Pink Lotus Essential Oil and Alleviates on Free Fatty Acid Induced Steatosis in HepG2 Cells via PI3K/Akt and NF- κ B Pathways

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Abstract: Pink lotus essential oil (PLEO) is the volatile components extracted from lotus flowers and there are few relevant research. The purpose of this study was to observe the effect of PLEO on NAFLD *in vitro* model and its possible mechanism. The ingredients of PLEO were determined by gas chromatography–mass spectrometry (GS-MS) and its lipid-lowering and hepatoprotective activities were investigated. HepG2 cells were treated with free fatty acid (FFA) to establish a cell model of NAFLD. Cell viability was evaluated by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Total cholesterol (TC), triglyceride (TG), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) were determined by Enzyme-Linked Immune Sorbent Assay (ELISA). Oil red O staining was performed to observe the lipid accumulation in the HepG2 cells. Lipid metabolism enzymes including fatty acid synthase (FAS), acetyl-coA carboxylase (ACC), stearoyl-CoA desaturase 1 (SCD-1), and carnitine palmitoyltransferase-1 (CPT-1), insulin signaling pathways including phosphatidylinositol 3 kinase (PI3K) and protein kinase B Akt, inflammatory signaling pathways such as nuclear factor kappa-B (NF- κ B), were determined by Western blotting. There were 46 components determined in PLEO with many terpenoids compounds. PLEO decreased TC and TG contents in the FFA-treated HepG2 cells. Furthermore, PLEO inhibited TNF- α , IL-6 and IL-1 β excretion, decreased NF- κ B, FAS, ACC and SCD-1 while increased phosphorylation of NF- κ B, PI3K, Akt, and CPT-1 expression. It is the first time to reveal that PLEO alleviates FFA-induced steatosis in HepG2 cells by regulating lipid metabolism, inhibiting inflammatory response, and improving insulin sensitivity.

Key words: pink lotus essential oil, PLEO, NAFLD, HepG2 cells, PI3K/Akt, NF- κ B

1 Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) has ballooned significantly in recent years with the global epidemic of obesity. The disease begins with liver steatosis and may develop into steatohepatitis with liver inflammation¹⁾. At present, multiple-hit theory is well recognized as the pathogenesis of NAFLD²⁾. Multiple hits, including insulin resistance, nutritional factors, hormones secreted from the adipose tissue, gut microbiota and genetic and epigenetic factors, act together to induce NAFLD in genetically predisposed subjects. However, there are no specific and effective drugs to treat NAFLD. Several antioxidants, insulin sensitizers, and lipid-lowering drugs have been applied in clinical trials to treat NAFLD/non-al-

coholic steatosis (NASH)^{3,4)}. It is expected to achieve satisfied therapeutic effect. Treatment of HepG2 cells with free fatty acid (FFA) is a commonly used cell model for NAFLD, which seems to reproduce the major characters of NAFLD in humans⁵⁾. Excessive FFA induces cellular steatosis and toxicity, insulin resistance, oxidative stress, and inflammation, which is consistent with *in vivo* observations of NAFLD.

Lotus, a member of the *Nelumbonaceae* family, is a widely cultivated perennial aquatic plant in Asia. Its various parts, including roots, leaves, and flowers, have been used as food and herbal medicine for a long history^{6,7)}. Studies have shown that flavonoids in lotus leaf have an obvious protective effect on liver injury induced by carbon tetra-

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chloride (CCl₄) in mice⁸). Nonedible lotus pods is a source of natural antioxidants and anticancer agents⁹). Lotus seedpod extracts have anti-inflammatory effects on lipopolysaccharide (LPS) induced liver inflammation¹⁰). However, there has been little research on lotus flowers. Pink lotus essential oil (PLEO) is a volatile component extracted from lotus flowers that gives it a special aroma. PLEO might have a variety of activities due to its complex composition. Therefore, it is necessary to explore the composition and its pharmacological activity for further development.

In the present study, we explored the protective effects of PLEO on lipid accumulation and inflammation induced by FFA in HepG2 cells. Furthermore, the potential molecular mechanisms involving in the inflammation and insulin signal pathway were discussed as well.

2 Materials and Methods

2.1 Chemicals and reagents

PLEO was obtained from Healzone Co. Ltd. (Qingdao, China). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin solution were purchased from Gibco (Carlsbad, CA, USA). The HepG2 cells were purchased from Fuheng Biology, Co. Ltd. (Shanghai, China). Total cholesterol (TC), triglyceride (TG), tumor necrosis factor α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) ELISA kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rabbit antibodies for fatty acid synthase (FAS) (ab133619), acetyl-coA carboxylase (ACC) (ab45174), stearoyl-CoA desaturase-1 (SCD-1) (ab236868), carnitine palmitoyltransferase-1 (CPT-1) (ab234111), phosphatidylinositol 3 kinase (PI3K) (ab131067), phosphorylated PI3K (p-PI3K) (ab278545), protein kinase B (Akt) (ab8805), phosphorylated Akt (p-Akt) (ab38449), nuclear factor kappa-B p65 (NF- κ B p65) (ab16502), phosphorylated nuclear factor kappa-B p65 (p-NF- κ B p65) (ab183559), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab245355) were obtained from Abcam (Cambridge, UK). RIPA lysis buffer, BCA kit, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), polyvinylidene difluoride (PVDF) membranes, ECL substrate kit, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phosphate buffer saline (PBS) powder, dimethyl sulfoxide (DMSO), Oil red O were purchased from Beijing Bioss Biotechnology Co. Ltd. (Beijing, China). Palmitate and oleate were obtained from Shanghai Aladdin Bio-Chem (Shanghai, China).

2.2 Gas chromatography-mass spectrometry

Coupled gas chromatography-mass spectrometry (GC-MS) analysis was performed on a Shimadzu system/GC

2010 coupled to a self-gun Shimadzu/AOC-5000 and mass detector (Shimadzu MS 2010 Plus). The column used in the present study was HP-5, and the carrier gas was high purity nitrogen. The injection port temperature was 280°C. The programmed warming process of the column was as follows: initial temperature was 50°C maintaining for 2 min, and then programmed warming at 10°C per minute until 280°C for 10 min. The column pressure was 10 psi, the shunt injection ratio was 20:1, and the injection volume was 1 μ L. Mass spectrum conditions were as follows: the ion source was electron bombardment ion, the electron energy was 70 eV, the interface temperature was 250°C, the mass scanning range was m/z 50-600, and the scanning interval was 1.0 s.

2.3 Cell culture and drug treatments

Human hepatic carcinoma cell line HepG2 was cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin solution. The culture was maintained at 37°C in a humidified 5% CO₂ incubator. When the cells reached 50-70% confluence, the MTT assay and the lipid-lowering experiment were conducted. PLEO was dissolved in DMEM medium containing 0.1% DMSO at a concentration of 0.01, 0.1, 1 μ g/mL, respectively. In the lipid-lowering experiment, HepG2 cells were treated with FFA mixture composed of oleate acid (OA) and palmitate acid (PA) (2:1) for 48 h to induce the hepatic steatosis model *in vitro*. After that, the experiment group was added 100 μ L different concentration of PLEO. The control group and FFA group were added the same volume of DMEM after treated without or with FFA, respectively. Subsequently, the lipid and cytokines in the cell culture supernatant, lipid accumulation, the expression of lipid metabolism associated enzyme and PI3K/Akt and NF- κ B p65 were determined by ELISA, oil red O staining and Western blotting analysis, respectively.

2.4 MTT assay

Cell viability was measured using the MTT assay. The cells (2×10^4 cells/well) were seeded in a 96-well cell culture dish, and then treated with different concentrations of 100 μ L PLEO (0.01, 0.1, 1 μ g/mL) for 48 h. The control group was treated with the same volume of DMEM containing 0.1% DMSO. Every group was set five duplicated wells. After incubation, the medium was discarded, and the cells were treated with 100 μ L MTT solution (1 mg/mL) for 1 h. The solution was then removed, and DMSO was added to dissolve the insoluble formazan crystals. The absorbance at 570 nm was measured using a full-wave-length Microplate Reader (Infinite m plex, Tecan, Switzerland). The cell viability was calculated as the following:

$$\text{cell viability (\%)} = \frac{OD_{\text{sample}}}{OD_{\text{control}}} \times 100\%$$

2.5 ELISA for quantification of lipids and cytokines

After treatment, the HepG2 cells (2×10^5 cells per well in a 24-well plate) supernatants were centrifuged at $10,000 \times g$ at 4°C for 10 min to remove debris. The contents of TC, TG, TNF- α , IL-6, and IL-1 β in the supernatants were detected with ELISA kits according to the manufacturer's instructions.

2.6 Oil Red O staining

The treated HepG2 cells were washed with PBS and fixed in 4% paraformaldehyde for 30 min. After washed with distilled water, the cells were added 200 μL 5% oil red O and stained at room temperature for 30 min. Then the cells were washed with tap water and dyed with hematoxylin. The staining was observed with a microscope (BX53, Olympus, Japan) and quantified with Image J software (v1.44, Bethesda, Rockville, MD, USA).

2.7 Western blotting analysis

The HepG2 cells (1×10^6 cells per well in a 6-well plate) were collected and the total protein was extracted using a RIPA buffer containing protease inhibitors or phosphatase inhibitors. Bicinchoninic acid (BCA) protein assay was used to determine the protein concentrations, and 30 μg of protein was loaded per sample. The total protein was separated by SDS-PAGE (10% separating gel-5% concentrating gel) and transferred to PVDF membranes. The membranes were blocked with 5% nonfat skim milk for 2 h at room temperature. After washed with TBST for 3 times, the membranes were incubated with specific antibodies for Akt, p-Akt, PI3K, p-PI3K, NF- κB p65, p-NF- κB p65, GAPDH, FAS, SCD-1, CPT-1, ACC-1 (1:1,000) for 2 h. The membranes were then incubated with goat anti-rabbit HRP secondary antibodies (1:5,000) for 1.5 h. The chemiluminescence detection was performed using an ECL reagent (Thermo Scientific, Massachusetts, America) and bands were developed with a gel imager (TANON-4600 Chemiluminescence Imager, Shanghai Tianneng Technology Co., Ltd. Shanghai, China). Specific bands were detected, analyzed, and quantified by Image J Software (v1.44, Bethesda, Rockville, MD, USA).

2.8 Statistical analysis

Statistical analysis was performed using SPSS 23.0. Data were expressed as mean $\pm$ standard deviation (SD). The one-way ANOVA followed with multiple comparison was used to determine significant differences among groups. Values of P less than 0.05 were considered to be significant.

3 Results

3.1 GC-MS analysis of PLEO

There were 46 components in the PLEO identified by

GC-MS, representing 99.85% of the total (Table 1, Fig. 1). As a complex mixture, PLEO were mainly composed of 4 groups of ingredients: aliphatic aldehydes (26.57%), terpenoids (25.64%), lipids (14.89%), and aromatic hydrocarbons (14.23%). 2-(phenylmethylene)-Octanal (14.85%) is the most abundant aliphatic compound detected. The identified terpenoids mainly included isolongifolol (5.83%), L-alpha-terpineol (4.56%), citronellol (3.63%), geraniol (2.64%), linalool (2.45%), etc. It was deduced that the possible biological activities of PLEO may be attributed to terpenoids.

3.2 Effects of PLEO on cell viability of HepG2 cells

The effects of PLEO treatment on HepG2 cell viability were examined and shown in Fig. 2. When the cell viability is above 80%, the drug can be thought to have no significant cytotoxicity on cell growth. As expected, cell viability after treated with PLEO maintained at above 80%. Otherwise, it is amazing that the cell viability increased with the increase of PLEO concentration.

3.3 Effects of PLEO on FFA-induced lipid accumulation in HepG2 cell

The effects of PLEO treatment on lipid content in the FFA pre-treated HepG2 cells were examined and shown in Fig. 3. The results indicated that FFA pretreatment dramatically increased the intracellular TC and TG contents compared with the control (Fig. 3, $p < 0.01$). Meanwhile, PLEO treatment dose-dependently decreased TC and TG contents in the HepG2 cells compared with the FFA group (Fig. 3, $p < 0.05$ or 0.01). PLEO exhibited the strongest inhibition effect at a concentration of 1 $\mu\text{g}/\text{mL}$ (Fig. 3).

To further determine whether PLEO decreased the hepatocyte lipid accumulation in FFA-induced steatosis of HepG2 cells, Oil Red O staining were performed and quantified. There were multiple of red lipid droplets in the FFA treated HepG2 cells compared with the control group (Figs. 4A and 4B, $p < 0.01$), indicating an accumulation of lipid in the cells. Compared with the FFA group, the PLEO-treated HepG2 cells showed a decrease in cellular lipid accumulation (Figs. 4B-4E, $p < 0.05$ or 0.01). Combined with the above results, it is revealed that PLEO ameliorated the hepatic lipid droplets induced by FFA treatment.

3.4 Effects of PLEO on FFA-induced expression of inflammatory mediators in HepG2 cells

The effect of PLEO on inflammatory cytokines secreted by HepG2 cells was also observed. The results showed that TNF- α , IL-6, and IL-1 β in the HepG2 significantly increased after FFA treatment compared with the control group (Figs. 5A-5C, $p < 0.01$). As expected, FFA induced an inflammation in addition to a lipid accumulation. Moreover, PLEO inhibited TNF- α , IL-6, and IL-1 β secreted by FFA treated HepG2 cells in different degrees (Figs. 5A-5C, $p <$

Table 1 Analysis results of PLEO by GC-MS.

No.	Compounds	Rt, min	Chemical formula	MW, g/mol	Content, %
1	D-Limonene	6.533	C ₁₀ H ₁₆	136.234	0.087
2	Benzyl alcohol	6.723	C ₇ H ₈ O	108.138	0.069
3	Benzoic acid, methyl ester	7.684	C ₈ H ₈ O ₂	136.148	0.084
4	Linalool	7.759	C ₁₀ H ₁₈ O	154.249	2.445
5	Phenylethyl alcohol	8.025	C ₈ H ₁₀ O	122.164	5.872
6	(1R,2R,4R)-2,7,7-Trimethylbicyclo[2.2.1]heptan-2-ol	8.535	C ₁₀ H ₁₈ O	154.249	0.183
7	Citronellal	8.632	C ₁₀ H ₁₈ O	154.249	0.084
8	Acetic acid, phenylmethyl ester	8.871	C ₉ H ₁₀ O ₂	150.174	10.374
9	endo-Borneol	8.933	C ₁₀ H ₁₈ O	154.249	0.161
10	L- α -Terpineol	9.296	C ₁₀ H ₁₈ O	154.249	4.560
11	Cyclohexanol, 1-methyl-4-(1-methylethylidene)-	9.389	C ₁₀ H ₁₈ O	154.249	0.732
12	Citronellol	9.664	C ₁₀ H ₂₀ O	156.265	3.628
13	Geraniol	10.249	C ₁₀ H ₁₈ O	154.249	2.643
14	Octanal, 7-hydroxy-3,7-dimethyl-	10.807	C ₁₀ H ₂₀ O ₂	172.265	11.728
15	2-Propen-1-ol, 3-phenyl-	11.055	C ₉ H ₁₀ O	134.175	1.465
16	α -Terpinyl acetate	11.662	C ₁₂ H ₂₀ O ₂	196.286	0.740
17	2(3H)-Furanone, dihydro-5-pentyl-	11.843	C ₉ H ₁₆ O ₂	156.222	0.130
18	(E)-3,7-Dimethylocta-2,6-dienyl ethyl carbonate	12.096	C ₁₃ H ₂₂ O ₃	226.312	0.153
19	Longifolene	12.543	C ₁₅ H ₂₄	204.351	0.066
20	Caryophyllene	12.72	C ₁₅ H ₂₄	204.351	0.082
21	2-Propenoic acid, 3-phenyl-, ethyl ester	13.265	C ₁₁ H ₁₂ O ₂	176.212	0.376
22	Lilial	14.089	C ₁₄ H ₂₀ O	204.308	0.828
23	Diethyl Phthalate	14.922	C ₁₂ H ₁₄ O ₄	222.237	0.272
24	Tricyclo[3.1.0.0(2,4)]hexane,3,3,6,6-tetraethyl-,trans-	15.732	C ₁₄ H ₂₄	192.340	1.197
25	11,11-Dimethyl-4,8-dimethylenebicyclo[7.2.0]undecan-3-ol	15.817	C ₁₅ H ₂₄ O	220.350	3.441
26	Caryophylla-4(12),8(13)-dien-5 α -ol	15.901	C ₁₅ H ₂₄ O	220.350	0.261
27	Phenol, 2-(1,2-dimethyl-2-cyclopenten-1-yl)-, acetate	16.224	C ₁₅ H ₁₈ O ₂	230.302	0.092
28	Octanal, 2-(phenylmethylene)-	16.831	C ₁₅ H ₂₀ O	216.319	14.847
29	6-epi-shyobunol	16.915	C ₁₅ H ₂₆ O	222.366	0.502
30	Isolongifolol	17.208	C ₁₅ H ₂₆ O	222.366	5.826
31	6-Isopropenyl-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-1H naphthalen-2-one	17.398	C ₁₅ H ₂₂ O	218.335	4.127
32	Methyl 3- <i>cis</i> ,9- <i>cis</i> ,12- <i>cis</i> octadecatrienoate	17.451	C ₁₉ H ₃₂ O ₂	292.456	3.939
33	<i>trans</i> -Geranylgeraniol	17.518	C ₂₀ H ₃₄ O	290.483	1.427
34	1,1,6-trimethyl-3-methylene-2-(3,6,9,13-tetramethyl-6-ethenyl-10,14-dimethylenepentadec-4-enyl)cyclohexane	17.664	C ₃₃ H ₅₆	452.798	0.432
35	Spiro[2.5]octane, 5,5-dimethyl-4-(3-oxobutyl)-	17.748	C ₁₄ H ₂₄ O	208.340	12.599
36	4-Hexen-1-ol, 6-(2,6,6-trimethyl-1-cyclohexenyl)-4-methyl-, (E)-	17.907	C ₁₆ H ₂₈ O	236.393	1.343
37	3a,9-Dimethyldodecahydrocyclohepta[d]inden-3-one	18.067	C ₁₆ H ₂₆ O	234.377	0.590
38	3-Hexen-1-ol, 6-(2,6,6-trimethyl-1-cyclohexenyl)-4-methyl-, (E)-	18.116	C ₁₆ H ₂₈ O	236.393	0.386
39	1-Naphthalenepropanol, α -ethenyldodecahydro- α ,5,5,8a-tetramethyl-2-methylene-, [1S-[1 α (R*),4 $\alpha\beta$,8 $\alpha\alpha$]]-	18.266	C ₂₀ H ₃₄ O	290.483	0.598
40	Phenanthrene, 7-ethenyl-1,2,3,4,4a,5,6,7,9,10,10adodecahydro-1,1,4a,7-tetramethyl-, [4aS-(4 $\alpha\alpha$,4 $\beta\beta$,7 β ,10 $\alpha\beta$)]-	18.426	C ₂₀ H ₃₂	272.468	0.357
41	Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl-	18.665	C ₂₀ H ₃₄ O	290.483	0.239
42	(2,6,6-Trimethylcyclohex-1-enylmethanesulfonyl)benzene	18.785	C ₁₆ H ₂₂ O ₂ S	278.410	0.290
43	Methyl 10- <i>trans</i> ,12- <i>cis</i> octadecadienoate	20.366	C ₁₉ H ₃₄ O ₂	294.472	0.082
44	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	20.428	C ₁₉ H ₃₂ O ₂	294.472	0.142
45	6-(4-Methoxy-phenyl)-2H-[1,3]thiazin	22.093	C ₁₁ H ₁₁ NOS	205.276	0.333
46	Tetramethrin	24.233	C ₁₉ H ₂₅ NO ₄	331.406	0.188

Fig. 1 GC-MS analysis results of PLEO. There were 46 components in the lotus essential oil identified by GC-MS.

Fig. 2 Effects of PLEO on cell viability in HepG2 cells (A). There was no significant change of cell viability in HepG2 cells after treated with PLEO, even at the highest concentration of 1 µg/mL.

0.05 or 0.01). The inhibition on inflammation is beneficial for PLEO to intervene the FFA induced steatosis.

3.5 Effects of PLEO on expression of PI3K/Akt, NF-κB and lipid metabolism associated enzymes in HepG2 cells

In order to reveal the possible mechanism of PLEO on FFA induced steatosis, the expression of PI3K/Akt, NF-κB and lipid metabolism associated enzymes was determined in the present study. After treated with FFA, the expression of PI3K, Akt and their phosphorylation significantly decreased (Figs. 6A and 6B, compared with control group, $p < 0.05$ or 0.01). PI3K/Akt signal pathway is an important one involving in the insulin signaling and is related with the insulin sensitivity. The results revealed that FFA induced insulin resistance in the HepG2 cells via inhibition of PI3K/Akt signal pathway. PLEO significantly increased the expression of PI3K, Akt and their phosphorylation at a concentration of 0.1 and 1 µg/mL, respectively (Figs. 6A and 6B, compared with FFA group, $p < 0.05$ or 0.01), indicating an improvement of insulin resistance. The expression of NF-κB p65 increased significantly while p-NF-κB p65 decreased in FFA treated HepG2 cells (Figs. 6A and 6C, compared with control group, $p < 0.01$). After treated with PLEO, the expression of NF-κB p65 decreased while p-NF-κB p65 increased (Figs. 6A and 6C, compared with FFA group, $p < 0.05$ or 0.01). The inhibition on NF-κB p65

Fig. 3 Effects of PLEO treatment on TC and TG contents in the FFA treated HepG2 cells. PLEO significantly decreased TC (A) and TG (B) contents in the HepG2 cells treated with FFA, showing a dose dependent manner. * $p < 0.05$, ** $p < 0.01$ versus Control group; # $p < 0.05$, ## $p < 0.01$ versus FFA treated group; Δ $p < 0.05$, ΔΔ $p < 0.01$ versus PLEO (0.01 µg/mL); & $p < 0.05$, && $p < 0.01$ versus PLEO (0.1 µg/mL).

Fig. 4 Oil Red O staining of HepG2 Cells(A - E) and quantitative analysis(F). When compared with the untreated cells(A), a large amount of lipid accumulated in the hepatic cells(B). After treated with PLEO at a concentration of 0.01(C), 0.1(D) and 1 μg/mL(E), lipid accumulation significantly decreased to varying degrees. Red dot shows lipid droplet; pale blue circle shows cell nucleus. * $p < 0.05$, ** $p < 0.01$ versus Control group; # $p < 0.05$, ## $p < 0.01$ versus FFA treated group; ^ $p < 0.01$ versus PLEO (0.01 μg/mL); & $p < 0.05$ versus PLEO (0.1 μg/mL). Bars: 20 μm.

Fig. 5 Effects of PLEO treatment on inflammatory mediators in the FFA treated HepG2 cells. PLEO significantly decreased TNF-α(A), IL-6(B) and IL-1β(C) contents in the HepG2 cells treated with FFA, showing a dose dependent manner. * $p < 0.05$, ** $p < 0.01$ versus Control group; # $p < 0.05$, ## $p < 0.01$ versus FFA treated group; ^ $p < 0.05$, △ $p < 0.01$ versus PLEO (0.01 μg/mL); & $p < 0.05$ versus PLEO (0.1 μg/mL).

expression and activation on p-NF-κB p65 of PLEO attribute to the inhibition of inflammation induced by FFA. Furthermore, the expression of FAS, ACC and SCD-1 in FFA treated HepG2 cells increased, while the expression of CPT-1 decreased (Figs. 6A and 6D, compared with the control group, $p < 0.05$ or 0.01), which might be involved in cellular lipid accumulation. After PLEO treatment, the expression of FAS, ACC and SCD-1 decreased, while the expressions of CPT-1 increased (Figs. 6A and 6D, compared with the FFA group, $p < 0.05$ or 0.01) which resulted in decreased lipid accumulation.

4 Discussion

The liver, the body's largest solid organ, plays a key role in nutrient storage, metabolism, synthesis of new molecules, purification of toxic chemicals and other physiologi-

cal processes¹¹. In recent decades, NAFLD has become the most common cause of chronic liver dysfunction. NAFLD represents a wide spectrum of disease stages, extending from simple steatosis (triglycerides accumulation in the liver), nonalcoholic steatohepatitis (steatosis with inflammation) to fibrosis, which may eventually progress to cirrhosis and hepatocellular carcinoma¹². Therefore, it is urgent to develop new drugs for NAFLD treatment. FFA treated HepG2 cells is an *in vitro* model of steatosis commonly applied to study the hepatocellular consequences of lipid accumulation. HepG2 cells have the advantages of high availability, simple operation, almost infinite life, stable phenotype and so on¹³. The functional properties of HepG2 cells have been extensively studied, expressing many different liver functions, such as synthesis and secretion of plasma protein and bile acid, metabolism of cholesterol, triglyceride and lipoprotein, glycogen synthesis or insulin signaling^{14, 15}. In the present study, the mixture of

Fig. 6 Effects of PLEO treatment on the protein expressions of PI3K/Akt, NF-κB and lipid metabolism associated enzymes in the FFA treated HepG2 cells. WB bands were shown in (A) and the expression of PI3K/Akt (B), NF-κB (C) and lipid metabolism associated enzymes (D) was quantitatively analyzed. There was an inhibition of p-PI3K, p-Akt, p-NF-κB, SCD-1, CPT-1 expressions while an activation of NF-κB expression induced by FFA. After treated with PLEO, the above protein expressions were restored in different degrees. ** $p < 0.05$, * $p < 0.01$ versus Control group; # $p < 0.05$, ## $p < 0.01$ versus FFA treated group; $\Delta p < 0.05$ versus PLEO (0.01 μg/mL).

OA and PA (2:1) was employed to induce liver cellular steatosis in the HepG2 cells. As expected, the contents of TC and TG increased significantly after treatment of FFA, indicating a lipid accumulation occurred. Oil red O staining exhibited a typical pathomorphologic changes of steatosis. Furthermore, the contents of TNF- α , IL-6 and IL-1 β increased significantly, indicating that there accompanied by inflammation after FFA mixture induced lipid accumulation. All the above changes in the FFA treated HepG2 cells were similar with the key pathological characters of NAFLD in humans, which was an ideal *in vitro* model of NAFLD. Subsequently, we detected the effect of PLEO on lipid accumulation and inflammation induced by FFA mixture. The results revealed that PLEO significantly decreased TG, TC, TNF- α , IL-6 and IL-1 β contents and alleviated cellular steatosis induced by FFA, showing a potential therapeutic effect on NAFLD. There were four groups of ingredients in PLEO recognized by GC-MS, including aliphatic aldehydes, terpenoids, lipids and aromatic hydrocarbons. 2-(phenylmethylene)-Octanal was the most abundant aliphatic compound detected. The identified terpenoids mainly included isolongifolol (5.83%), L-alpha-terpineol (4.56%), citronellol (3.63%), geraniol (2.64%), linalool (2.45%), etc. Terpenoids represent the largest

group of ingredients consisting of essential oil which account for its pharmaceutical activity¹⁶⁾. Alpha-terpineol is a monoterpenoid widely found in essential oil with various bioactive such as antioxidant and anti-inflammatory activity, accounting for 4.56% in LEO. Recent report suggested that alpha-terpineol improved the insulin sensibility and reduced serum TNF- α and IL-1 β levels in rats fed a high-fat diet¹⁷⁾. Geraniol is an acyclic isoprenoid monoterpene isolated from LEO and accounts for 2.64%. Geraniol is potentially applied as a drug based on its various pharmacological properties, including anti-inflammatory, anti-cancer, antimicrobial, antioxidative, cardioprotective, hepatoprotective and neuroprotective activities¹⁸⁾. Linalool, a monoterpene alcohol, accounts for 2.45% in LEO. Linalool inhibited the production of TNF- α and IL-6 induced by LPS both *in vitro* and *in vivo*¹⁹⁾. Furthermore, linalool significantly inhibited lipid production, decreased cholesterol and maintained body weight through multiple mechanisms^{20, 21)}. Based on these reports, it was speculated that terpenoids in LEO, especially L-alpha-terpineol, geraniol and linalool, were the key potent part contributing to free fatty acid induced steatosis. However, the exact active ingredients and detailed possible mechanisms need for further research.

The accumulation of a large amount of fat in the liver leads to steatosis of liver parenchymal cells, which is an important inducer of NAFLD. FAS, ACC, and SCD-1 are three important enzymes participate in fatty acid synthesis. ACC catalyzes malonyl-CoA, an intermediate metabolite of lipid formation and oxidation²²⁾. FAS is a rate-limiting enzyme to catalyze the final step of lipid synthesis in the liver²³⁾. SCD-1 is another key enzyme involving in lipid synthesis, which catalyzes fatty acid elongation and desaturation²⁴⁾. At present, there are two signal pathways involving in insulin signaling that up-regulate the expression of these enzymes. One is the sterol regulated element-binding protein-1C (SREBP-1C), and the other is carbohydrate responsive element binding protein (ChREBP)²⁵⁾. CPT-1 is the rate-limiting enzyme of fatty acid oxidation. In the process of fatty acid oxidation, CPT-1 is a key regulatory site for fatty acid flow into mitochondria, and abnormal fatty acid oxidation process is also an important pathogenic factor of NAFLD. CPT-1 controls β -oxidation of fatty acids²⁶⁾. In our present study, the expression of FAS, ACC and SCD-1 in the HepG2 cells treated with FFA increased significantly, while the expression of CPT-1 decreased when compared with the control group. These changes resulted in lipid accumulation in the cells. After PLEO was applied, the expression of FAS, ACC and SCD-1 decreased, while the expression of CPT-1 increased, indicating that PLEO inhibited fatty acid synthesis and promoted its oxidation. The regulation of PLEO on the lipid metabolism associated enzymes might be involved in the therapeutic effect of PLEO on steatosis induced by FFA.

The PI3K/Akt signaling pathway is involved in the regulation of various cell functions, such as proliferation, apoptosis and autophagy. PI3Ks are members of the intracellular lipid kinase family that phosphorylate the hydroxyl groups of 3'-phosphatidylinositol and phosphoinositides²⁷⁾. Akt is a key downstream target of PI3K and a central mediator of the PI3K pathway. It plays a variety of downstream effects through phosphorylation of many substrates involved in cell survival, proliferation, metabolism and movement. Furthermore, PI3K/Akt is an important pathway involving in insulin signaling. Insulin activates PI3K and downstream enzymes via its receptor to regulate glucose and lipid metabolism²⁸⁾. Damage of PI3K/AKT results in insulin resistance, which increases *de novo* lipogenesis and promotes FFA flux to the liver, therefore leads to metabolic disorders such as NAFLD, obesity and type 2 diabetes^{29, 30)}. Cumulative evidences suggest that the PI3K/Akt signaling pathway plays a role in lipid metabolism and liver injury which is related with insulin resistance³¹⁾. The activation of PI3K/Akt can inhibit fat generation, delay excessive fat deposition, and reduce liver steatosis³²⁾. Phosphorylation of Akt up-regulates lipase production and promotes lipid metabolism in hepatocytes³³⁾. Therefore, PI3K/Akt has been an important target for ameliorating insulin resistance in

the treatment of NAFLD^{34–36)}. Here we confirmed that there was a significant decrease in expression of Akt, PI3K and their phosphorylation levels in the FFA treated HepG2 cells. Moreover, PLEO significantly increased the expression of p-Akt and p-PI3K^{37, 38)}, which resulted in alleviation of liver cellular steatosis through improving insulin resistance and was a new strategy for NAFLD treatment.

NF- κ B is a major transcriptional regulator of inflammation and cell death in the development of chronic liver disease³⁹⁾. It is always activated in NAFLD in response to inflammation and regulates the functions of hepatocytes, Kupffer cells, and hepatic stellate cells by inducing the expression of inflammation-related genes, essentially promoting inflammation of the liver during the progression of NAFLD⁴⁰⁾. Our Western blotting analysis data showed that expression of NF- κ B p65 increased while its phosphorylation decreased in the HepG2 cells treated with FFA, indicating that NF- κ B p65 activation mediated the inflammatory injury in the cellular model of NAFLD. After treated with PLEO, p-NF- κ B p65 expression increased significantly while NF- κ B p65 expression decreased, indicating an inhibition of inflammatory injury induced by FFA.

5 Conclusion

In conclusion, PLEO is a complex essential oil extracted from pink lotus flowers. It decreased TC and TG contents and inhibited lipid accumulation in the FFA treated HepG2 cells, exhibiting a potential therapeutic effect on NAFLD. The underlying mechanism might be involved in its regulation on the lipid metabolism associated enzymes, inhibition of NF- κ B signals and inflammatory mediators release, activation of PI3K/Akt signal to improve insulin resistance. PLEO is a promising natural plant essential oil deserving for further research and development.

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Contributions

Runzhou Sun: performed research and wrote the manuscript. Ruixin Xiao: performed research. Pengfei Lv: performed research. Feifei Guo: analyzed data. Yanling Gong: designed research. Meixing Yan: provided financial support.

Conflict of Interest

None of the authors has personal or financial conflicts of interest.

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