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Alpiniae oxyphyllae Fructus ameliorates renal lipid accumulation in diabetic kidney disease *via* activating PPAR α Zi-Jie Yan^{1#}, Lin Zhang^{1#}, Xin-Yao Han², Yu Kang³, Shu-Man Liu¹, Tian-Peng Ma¹, Man Xiao^{4✉}, Yi-Qiang Xie^{1✉}¹College of Traditional Chinese Medicine, Hainan Medical University, Haikou 571199, China²First Clinical College of Medicine of Guizhou University of Traditional Chinese Medicine, Guiyang 550002, China³Heilongjiang Academy of Traditional Chinese Medicine, Harbin 150001, China⁴Key Laboratory of Tropical Translational Medicine of Ministry of Education, School of Basic Medicine and Life Sciences, Hainan Medical University, Haikou 571199, China

ABSTRACT

Objective: To investigate the effects of *Alpiniae oxyphyllae* Fructus (AOF) on renal lipid deposition in diabetic kidney disease (DKD) and elucidate its molecular mechanisms.

Methods: The mechanism of AOF in treating DKD was explored by network pharmacological enrichment analysis, molecular docking, and molecular dynamics simulation. The effects of AOF on renal function and lipid deposition were assessed in a mouse model of DKD and high glucose-stressed HK-2 cells. Cell viability and lipid accumulation were detected by CCK8 and oil red O staining. The expressions of PPAR α and fatty acid oxidation-related genes (*ACOX1* and *CPT1A*) were detected by quantitative RT-PCR, Western blot, and immunofluorescence. Furthermore, PPAR α knockdown was performed to examine the molecular mechanism of AOF in treating DKD.

Results: Network pharmacological enrichment analysis, molecular docking, and molecular dynamics simulation showed that the active compounds in AOF targeted PPAR α and thus transcriptionally regulated *ACOX1* and *CPT1A*. AOF lowered blood glucose, improved dyslipidemia, and attenuated renal injury in DKD mice. AOF-containing serum accentuated high glucose-induced decrease in cell viability and ameliorated lipid accumulation. Additionally, it significantly upregulated the expression of PPAR α , *ACOX1*, and *CPT1A* in both *in vivo* and *in vitro* experiments, which was reversed by PPAR α knockdown.

Conclusions: AOF may promote fatty acid oxidation *via* PPAR α to ameliorate renal lipid deposition in DKD.

KEYWORDS: Diabetic kidney disease; *Alpiniae oxyphyllae* Fructus; Natural medicine; Lipid accumulation; PPAR α ; Fatty acid oxidation

1. Introduction

Diabetic kidney disease (DKD) is a highly prevalent microvascular complication caused by diabetes mellitus (DM) and the leading cause of end-stage renal disease[1,2]. The pathogenesis of DKD is complex. The focus of research has been on the effect of lipid accumulation in the kidney to identify new therapeutic ways. The excessive

Summary

Question: Is it possible that *Alpiniae oxyphyllae* Fructus (AOF) improves renal lipid deposition in diabetic kidney disease by modulating PPAR α ?

Findings: In *in vivo* and *in vitro* studies, AOF significantly attenuated renal damage and lipid deposition. Moreover, the active compounds in AOF may bind to PPAR α to transcriptionally regulate the expression of *ACOX1* and *CPT1A*, promoting fatty acid oxidation and improving lipid deposition.

Meaning: AOF may offer a therapeutic strategy for reducing renal lipid deposition in diabetic kidney disease by activating PPAR α .

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accumulation of lipids causes the development of lipotoxicity, which affects renal function and leads to renal tubulopathy[3,4]. Fatty acid oxidation (FAO) plays an important role in energy metabolism and fatty acid utilization, and reduced FAO function is associated with lipid accumulation as well as renal damage[5,6]. Therefore, improving FAO and renal lipid deposition in patients with DKD may be important for delaying disease progression. DKD is characterized by rapid disease progression and a high mortality rate, but due to the side effects of the drugs and their limitations in application[7], there is a need to find new drugs to target the relevant molecular mechanisms to delay disease progression.

In recent years, natural botanicals have become a research hotspot due to their relative safety, low toxicity, and side effects, and the complexity and diversity of their components, which can interact with multiple targets to delay disease progression[8,9]. According to the Chinese pharmacopeia and flora of China, *Alpiniae oxyphyllae* Fructus (AOF, Yizhiren in Chinese), the dried and ripe fruits of *Alpinia oxyphylla* Miq. (Zingiberaceae), has been widely used for the treatment of chronic glomerulonephritis, nephrotic syndrome, and other kidney diseases for thousands of years in China. AOF has been verified by modern pharmacology to contain a variety of anti-inflammatory, anti-cellular senescence, and anti-diabetic components, which may be an effective candidate for improving renal lipid deposition in patients with DKD[10–12].

Peroxisome proliferator-activated receptor alpha (PPAR α) is a nuclear transcription factor that plays an essential role in the regulation of lipid metabolism[13]. Beta-oxidation in peroxisomes is one of the FAO pathways, which is largely regulated by PPAR α activity[14]. It was found that activation of PPAR α could promote FAO, uptake and reduce lipid levels in the blood, which not only could reduce lipid accumulation in the kidney but also might reduce inflammation and oxidative stress and protect kidney function[15]. Therefore, targeting PPAR α may be an effective therapeutic approach for DKD.

In this experiment, we aimed to investigate whether AOF could ameliorate lipid deposition by modulating the expression of PPAR α in DKD mice and HK-2 cells under high glucose (HG) conditions.

2. Materials and methods

2.1. Chemicals and reagents

The following chemicals and reagents were used in the study: AOF [24011204, Tongrentang Long Kunnan store, Haikou, China; it was authenticated by Prof. Xie Yiqiang (College of Traditional Chinese Medicine, Hainan Medical University) in accordance with the 2020 edition of the Chinese Pharmacopoeia]; Canagliflozin (MedChemExpress, Shanghai, China); Fenofibrate (Topscience, Shanghai, China); MEM medium, fetal bovine serum, and penicillin-

streptomycin (P/S) solution (Pricella, Wuhan, China); 45% D-(+)-dextrose solution (Sigma, Shanghai, China); CCK-8 detection kit (Biosharp, Beijing, China); urine microalbumin (mAlb) assay kit (Shfksc, Shanghai, China); serum creatinine (Scr), blood urea nitrogen (BUN), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) assay kits (Nanjing Jiancheng, Nanjing, China); Eastep[®] Super total RNA extraction kit (Promega, Beijing, China); RIPA lysate, HRP-labeled Goat Anti-Rabbit IgG (H+L) (Beyotime, Shanghai, China); Omni-Easy[™] One-Step PAGE Gel Rapid Preparation Kit, Omni-Easy[™] Instant Protein Sampling Buffer (denaturing, reducing, 5 $\times$) (Epizyme, Shanghai, China); Oil Red O Staining Kit (Kaiji, Nanjing, China); CoraLite594-Goat Anti-Rabbit IgG (H+L) (Proteintech, Wuhan, China); PPAR α (#AF5301, 1:2000) and β -actin (#AF7018, 1:10000) antibodies (Affinity Biosciences, Changzhou, China).

2.2. AOF decoction

A decoction of 400 mL of water with 50 g of AOF was made for 60 min after soaking for 1 h. After the above process was repeated 2 times, the AOF decoction mixture was concentrated to a concentration of 1 g/mL using a rotary evaporator.

2.3. Animals

In this study, 35–48 day-old male SPF grade SD rats, 4-week-old C57BKS-DB (+/+) (*db/m*) mice, and 4-week-old C57BKS-DB (-/-) (*db/db*) mice were used and purchased from Gempharmatech (Nanjing, China). The animals were housed in a specific pathogen-free barrier facility under controlled conditions of constant humidity (50% $\pm$ 5%), temperature (22 $\pm$ 2) $^{\circ}$ C, and a 12-hour light/dark cycle. All mice were fed a standard diet with free access to water.

2.4. AOF-containing serum

The drug-containing serum was prepared by gavage of AOF extract in SD rats. The standard dose of AOF extract was 9 g/kg/day and the normal group was given an equivalent amount of sterilized water according to our previous study[16]. Blood was collected under anesthesia after gavage once a day for 7 consecutive days. The blood was then allowed to stand for 2 h and centrifuged at 3000 rpm at 4 $^{\circ}$ C for 10 min to extract the upper serum layer. Finally, the serum was inactivated in a water bath at 56 $^{\circ}$ C for 30 min and stored at -80 $^{\circ}$ C for subsequent experiments.

2.5. Animal experiments

2.5.1. Experimental grouping

The *db/m* mice were used in normal groups and given sterilized water for 8 weeks and the *db/db* mice were randomly divided into

five groups and treated orally for 8 weeks as follows: 1) Model group given sterilized water; 2) LAOF group receiving 0.625 mg/g/day AOF; 3) MAOF group treated with 1.25 mg/g/day AOF; 4) HAOF group receiving 2.5 mg/g/day AOF; 5) Canagliflozin group administered with 12.5 mg/kg/day Canagliflozin.

2.5.2. Biochemical index analysis

After 8 weeks of drug intervention, a 24-hour urine volume was collected and a blood sample was taken. Random blood glucose (GLU), mAlb, Scr, BUN, TG, TC, and LDL-C were measured using the corresponding kits by a blood glucose meter or MicroplateReader.

2.5.3. Histopathological examination

The kidneys were fixed in 4% paraformaldehyde to prepare paraffin sections. Hematoxylin and eosin (H&E) staining was then performed according to the kit instructions, and images were obtained at 40× magnification using CaseViewer software.

2.6. Cell experiments

2.6.1. Cell culture

HK-2 cells (Pricella, Wuhan, China) were cultured in 5% CO₂ and 37°C incubator with MEM medium containing 1% P/S, 10% fetal bovine serum, or drug-containing serum or blank serum. The cells were divided into 6 groups: 1) Normal group; 2) Model (60 mmol/L glucose); 3) LAOF (60 mmol/L glucose, 2% AOF-containing serum); 4) MAOF (60 mmol/L glucose, 5% AOF-containing serum); 5) HAOF (60 mmol/L glucose, 10% AOF-containing serum); 6) Canagliflozin (60 mmol/L glucose, 10 µmol/L Canagliflozin). The glucose intervention was done for 72 h and the AOF-containing serum intervention for 24 h. To detect the knockdown efficiency of PPARα, HK-2 cells were inoculated in 6-well plates and divided into 5 groups: 1) Normal; 2) si-negative control (NC); 3) si-PPARα (93); 4) si-PPARα (143); 5) si-PPARα (378). Si-RNA oligonucleotides were synthesized by IBSBIO (Shanghai, China), and the si-RNA sequences are shown in Supplementary Table 1. The sequences with the lowest interference efficiency by quantitative RT-PCR and Western blot were selected for further experiments. Then, cells were divided into 5 groups as follows: 1) Normal; 2) Model (60 mmol/L glucose); 3) AOF (60 mmol/L glucose, 10% AOF-containing serum); 4) AOF+si-PPARα [60 mmol/L glucose, 10% AOF-containing serum, si-PPARα (378)]; 5) AOF+si-NC (60 mmol/L glucose, 10% AOF-containing serum, si-NC). Since the downregulation effect of si-RNA on target genes could only be detected at about 48 h, the experiment was performed after 24 hours of intervention with glucose in HK-2 cells, and then the cells were cultured for 8 h in a basic medium with the addition of the corresponding siRNA and transfection reagents, before modeling and subsequent drug intervention. In addition, a rescue experiment was carried out and

there were 4 groups in this experiment: 1) Control; 2) si-PPARα; 3) si-PPARα+Fenofibrate; 4) si-PPARα+AOF.

2.6.2. Cell viability assay

The cell viability was determined using CCK8 assay. In brief, the cell culture medium was removed, then 100 µL MEM medium and 10 µL CCK8 solution were added to each well, and cells were incubated for 2 h. Finally, the absorbance at 450 nm was measured.

2.6.3. TC and TG assays

Cells were collected and resuspended in PBS and centrifuged at 4 °C, 1 000 rpm for 10 min, and the supernatant was discarded. The cells were lysed with 200 µL of lysate for 30 min and the absorbance was measured at 500 nm according to the instructions.

2.6.4. Oil red O staining

HK-2 cells were inoculated onto slides for modeling and drug treatment and then stained with oil-red O according to the kit instructions. After staining, the slides were sealed with a glycerol-gelatin sealing solution and finally observed and photographed under an orthostatic microscope (Olympus, BX53).

2.6.5. Immunofluorescence staining

Modeling and drug interventions were performed on HK-2 cells inoculated into 24-well plates. Before staining, the cells were washed 3 times with PBS, fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.2% Triton X-100 for 10 min. After blocking with 10% goat serum, primary antibodies were applied and incubated overnight at 4°C. On the following day, cells were incubated with a fluorescent secondary antibody for 1 h at room temperature. Finally, an anti-fluorescence quenching blocking solution (containing DAPI) was applied, and cells were imaged using a fluorescence microscope (Olympus, IX51).

2.7. Quantitative RT-PCR analyses

Total RNA was extracted from cellular or renal tissue samples and reverse transcribed to cDNA. Subsequently, quantitative RT-PCR analyses were performed using primers synthesized by Tsingke (Beijing, China), and the primer sequences are shown in Supplementary Table 2. Gene expression was detected following a previous methodology[11].

2.8. Western blot analysis

Cells or kidney tissues were lysed with RIPA and PMSF. Western blot was performed using antibodies obtained from Affinity. Protein band signals were detected in e-Blot using ECL developer and the gray scale of protein bands was scanned with imageJ. Detection was conducted following a previous methodology[11].

2.9. Network pharmacology analysis

Based on the AOF active compounds and related targets of DKD collected in our previous study[11], we intersected the drug with the disease targets and then entered the intersecting targets into the DAVID (<https://david.ncifcrf.gov/>) for enrichment analysis, and the results were exported *via* KEGG (<https://www.genome.jp/kegg/>) and visualized as bubble plots.

2.10. Molecular docking

The crystal structure of PPAR α protein (6KAX) was obtained from the PDB (<http://www.rcsb.org/>). Structures of small molecule compounds were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). Molecular docking was performed after processing of proteins and small molecule compounds using PyMOL, AutoDock Vina 1.1.2, and Open Babel GUL. Finally, the region where the compound binds most stably to the protein is selected as the final conformation and the ligand-protein complex was visualized using PyMOL software.

2.11. Molecular dynamics simulation

In order to run molecular dynamics simulations in Amber 24 (San Francisco, CA, USA), each molecular interaction will be described by a molecular force field, with specific parameters defined for each molecule using the FF19SB force field. Solvation was carried out using the TIP3P water model and counterbalance ions were added to neutralize the system and energy was minimized. Then, the system was heated from 0 K to 310.15 K within 500 ps and the system was pre-equilibrated. Finally, 100 ns molecular dynamics simulations were performed in an isothermal isobaric system. The results were analyzed using AmberTools23 and visualized using Qtgrace. The root mean square deviation (RMSD) curves indicate the fluctuation of protein conformation. The root mean square fluctuation (RMSF) responds to how much each residue in the protein fluctuates during the simulation. Solvent accessible surface area (SASA) analysis reflects the relative exposure of the ligand to the solvent, and thus indirectly reflects how tightly the protein wraps around the ligand. The tightness and rigidity of the protein backbone can be assessed by the radius of gyration (Rg). Hydrogen bonds play an important role in the maintenance of protein conformation. There is a positive correlation between the change in the number of hydrogen bonds and the binding strength during the simulation. The free energy landscape topography can be used to study the transition behavior between the multiple conformational state clusters that Nabai may exhibit and to extract the steady state conformation of the protein at the energy low point. The blue regions in the figure are lower in energy and are regions of conformational concentration; the red regions are higher in energy and are regions of conformational rarity.

2.12. Statistical analysis

Data are presented as mean $\pm$ standard deviation. Statistical comparisons among multiple groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test for pairwise comparisons. All analyses were conducted using GraphPad Prism 9.4.1, with $P < 0.05$ considered statistically significant.

2.13. Ethical statement

The study was approved by the Animal Care and Ethics Committee of Hainan Medical University on 16 February 2021 (approval ID: HYLL-2021-389).

3. Results

3.1. Transcriptional regulation of ACOX1 and CPT1A by PPAR α may be an important pathway of AOF in improving DKD

We screened the active compounds of AOF by UHPLC-QTOF-MS in a previous study[11]. In this study, a network pharmacological analysis of 26 AOF active components was performed. KEGG enrichment analysis showed that PPAR α was involved in 10 signaling pathways, including adipocytokine signaling pathway, glucagon signaling pathway, and PPAR signaling pathway (Figure 1A-B). Figure 1A demonstrates the upstream and downstream positions and roles of PPAR α in the PPAR signaling pathway, which may act as a transcription factor to regulate the expression levels of CPT1A and ACOX1 and thus affect FAO, thereby ameliorating the abnormalities of lipid metabolism. Additionally, the transcriptional regulation of CPT1A and ACOX1 by PPAR α was predicted through the TRRUST database. Next, we performed molecular docking of the active components with PPAR α . Molecular docking showed all binding energies were ≤ -1.2 kcal/mol (Supplementary Table 3), indicating that these active ingredients have good binding activity with PPAR α and can target binding proteins, and the lower the binding energy, the higher the likelihood of binding. Supplementary Figure 1 shows a schematic representation of the lowest binding energy of PPAR α to AOF active compounds. Subsequently, based on the above molecular docking results, we selected the two compounds with the lowest binding energies to perform molecular dynamics simulations with the protein conformations to further confirm their binding ability to PPAR α .

The RMSD curves show that PPAR α -abscisic acid and PPAR α -matricin were stable after 70 ns and 60 ns, respectively (Figure 1C), which means that the protein conformation of PPAR α did not change much after binding to these two compounds, and the binding was more stable. The absence of interruptions in the RMSD curves indicates that the small molecule compounds can firmly attach to PPAR α without dissociating from the protein pocket during the

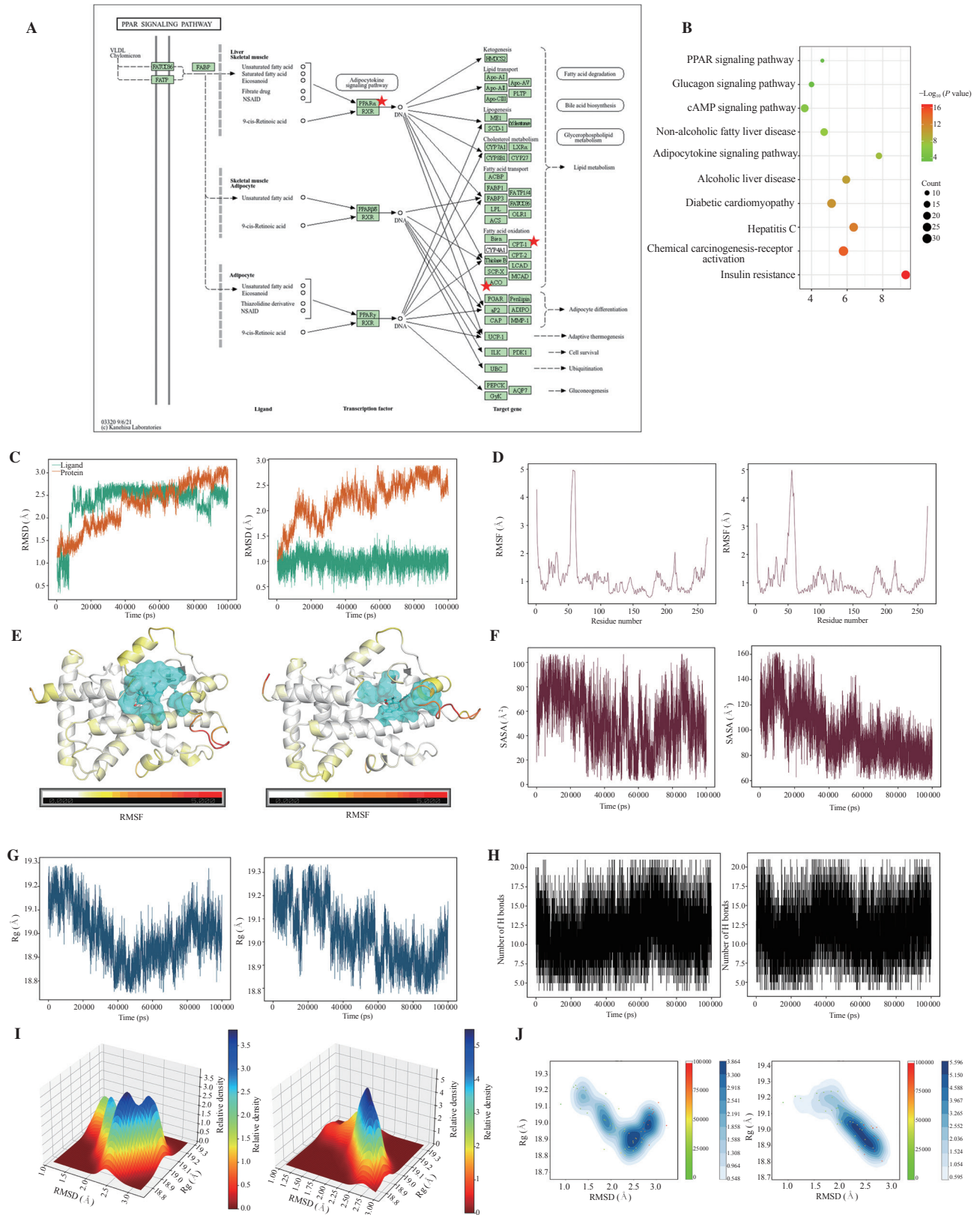


Figure 1. PPAR signaling pathway (A); PPAR α -involved signaling pathways by KEGG enrichment analysis (B). Results of molecular dynamics simulations about root mean square deviation (RMSD) (C), root mean square fluctuation (RMSF) (D), complex trajectory diagrams and RMSF values (E), solvent accessible surface area (SASA) (F), radius of gyration (Rg) (G), number of hydrogen bonds (H), 3D (I) and 2D (J) landscape maps on free energy. PPAR α -abscisic acid on the left side of each subfigure, and PPAR α -matricin on the right side of each subfigure for C-J.

simulation. The similarity of the RMSF peaks and values of these two complexes suggests that they have roughly the same effective site of action, and it may also imply that abscisic acid and matricin act by a similar mechanism (Figure 1D-E). SASA analysis shows that PPAR α -abscisic acid and PPAR α -matricin decreased throughout the simulation, suggesting that the binding between them gradually

increases (Figure 1F). Moreover, the average value of Rg for both complexes was about 19 Å, indicating a tightly bound and rigid structure of the protein (Figure 1G). The hydrogen bonding change curves indicate that the interaction between these two compounds and PPAR α is through hydrogen bonding (Figure 1H). Figure 1I-J shows the free energy landscape changes for two ligands

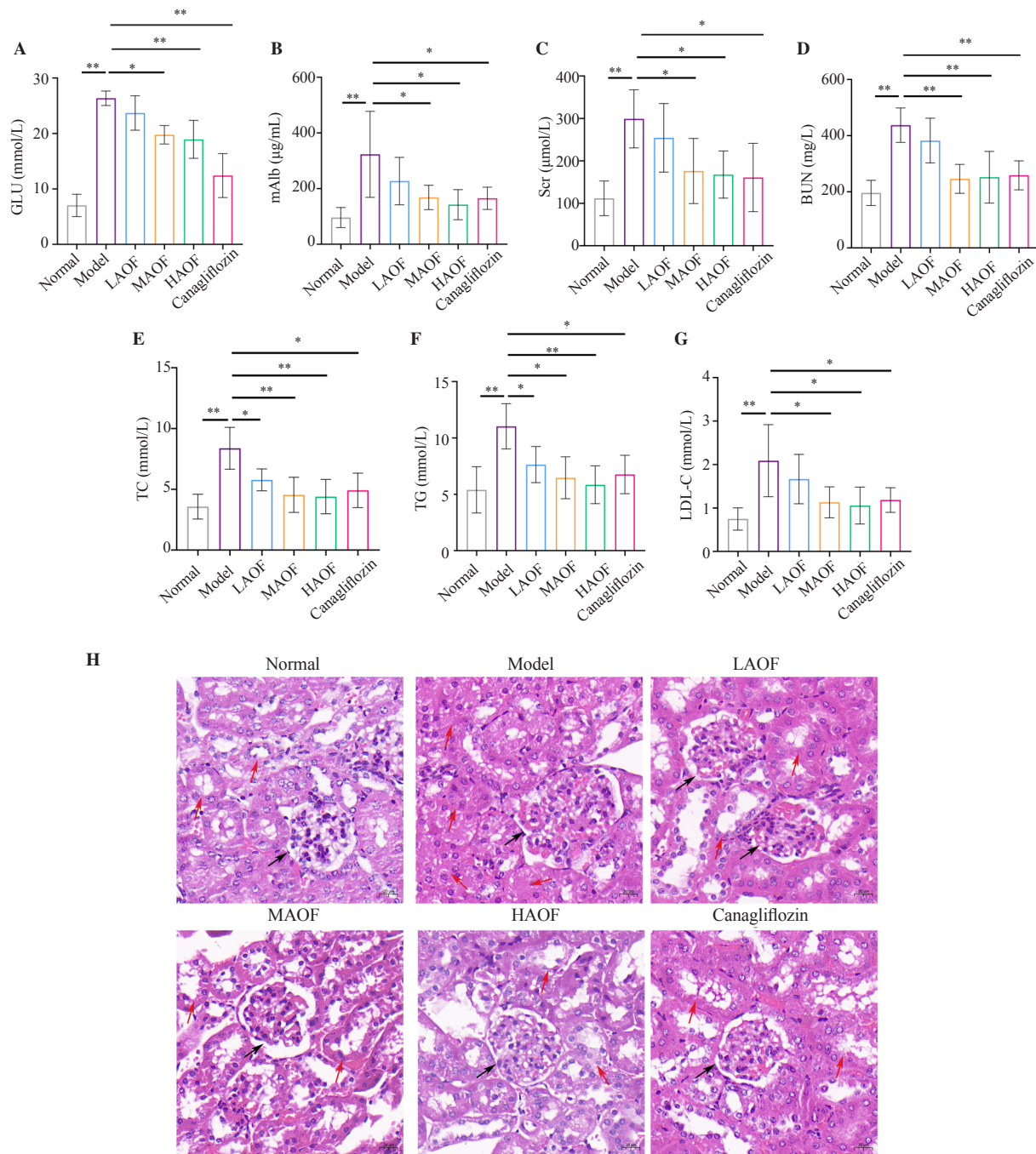


Figure 2. *Alpiniae oxyphyllae* fructus (AOF) decoction ameliorates abnormal biochemical indices and renal damage in mice with diabetic kidney disease. Effect of AOF on the random blood glucose (GLU) (A), urine microalbumin (mAlb) (B), serum creatinine (Scr) (C), blood urea nitrogen (BUN) (D), total cholesterol (TC) (E), triglyceride (TG) (F), low-density lipoprotein cholesterol (LDL-C) (G). Histopathological changes in renal tissues by H&E staining (magnification ×40; scale bar: 20 μm) (H). Red arrows indicate tubules and black arrows indicate glomeruli. All data are expressed as mean ± SD ($n = 6$). * $P < 0.05$, ** $P < 0.001$.

bound to PPAR α . In the plot for PPAR α -abscisic acid, there were four free energy grooves, indicating four relatively stable low-energy conformations during the molecular dynamics simulation process; however, there was only one free energy groove in the free energy landscape plot for PPAR α -matricin, indicating one stable conformation.

3.2. AOF decoction ameliorates abnormal biochemical indices and renal pathological tissue damage in DKD mice

Compared with the normal group, GLU, mAlb, Scr, and BUN levels of the model group were significantly elevated, which were significantly decreased in the MAOF and HAOF groups ($P < 0.05$) (Figure 2A-D). In order to investigate whether the AOF

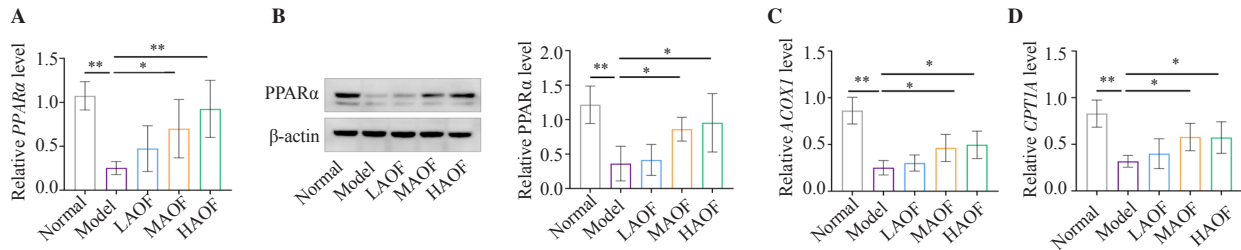


Figure 3. AOF decoction increases PPAR α (A-B), ACOX1 (C) and CPT1A (D) expression levels. β -actin was used as an internal control. All data are expressed as mean $\pm$ SD ($n = 6$). * $P < 0.05$, ** $P < 0.001$.

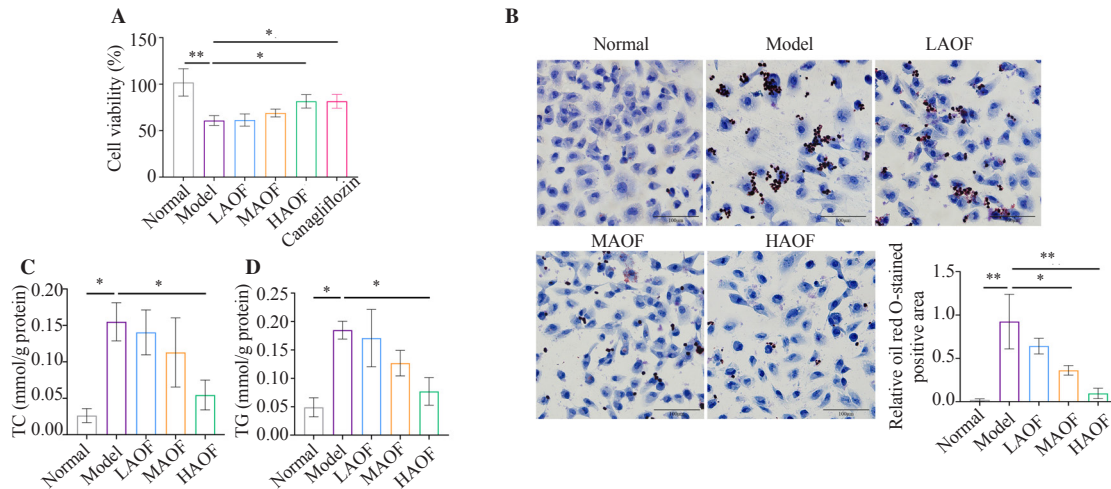


Figure 4. AOF-containing serum increases HK-2 cell viability and lipid deposition under high glucose conditions. Cell viability (A); red oil O-stained positive area (magnification $\times 10$; scale bar: 100 μ m) (B); TC (C) and TG (D) contents. All data are expressed as mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.001$.

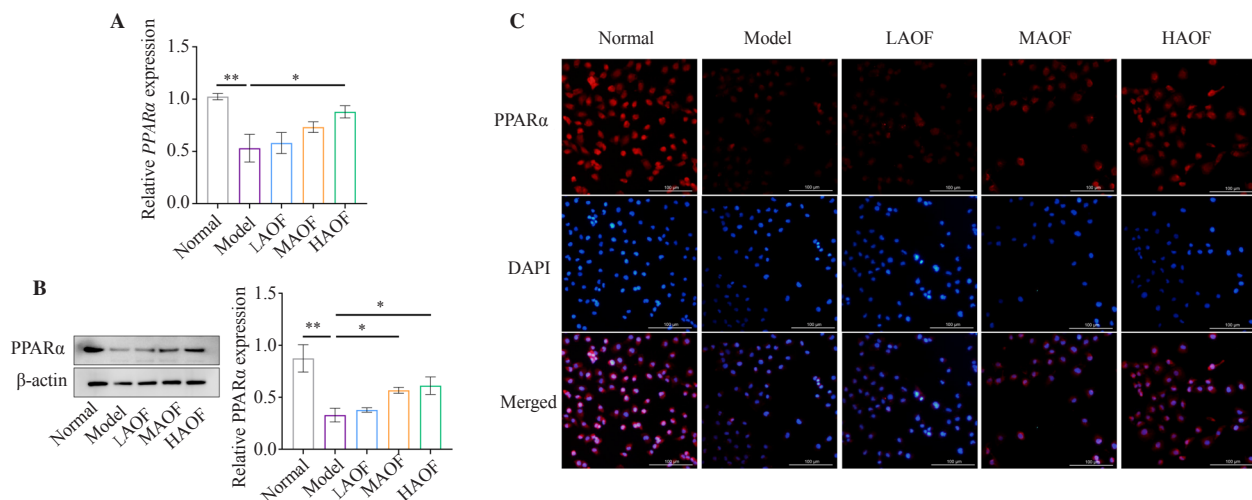


Figure 5. AOF-containing serum promotes PPAR α expression in HK-2 cells under high glucose conditions. The mRNA and protein expression of PPAR α (A-B) and its fluorescence intensity (magnification $\times 10$; scale bar: 100 μ m) (C). All data are expressed as mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.001$.

decoction improves lipid levels, changes in relevant serum assays were examined. TC, TG, and LDL-C in the serum of DKD mice were significantly higher than those of mice in the normal group

($P < 0.001$). Treatment with AOF decreased TC, TG, and LDL-C dose-dependently (Figure 2E-G). H&E staining was used to observe the pathological changes in the kidneys of mice. It was seen that

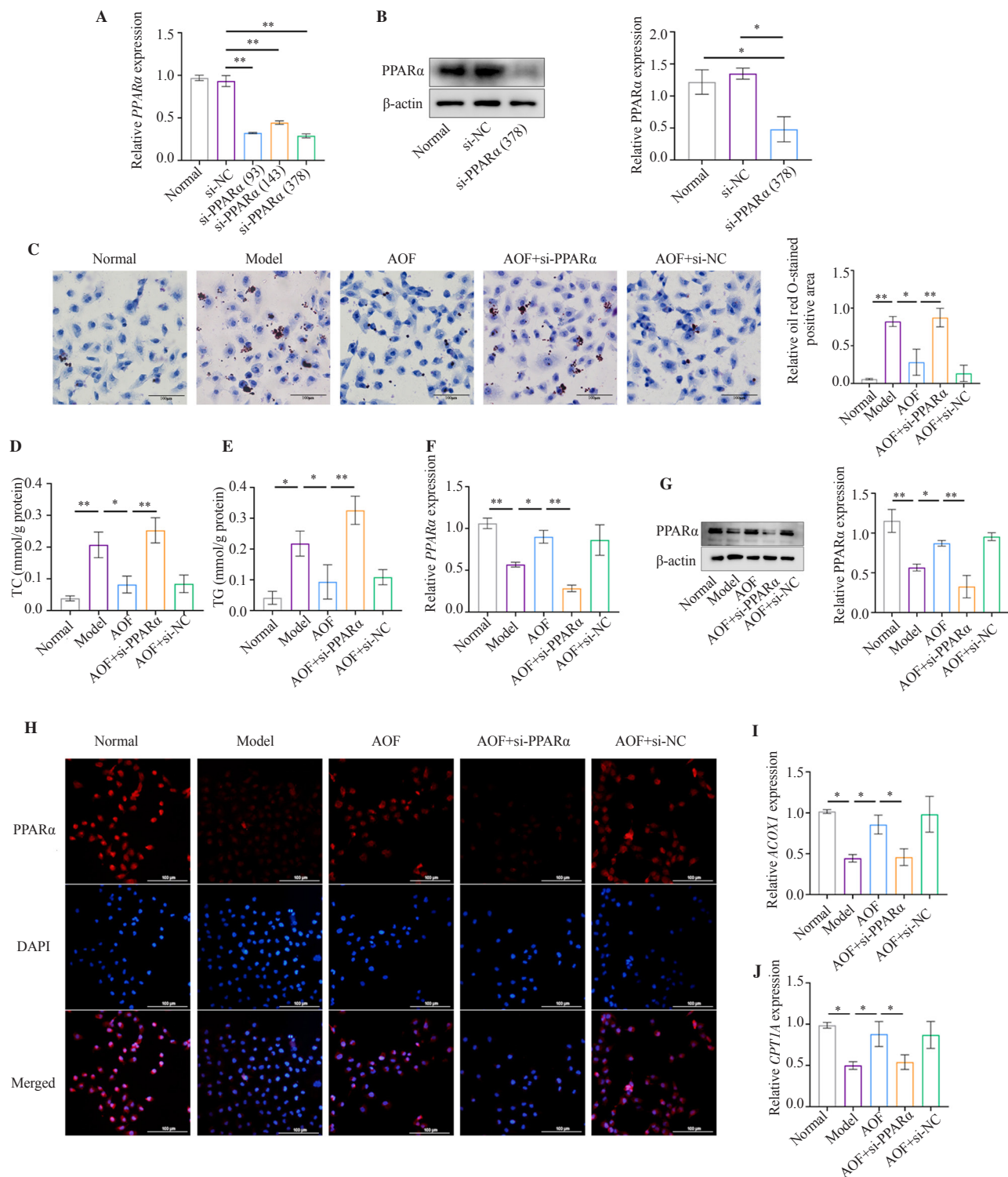


Figure 6. AOF-containing serum ameliorates lipid deposition in HK-2 cells under high glucose conditions by promoting PPARα. PPARα mRNA (A) and protein (B) expression levels were detected by quantitative RT-PCR and Western blot, respectively after administration of 100 pmol si-PPARα synthesized by IBSBIO. Red oil O-stained positive area (magnification ×10; scale bar: 100 μm) (C); TC (D) and TG (E) contents; PPARα mRNA and protein expression (F-G) as well as its fluorescence intensity (magnification ×10; scale bar: 100 μm) (H); *ACOX1* mRNA expression (I); *CPT1A* mRNA expression (J). All data are expressed as mean ± SD ($n = 3$). * $P < 0.05$, ** $P < 0.001$.

the kidney tissues of mice in the normal group were structurally intact and morphologically clear under the microscope, while the kidney tissues of the model group showed an increase in the area of glomerulonephritis, thickening of the basement membrane, and the renal tubules were swollen with a narrowing of the tubular lumen. After treatment with AOF, the above renal changes in the DKD mice were significantly alleviated (Figure 2H).

3.3. AOF decoction increases *PPARα*, *ACOX1*, and *CPT1A* expression levels

The results showed that AOF increased the expression levels of *PPARα* mRNA and protein in the kidneys of DKD mice in a dose-dependent manner (Figure 3A-B). In addition, AOF could upregulate the expression levels of *ACOX1* and *CPT1A* mRNA, and the effect of medium and high doses was better than that of a low dose (Figure 3C-D).

3.4. AOF-containing serum increases HK-2 cell viability and ameliorates lipid deposition under HG conditions

CCK8 assay showed that the cell viability of the model group was significantly decreased compared with that of the normal group ($P < 0.001$). After the administration of different doses of AOF-containing serum, the cell viability was increased to different degrees (Figure 4A). Oil red O staining showed that lipid droplets of HK-2 cells increased under HG conditions, while they decreased significantly after administration of AOF-containing serum (Figure 4B). In addition, intracellular TC and TG content increased significantly under HG conditions ($P < 0.05$), which were markedly reduced by treatment with medium and high doses of AOF-containing serum ($P < 0.05$) (Figure 4C-D). The efficacy of the drug-containing serum increased with increasing concentration.

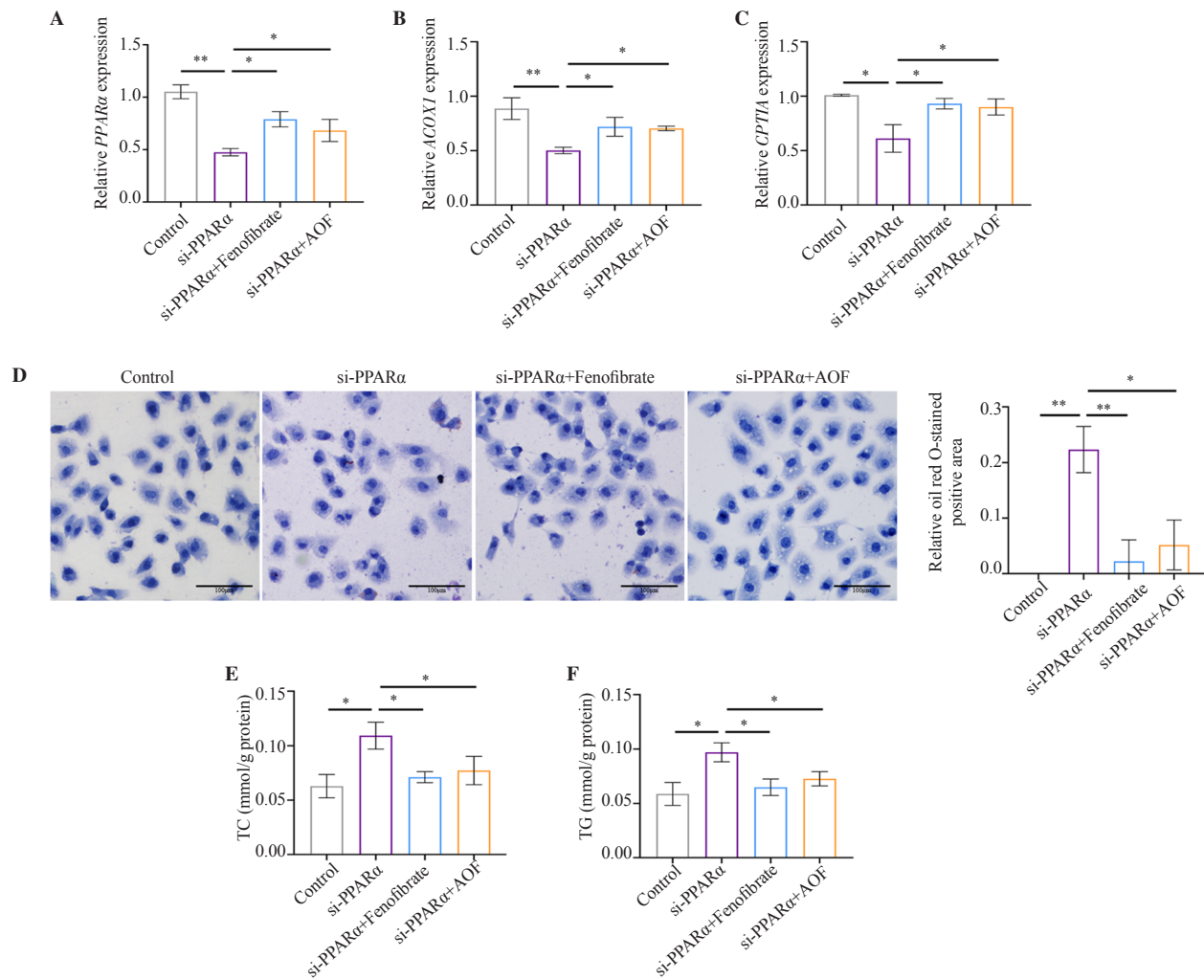


Figure 7. Rescue experiment results. *PPARα* (A), *ACOX1* (B) and *CPT1A* (C) mRNA expressions were determined by quantitative RT-PCR assay. Red oil O-stained positive area (magnification $\times 10$; scale bar: 100 μm) (D); TC (E) and TG (F) contents. All data are expressed as mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.001$.

3.5. AOF-containing serum promotes *PPARα* expression in HK-2 cells under HG conditions

The results showed that compared with the normal group, the mRNA and protein expression of *PPARα*, and its fluorescence intensity were significantly reduced in the model group ($P<0.001$). However, AOF-containing serum enhanced its expression levels and fluorescence intensity ($P<0.05$) (Figure 5).

3.6. AOF-containing serum upregulates *CPT1A* and *ACOX1* mRNA expression and ameliorates lipid deposition in HK-2 cells under HG conditions by promoting *PPARα*

To further investigate the molecular mechanism of the ameliorative effect of AOF on lipid deposition in DKD, AOF-containing serum was administered along with *PPARα* knockdown for reverse validation under HG conditions. Firstly, we detected the interference efficiency of *PPARα* by quantitative RT-PCR and found that *PPARα* (378) knockdown was the most efficient, which was further verified in Western blot analysis (Figure 6A-B). Since previous results showed that 10% AOF-containing serum had the best efficacy in mitigating DKD, we chose the interfering sequence of si-*PPARα* (378) to intervene in 10% AOF-containing serum for further experiments. The results showed that *PPARα* knockdown significantly reversed the ameliorative effect of AOF on lipid droplet accumulation ($P<0.001$) (Figure 6C). Moreover, *PPARα* knockdown remarkably abrogated the effect of AOF on lowering TG and TC ($P<0.001$) (Figure 6D-E). Quantitative RT-PCR, Western blot, and

immunofluorescence staining showed that compared with the AOF group, the AOF + si-*PPARα* group showed *PPARα* mRNA, protein, and fluorescence intensity was significantly reduced ($P<0.05$) (Figure 6F-H). Previous results have shown that *PPARα* may act as a transcription factor for *ACOX1* and *CPT1A* and thus regulate their expression levels; therefore, we examined by quantitative RT-PCR whether AOF regulates these two target genes by promoting *PPARα*. Quantitative RT-PCR showed that *ACOX1* and *CPT1A* mRNA expression levels were increased by AOF-containing serum compared to the model group, which were abrogated by knockdown of *PPARα* ($P<0.05$) (Figure 6I-J). In addition, to further validate the effects of *PPARα* on the regulation of *CPT1A*, *ACOX1* and lipids, we performed a rescue experiment in which a *PPARα* agonist (fenofibrate) and AOF were administered along with the addition of si-*PPARα*. The results revealed that fenofibrate and AOF reversed the effects of *PPARα* knockdown on *ACOX1* and *CPT1A* reduction as well as increased TC and TG content and lipid droplet accumulation (Figure 7).

4. Discussion

The development of DKD is related to a variety of factors, such as family history, poor lifestyle habits, hypertension, persistent hyperglycemia, and abnormal lipid levels[17,18]. Among them, lipid metabolism disorders are an important cause of DKD progression and one of the major complications of DM[19]. However, the mechanism of action associated with lipid accumulation and renal

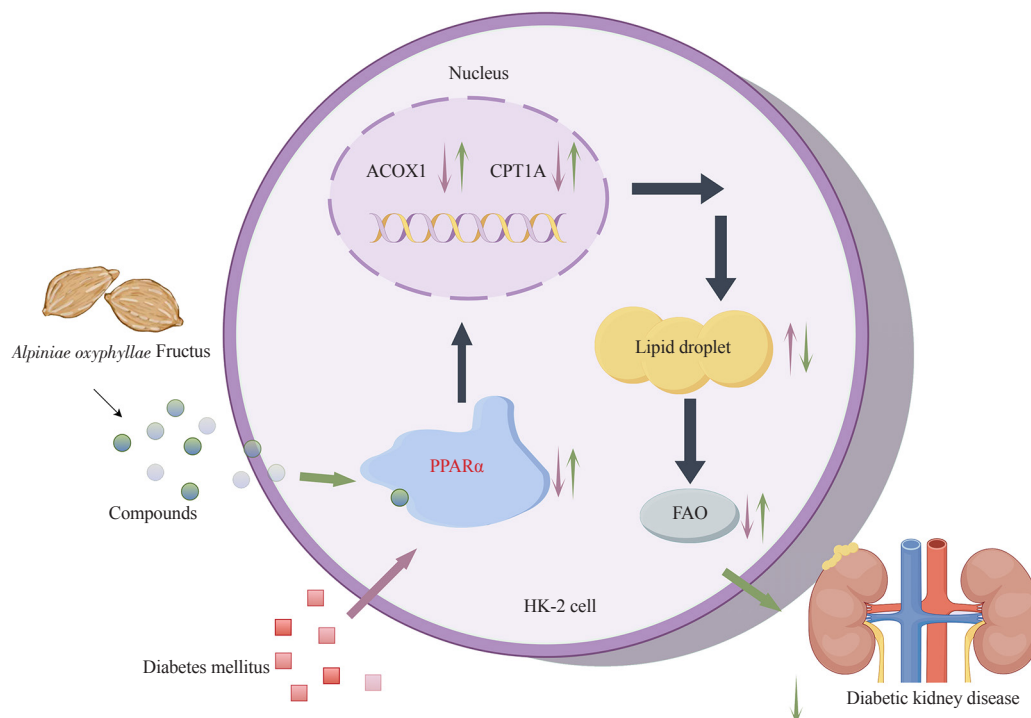


Figure 8. Mechanism of the effect of AOF on renal lipid deposition in diabetic kidney disease (by Figdraw). FAO: fatty acid oxidation.

injury due to lipid metabolism disorders in DKD has not been fully elucidated so far. Therefore, it is particularly important to seek the pathogenesis of DKD and explore new therapeutic approaches to delay the development of DKD.

Persistent hyperglycemic abnormalities in patients with DM progress to DKD, which is clinically characterized by increased urinary mAlb excretion and impaired renal function, including elevated Scr and urea nitrogen levels. In the early stages of DKD, increased urinary mAlb excretion occurs due to glomerular hyperfiltration caused by impaired renal function. Therefore, microalbuminuria is used as an early risk indicator of DKD and to predict the progression of DKD[20]. When DKD occurs, pathological changes are detected in the kidney tissues, including glomerular swelling and sclerosis, thickening of the basement membrane, and narrowing of the tubular lumen. Although a renal biopsy is not usually necessary for the diagnosis of DKD, it has been shown that functional changes in the kidneys are associated with specific histopathological findings in DM[21]. AOF is a commonly used clinical drug for the treatment of kidney diseases, which is rich in a variety of bioactive ingredients[22]. Previous clinical studies conducted by our group as well as literature reports have found that Chinese medicine compounds consisting of AOF as the monarch drug have good clinical efficacy in DKD patients[23,24].

In this study, biochemical indices of DKD mice showed increased levels of GLU, mAlb, Scr, BUN, and impaired renal function. H&E staining confirmed significant renal injuries in DKD mice. However, treatment with AOF decoction significantly improved renal function and pathological tissue damage in DKD mice. Typical glomerular structural damage has been reported in about 1% of patients with diabetic microalbuminuria, whereas 1/3 of patients have no or only very mild glomerular damage but with severe tubular epithelial cell damage[25]. Therefore, it is of greater importance to study the correlation between renal tubular epithelial cells and the deterioration of renal function by culturing HK-2 cells as an *in vitro* model. In the present study, we verified that AOF-containing serum had a protective effect on HG-induced HK-2 cell injury by CCK8 assay, which could reduce cell death and improve cell survival. The above characteristics are consistent with those reported in some studies[26,27], suggesting that AOF significantly ameliorates renal damage caused by the diabetic environment, especially renal tubular epithelial cells.

In recent years, a growing number of studies have shown that the occurrence of lipid deposition in the kidneys of DKD is associated with the progression of the disease[28]. High levels of lipids in the body can cause fatty deposits in renal tissues, glomerulosclerosis, and tubular dilatation, exacerbate the progression of proteinuria and tubulointerstitial fibrosis, and result in irreversible damage to renal function by directly damaging cells[29,30]. To further verify the relationship between lipid accumulation and kidney injury in DKD, we applied a DKD mouse model and observed that AOF significantly downregulated TC, TG, and LDL-C contents, and

improved the blood lipid level. In addition, when lipid metabolism disorders resulting from excessive energy intake cause lipid accumulation in important organs such as the liver and kidney, this accumulation is characterized by a positive manifestation of oil red O staining. In some studies, lipid deposition was found in the kidneys of DKD patients and DKD mice by oil red O staining, and the lipids were mainly located in the tubular site[31,32]. In our experiments, significant lipid droplet accumulation was found in HG-stressed HK-2 cells by oil red O staining, and lipid droplets were reduced after treatment with AOF-containing serum. Consistent with the oil red O results, intracellular TC and TG contents were significantly decreased and lipid levels were optimized by AOF treatment. These results suggest that AOF not only ameliorates renal injury, but also attenuates dyslipidemia and lipid accumulation in renal tubular epithelial cells in DM.

PPARs are a ligand-dependent nuclear receptor superfamily regulating lipid and glucose metabolism, of which PPAR α is one of the major members, mainly expressed in the liver, intestine, and kidney[33,34]. Numerous studies have shown that PPAR α plays a key role in regulating FAO and lipid metabolism as well as inflammation[35,36]. In the kidney, PPAR α , which is highly expressed in podocytes and tubular epithelial cells, regulates genes involved in FAO and is closely related to fatty acid uptake, esterification, and transport[37]. Therefore, a decrease in PPAR α causes lipid accumulation and exacerbates proteinuria and glomerular injury in kidney-related diseases[38]. In the clinic, PPAR α agonists such as fibrates are used to treat dyslipidemia and slow the progression of DKD through their lipid-related and lipid-independent effects[39]. Our study not only found that AOF has similar blood glucose-lowering and renal function-improving effects as canagliflozin but also similar lipid deposition-improving effects as fenofibrate, which further demonstrates the advantages of multiple targets and multiple pathways of traditional Chinese medicine in treating diseases. Moreover, fatty acid β -oxidation genes such as *ACOX1* and *CPT1A* were also found to be downregulated in a model of non-alcoholic fatty liver disease, affecting the progression of hepatic steatosis, whereas treatment with a PPAR α agonist ameliorated lipid accumulation in hepatocytes[40,41]. Some studies have found that the kidneys of DKD have lipid metabolism malfunctions accompanied by abnormal gene expression of PPAR α , *ACOX1*, and *CPT1A*, and DKD-associated renal lipid accumulation can be alleviated by regulating the expression of PPAR α , CPT1A, and ACOX1[42,43]. Therefore, PPAR α , CPT1A, and ACOX1 play critical roles in the regulation of abnormal lipid deposition, and its aberrant expression may be involved in lipid deposition in the kidneys of DKD. We found that the expression levels of PPAR α mRNA and protein and the expression levels of *ACOX1* and *CPT1A* mRNA were significantly reduced in DKD mice and HG-intervened HK-2 cell model, which were increased by AOF treatment.

In addition, a good binding ability was found between AOF active compounds and PPAR α by molecular docking and molecular

dynamics simulation, therefore, AOF has the potential to target and regulate the expression level of PPAR α . In subsequent experiments, the knockdown of PPAR α reversed the therapeutic effect of AOF, resulting in decreased levels of ACOX1 and CPT1A mRNA, increased TC and TG content, and accumulation of lipid droplets. These effects were abrogated by a PPAR α agonist (fenofibrate) as well as AOF, suggesting that PPAR α is a key target of AOF for the treatment of renal lipid deposition in DKD and may act as a transcription factor to regulate ACOX1 and CPT1A, promoting the FAO process and facilitating lipid clearance (Figure 8). However, there are some limitations of our study. The study only performed rescue experiments at the cellular level and lacked the reverse validation of PPAR α in animal experiments. In addition, the modulatory role of active components of AOF, such as abscisic acid and matricin, with PPAR α needs to be confirmed by further animal or cellular experiments.

In conclusion, AOF ameliorates DKD in *in vivo* and *in vitro* experiments by promoting PPAR α . It might be used as a preventive and therapeutic agent for DKD-induced renal lipid deposition. However, its efficacy needs further investigation.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

Conceptualization and design were performed by YQX, ZJY, and MX. Data collection, analysis and interpretation were done by ZJY, LZ, XYH, YK, SML, and TPM. Funding acquisition was from YQX. The first draft of the manuscript was written by ZJY and LZ. The previous version and revised version of the manuscript were commented by ZJY, LZ, YQX, MX and XYH. The final manuscript was read and approved by YQX, MX, ZJY and LZ.

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Alpiniae oxyphyllae Fructus ameliorates renal lipid accumulation in
diabetic kidney disease *via* activating PPAR α

Running title: Mechanism of alleviating renal lipid deposition by AOF

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Supplementary Table 1. si-RNA sequences.

si-RNA	Sense	Anti-sense
si-NC	5'-UUCUCCGAACGUGUCACGUTT-3'	5'-ACGUGACACGUUCGGAGAATT-3'
si-PPARA(93)	5'-GGGAAACAUCCAAGAGAUUUU-3'	5'-AAUCUCUUGGAUGUUUCCCUU-3'
si-PPARA(143)	5'-GCUUUGGCUUUACGGAAUAUU-3'	5'-UAUUCCGUAAAGCCAAAGCUU-3'
si-PPARA(378)	5'-GGCGAACGAUUCGACUCAAGC-3'	5'-UUGAGUCGAAUCGUUCGCCGA-3'

Supplementary Table 2. Primer sequences.

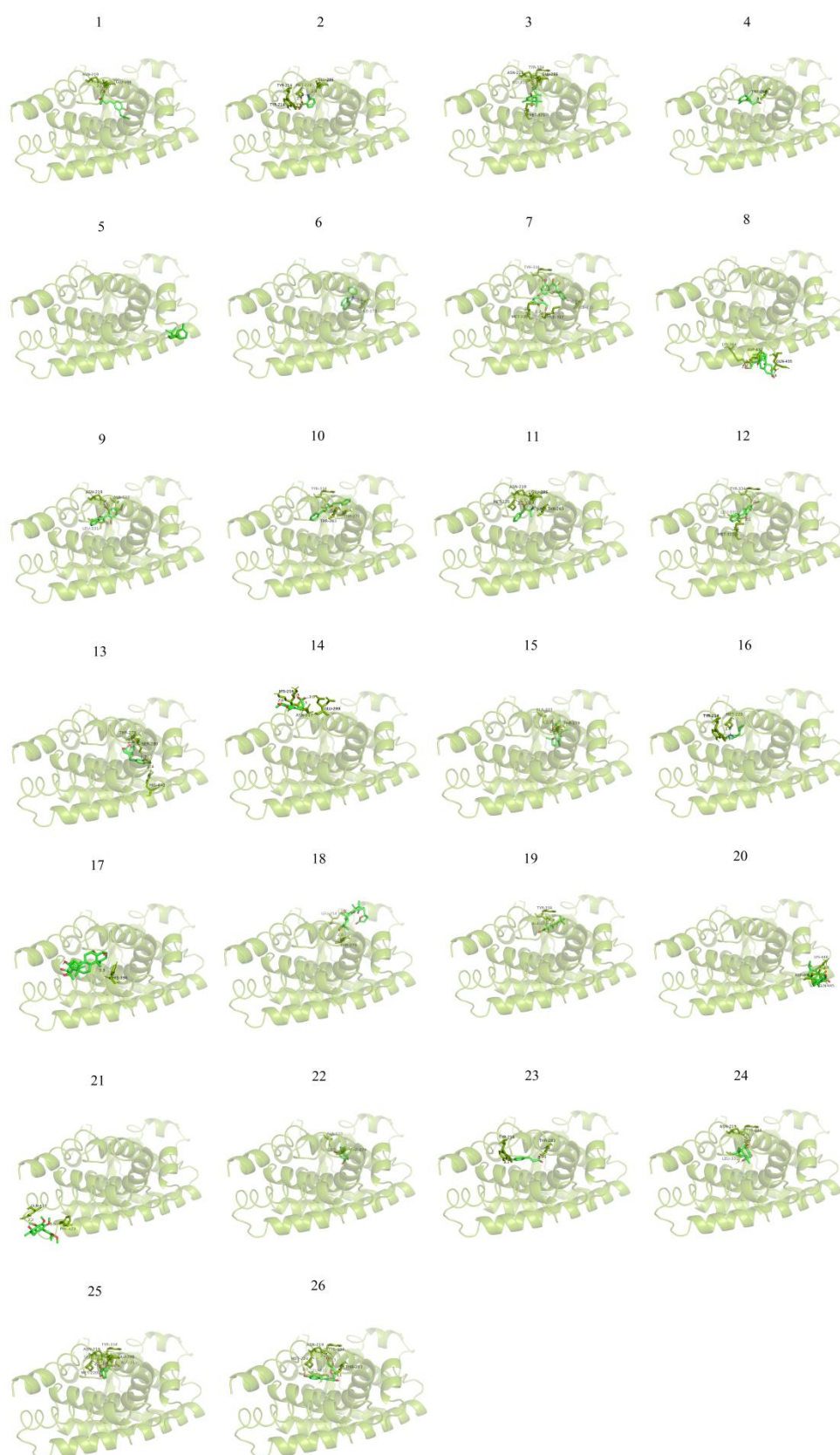
Gene	Forward	Reverse
<i>PPARA</i> (human)	5'-ATGGTGGACACGGAAAGCC-3'	5' -CGATGGATTGCGAAATCTCTTGG-3'
<i>ACOX1</i> (human)	5'-ACTCGCAGCCAGCGTTATG-3'	5-AGGGTCAGCGATGCCAAAC-3'
<i>CPT1A</i> (human)	5'-TCCAGTTGGCTTATCGTGGTG-3'	5-TCCAGAGTCCGATTGATTTTTGC-3'
<i>β-actin</i> (human)	5'- CATGTACGTTGCTATCCAGGC-3'	5'- CTCCTTAATGTCACGCACGAT-3'
<i>PPARA</i> (mouse)	5'- CCTCCGCTAGTGTCCGATAG-3'	5'- GCGCATCTCATCACCAAGAG-3'
<i>ACOX1</i> (mouse)	5'-TAACTTCCTCACTCGAAGCCA-3'	5'-AGTTCCATGACCCATCTCTGTC-3'

<i>CPT1A</i> (mouse)	5'-CTCCGCCTGAGCCATGAAG-3'	5'-CACCAGTGATGATGCCATTCT-3'
<i>β-actin</i> (mouse)	5'-GTGACGTTGACATCCGTAAAGA-3'	5'-GCCGGACTCATCGTACTCC-3'

Supplementary Table 3. Binding energy values for AOF active compounds and PPARA docking.

No.	Compounds	Binding energy (kcal/mol)	No.	Compounds	Binding energy (kcal/mol)
1	Chalcone base + 3O, 1Prenyl	-7.46	14	cis-Sinapic acid	-4.89
2	Methyl 2-aminobenzoate	-5.02	15	Matricin	-7.56
3	Abscisic acid	-7.90	16	DL-Coniine	-5.77
4	Dihydrodamascenone	-6.74	17	Ixocarpalactone A	-7.40
5	(1α,6α,7αH)-2,4(15)-Copadiene	-7.04	18	Lucidenolactone	-7.03
6	N-Phenyl-1-naphthylamine	-7.45	19	Luvangetin	-6.88
7	kaempferol-7-O-hexoside	-5.39	20	1,4a-dimethyl-9-oxo-7-propan-2-yl-3,4,10,10a-tetrahydro-2H-phenanthrene-1-carboxylic acid	-7.40
8	Reichsteins substance S	-6.38	21	Schizandrin A	-5.68
9	2',6'-dihydroxy-4'-methoxydihydrochalcone	-6.86	22	Isokobusone	-7.21
10	5-Methoxy-1,7-diphenyl-3-heptanone	-7.49	23	Methyl 4-hydroxycinnamate	-5.11

11	Norephedrine	-7.08	24	Azuleno(5,6-c)furan-1(3H)-one,4,4a,5,6,7,7a,8,9-octahydro-3,4,8-trihydroxy-6,6,8-trimethyl-	-6.84
12	Batatasin IV	-6.35	25	Ethyl caffeate	-5.34
13	Cubebin	-7.51	26	Coumaroyl Hexoside	-6.62



Supplementary Figure 1. Visualization of the molecular docking about PPAR α and AOF active compounds.