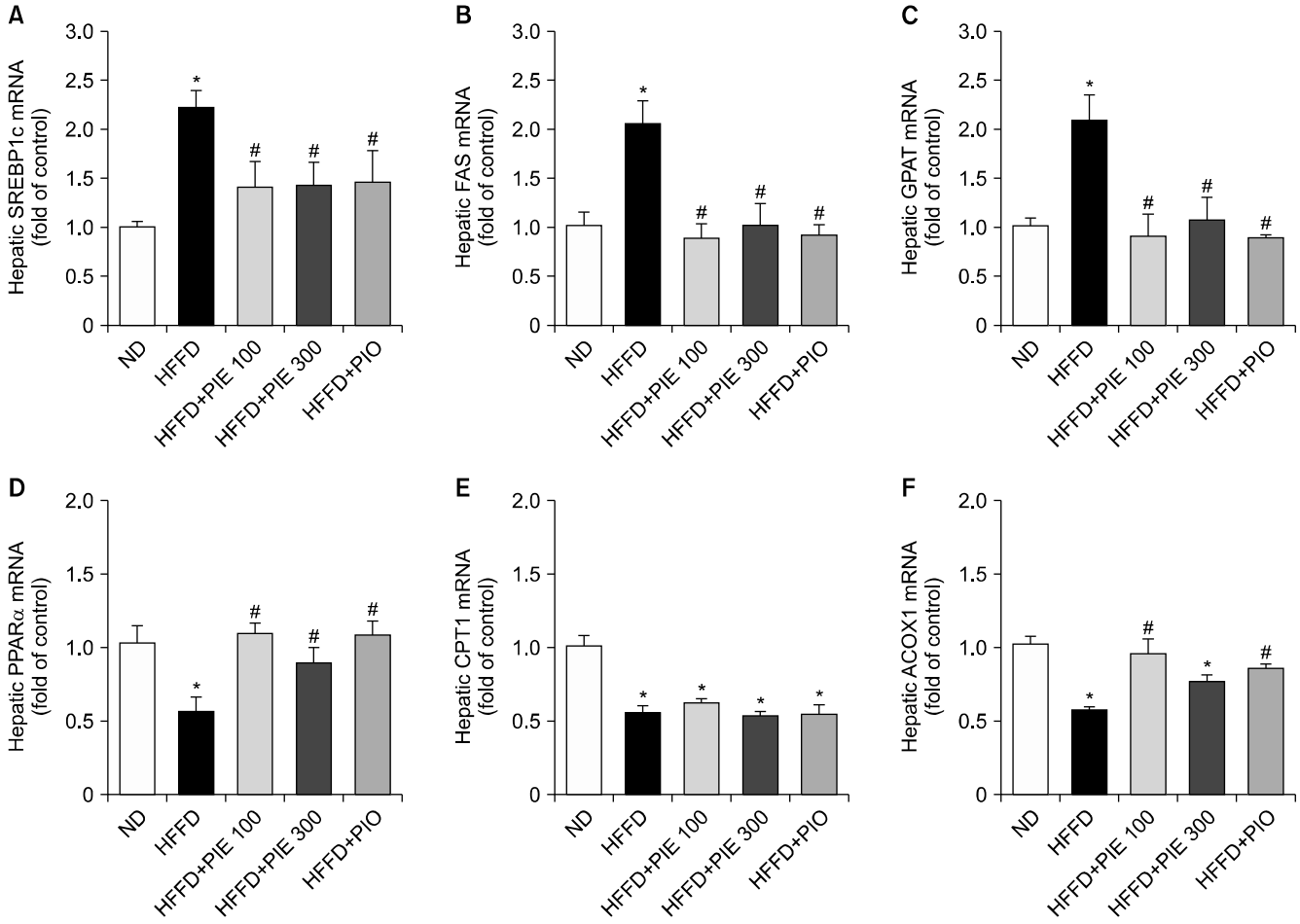

Fig. 6. Effects of *Pluchea indica* leaf extract (PIE) on the expression levels of hepatic *SREBP1c* (A), *FAS* (B), *GPAT* (C), *PPARα* (D), *CPT1* (E), and *ACOX1* (F) genes in high fat-high fructose diet (HFFD) rats. Data are presented as mean±SEM (n=6 per group). $^{*}P<0.05$ when compared with the normal diet (ND) group and $^{\#}P<0.05$ when compared with the HFFD group. HFFD + PIE 100 or 300, HFFD rats + PIE 100 or 300 mg/kg/d; HFFD + PIO, HFFD rats + pioglitazone 10 mg/kg/d. SREBP1c, sterol regulatory element binding protein 1; FAS, fatty-acid synthase; GPAT, glycerol-3-phosphate acyltransferase; PPARα, peroxisome proliferator-activated receptor α; CPT1, carnitine palmitoyl transferase 1; ACOX1, acyl-CoA oxidase 1.

erol, ceramides, and fatty acyl-CoA that could cause inflammatory pathways and impair insulin signaling, which precedes the development of insulin resistance (Sah et al., 2016). High fructose in the diet is metabolized principally in the liver and provides carbon atoms for glycerol and acyl portions of TGs, thus, increases *DNL*, leading to hepatic lipid accumulation and reduction of hepatic insulin sensitivity (Basciano et al., 2005). In our study, the long-term HFFD consumption in rats induced insulin resistance, characterized by increased FBG, impaired glucose tolerance, hyperinsulinemia, and elevated HOMA-IR index accompanied by hyperleptinemia. Interestingly, PIE treatment significantly reversed those changes nearly to the levels found in the ND group. These results show that PIE treatment enhances insulin sensitivity in HFFD-fed rats.

Leptin is an adipokine secreted from adipocytes that plays essential roles in regulating food intake and energy expenditure and is related to the impaired insulin sensitivity condition (Landecho et al., 2019). Leptin levels reportedly increase in rats fed an HFFD (Attia et al., 2019). Leptin resistance is closely associated with obesity. Leptin resistance refers to the state in which leptin fails to promote its anorectic and weight-reducing actions in the obese state, frequently coexisting with high circulating leptin levels (Andreoli et al., 2019). In this study, the HFFD-fed rats also indicated increase in BW and energy intake with high serum leptin levels. However, these parameters were restored to near-normal after six weeks of PIE treatment. Therefore, the decrease in BW, energy intake, and hyperleptinemia after PIE treatment may be related to the restoration of a leptin-sensitive state.

White adipose tissue (WAT) is a crucial site for storing excess fat and functions like an endocrine organ. During obesity development, WAT expands through an increase in fat-cell size (hypertrophy) and/or fat-cell number (hyperplasia). Subsequently, hypertrophic adipocytes indicate altered adipokine secretion and enhanced basal lipolysis causing increased FFA release (Longo et al., 2019; Herold and Kalucka, 2021). In this study, the HFFD-fed

rats increased in the weight of the epididymal fat pad and the size of epididymal fat cells, similar to a previous report (Liu et al., 2018). Interestingly, PIE treatment could reduce the weight of this tissue and the size of its cells, consistent with previous reports of *in vitro* and *in vivo* effects of *P. indica* (Sirichaiwetchakoon et al., 2018; Sirichaiwetchakoon et al., 2020). Additionally, PIE also reduced the FFA level in serum. These findings imply that PIE prevents HFFD-induced adipocyte hypertrophy.

FFAs in chylomicron remnants and those produced from *DNL* are re-esterified as TGs in the liver. Afterward, TGs are released as VLDL-C into circulation (Alves-Bezerra and Cohen, 2017). Similarly, insulin resistance induces adipose tissue lipolysis, resulting in FFA release into circulation and influx to the liver. The increased VLDL-C secretion led to hypertriglyceridemia and reduced HDL-C levels (Ormazabal et al., 2018). Long-term fructose overconsumption may promote the development of fatty liver, dyslipidemia, and insulin resistance, which result from an increase in hepatic DNL (Ter Horst and Serlie, 2017). Dyslipidemia, characterized by an elevation of serum total cholesterol (TC), LDL-C, or TG, and decreased serum HDL-C concentration, is a crucial risk factor in cardiovascular disease (Hedayatnia et al., 2020). In this study, HFFD-fed rats showed significant insulin resistance and dyslipidemia, and an increased AIP; $\log_{10}$ (TG/HDL-C), which is a good marker for predicting the risk of atherosclerosis and cardiovascular disease (Wu et al., 2018). PIE treatment improves dyslipidemia by lowering TG, VLDL-C, LDL-C, and FFA levels and increasing HDL-C levels. Thus, PIE may help protect against cardiovascular disease, especially considering its effect on decreasing AIP values. This alleviative effect of PIE on dyslipidemia in HFFD-fed rats was in the same direction as reported in a study using *P. indica* tea in high-fat diet-fed mice (Sirichaiwetchakoon et al., 2020).

Moreover, it was found that HFFD feeding for 16 weeks could cause NAFLD in rats, evidenced by a significant increase in hepatic TG levels with histological confirmation of lipid accumulation in the liver cells. PIE treatment effectively decreased hepatic TG levels and lipid accumulation in the HFFD-fed rats, demonstrating the NAFLD-alleviation activity of PIE. However, in this study, the lipid accumulation did not cause hepatocellular injury, as there was no significant change in serum AST and ALT levels.

In our study, PIO treatment led to a significant reduction in serum TG, VLDL-C, and FFA levels, and an increase in serum HDL-C level, but no significant change in serum LDL-C level. Our results are consistent with another report that used a similar diet-induced dyslipidemia model (Bhosale et al., 2013). There is evidence that PIO alleviates NASH in patients with T2DM (Kim et al., 2019). A recent study on PIO therapy for 18 months in patients with prediabetes and T2DM indicated that PIO could reduce liver fibrosis, intrahepatic TG content, and liver enzymes (Bril et al., 2018). Thus, we decided to use PIO as a positive control drug. Besides its hypoglycemic effect, most clinical studies have shown that PIO has a beneficial effect on lipid profiles, including a reduction in serum TG, LDL-C, and FFA and an increase in serum HDL-C (Chawla et al., 2013; Razavizade et al., 2013). However, the impact of PIO treatment on the lipid profiles in diabetic patients was found not to be entirely consistent. Rosenblatt et al. (2001) studied PIO's effect on glycemic control and atherogenic dyslipidemia in diabetic type 2 patients, showing that there was a significant improvement in TG and HDL-C, whereas, changes in the levels of TC and LDL-C were found to be insignificant. In another study in poorly controlled diabetic type 2 patients, PIO significantly reduced TGs, but there were no significant differences in values for TC, LDL-C, and HDL-C (Aghamohammadzadeh et al., 2015). In the point of increase in BW, a recognized side effect of PIO, and is proposed to be the result of an increase in subcutaneous fat mass with either no change or a small reduction in visceral fat mass and fluid retention (Lebovitz, 2019). This study also discovered that PIO-treated animals had a significant increase in BW with a reduction in epididymal adipose tissue weight, therefore, PIO-induced weight gain in this study may be a consequence of fluid retention.

Hepatic *DNL*, a biochemical process by which lipids are endogenously synthesized from dietary sources, plays an essential role in NAFLD development (Softic et al., 2016). SREBP1c is a critical transcription factor in hepatic lipogenesis by regulating the transcription of lipogenic genes, including *FAS* and *GPAT*, that contribute to fatty-acid and TG synthesis (Horton et al., 2002). Hyperinsulinemia stimulates hepatic SREBP1c transcription, leading to lipid synthesis and hepatic steatosis (Browning and Horton, 2004). Previous studies have clearly shown that hyperinsulinemia and the upregulated expression of *SREBP1c* and its downstream target genes, such as *FAS* and *GPAT*, cause the development of fatty liver in animals fed a high-fructose diet or high-fat diet (Zhang et al., 2015; Naowaboot et al., 2021). Following those findings, this study showed that HFFD consumption caused hyperinsulinemia and increased expression of *SREBP1c*, *FAS*, and *GPAT* genes. Intriguingly, PIE could reduce the expression levels of these lipogenesis genes: therefore, it is proposed that PIE may improve dyslipidemia and alleviates fatty liver by reducing hepatic *DNL*.

Additionally, hepatic lipid homeostasis is also controlled by intrahepatic fatty-acid β-oxidation (Ipsen et al., 2018). PPARα plays a crucial role in the transcriptional regulation of fatty acid oxidation. Among its downstream targets, CPT1 is the rate-limiting enzyme for mitochondrial fatty-acid β-oxidation, and ACOX1 is the enzyme

that catalyzes the first step in hepatic peroxisomal β-oxidation (Mandard et al., 2004; Reddy, 2004). Our results showed that PIE increased the expression of hepatic *PPARα* and *ACOX1*, but not *CPT1* genes, showing that PIE promotes fatty-acid oxidation in hepatic peroxisomes but not in the mitochondria. Our findings followed previous findings that downregulation of gene expression associated with lipogenesis and upregulation of gene expression associated with fat oxidation reduces abnormal hepatic lipid accumulation in mice fed a high-fat diet (Min et al., 2013; Chen et al., 2019; Li et al., 2019). However, *ACOX1* gene expression of the group treated with a high dose of PIE (300 mg/kg) showed an insignificant increase compared to the HFFD control group. It is probably because the crude extract has several compositions, such as different kinds of phenolic constituents, which may act on various receptors or pathways, giving antagonizing effects. These results strongly indicate that PIE may reduce hepatic *DNL* and increase fatty-acid oxidation, which then alleviates NAFLD and dyslipidemia. Leptin also plays an essential role in lipid homeostasis within the liver. Independent of leptin's ability to decrease food intake and BW, leptin could reduce hepatic lipid accumulation by inhibiting lipogenesis and promoting fatty-acid β-oxidation (Martínez-Uña et al., 2020). One study showed that leptin failed to alleviate hepatic steatosis in leptin-resistant rats (Fishman et al., 2007). Hence, the inhibitory effect of PIE on hepatic steatosis may, at least in part, owing to the increasing leptin sensitivity since PIE decreases leptin levels in HFFD-fed rats.

Phenolic compounds are ubiquitously distributed in most plants (fruits, vegetables, and cereals, etc.). They are a group of antioxidant phytochemicals and have many diseases alleviating effects, such as anti-cancer, anti-diabetes, anti-dyslipidemia, and anti-dementia effects (Guti Errez-Grijalva et al., 2016). The major phenolic composition in PIE comprised of tannic acid, rutin, quercetin, gallic acid, isoquercetin, and catechin. In many rodent models of obesity, rutin, quercetin, isoquercetin, and catechins have been shown to enhance blood lipid profile and protect the liver from excessive fat deposition and hepatic steatosis (Sandoval et al., 2020). Quercetin and rutin were frequently reported to improve NAFLD in animal models (Van De Wier et al., 2017). Tannic acid, the highest content in PIE, has been shown to have hypoglycemic and lipid-lowering effects in streptozotocin-induced diabetic rats (Esmaie et al., 2019), anti-adipogenic effect in 3T3-L1 cells via downregulation of PPARγ (Nie et al., 2015), and hepatic steatosis reducing activity in HFD-fed rats (Zou et al., 2014). Also, quercetin may exert anti-diabetic effects through several mechanisms, including enhancing insulin secretion and sensitivity (Yi et al., 2021). As previously mentioned, the metabolic-improving effects of PIE may be associated with the impact exerted by phenolic compounds in PIE.

Our study showed that PIE improved insulin-leptin resistance and dyslipidemia, reduced the hypertrophy of adipocytes, and attenuated hepatic steatosis in HFFD-fed rats. The mechanisms through which PIE alleviates HFFD-induced dyslipidemia, and hepatic steatosis may be related to (a) improvement of insulin-leptin sensitivity; (b) the downregulation of genes associated with hepatic lipogenesis: *SREBP1c*, *FAS*, and *GPAT1* genes; and (c) the upregulation of genes related to fatty-acid oxidation: *PPARα* and *ACOX1* genes (Fig. 7). Further details will be provided on the effect of PIE on insulin- and leptin-sig-

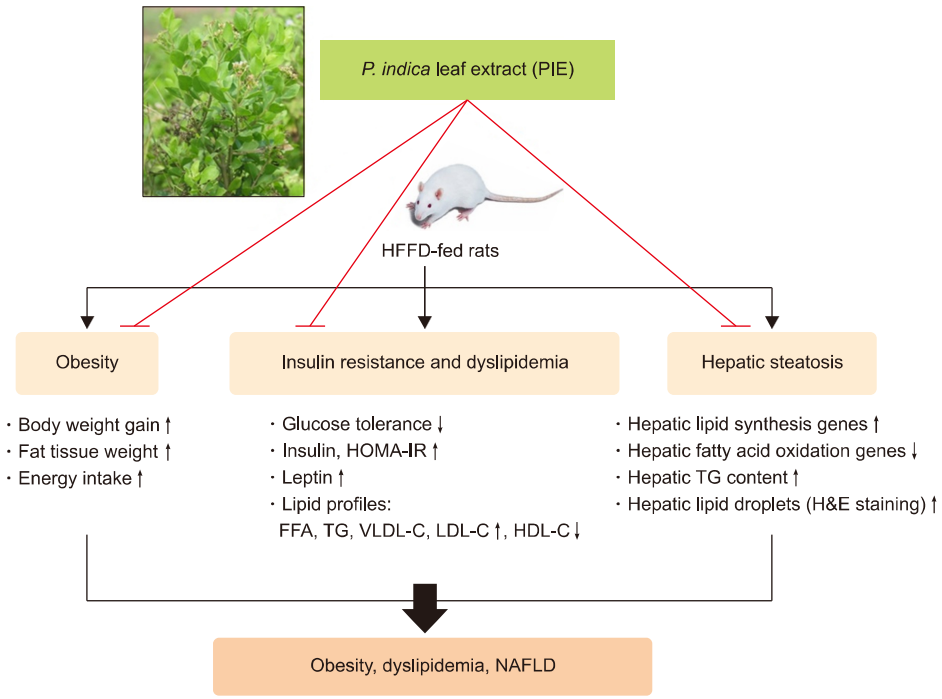

Fig. 7. Summary of all investigated activities of *Pluchea indica* leaf extract (PIE) in high fat-high fructose diet (HFFD)-fed rats. PIE could decrease body weight gain, energy intake, insulin resistance, and dyslipidemia, and could ultimately attenuate hepatic steatosis. PIE downregulated the genes associated with hepatic lipogenesis and upregulated genes related to fatty-acid oxidation in HFFD-fed rats. Collectively, these findings show beneficial roles of PIE on dyslipidemia and NAFLD. HOMA-IR, homeostasis model assessment of insulin resistance; FFA, free fatty acid; TG, triglyceride; VLDL-C, very-low-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; H&E, hematoxylin and eosin; NAFLD, non-alcoholic fatty liver disease.

naling pathways in the near future. Our results clearly showed that PIE is an effective natural or alternative medicine for preventing and treating NAFLD.

ACKNOWLEDGEMENTS

We would like to acknowledge Prof. David Blair for editing the manuscript via Publication Clinic Khon Kaen University.

FUNDING

This work was supported by Invitation Research Fund, Faculty of Medicine, Khon Kaen University (grant no. IN63348), and the Office of the Higher Education Commission of Thailand and partially funded by a scholarship under the Strategic Scholarships Fellowships Frontier Research Networks (specific for Southern Region) from the Ministry of Higher Education, Science, Research and Innovation.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Concept and design: PP, PS, JN. Analysis and interpretation: PP, PS, LS. Data collection: PS, KB. Writing the article: PP, PS. Critical revision of the article: PP. Final approval of the article: all authors. Statistical analysis: PP, PS, KB. Obtained funding: PP, PS. Overall responsibility: PP, PS.

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