ARTICLE

The anti-obesity effect of lotus leaves on high-fat-diet-induced obesity by modulating lipid metabolism in C57BL/6J mice

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Abstract
Lotus leaves (Nelumbo nucifera) are widely used in medicines and foods. The investigate systematically studied the anti-obesity effect of lotus leaf extracts. It could reduce body weight, alleviate liver damage, and inhibit fat accumulation in high-fat-diet-induced obese mice. Lotus leaf extracts reduced serum alanine aminotransferase (ALT), aspartate transaminase (AST), and alkaline phosphatase (AKP) levels; decreased total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) levels in the serum; and increased high-density lipoprotein cholesterol (HDL-C) levels to improve dyslipidemia. Lotus leaves also inhibited inflammation accompanied by obesity via decreasing inflammatory cytokine interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), interferon gamma (IFN-γ), and IL-6 levels and increasing anti-inflammatory cytokine IL-4 and IL-10 levels. qPCR analysis revealed that lotus leaves upregulated peroxisome proliferator-activated receptor alpha (PPAR-α), lipoprotein lipase (LPL), carnitine palmitoyltransferase 1 (CPT1), and cholesterol 7 alpha hydroxylase (CYP7A1) mRNA expressions and downregulated peroxisome proliferator-activated receptor gamma (PPAR-γ) and CCAAT/enhancer-binding protein alpha (C/EBP-α) mRNA expressions, to reduce adipocyte differentiation and fat accumulation, promote oxidation of fat and decomposition of triglyceride and cholesterol. So, lotus leaves effectively regulated lipid metabolism, alleviated inflammation and liver injury in obese mice; thus, lotus leaves could be further developed as a food to combat obesity.

Keywords: Lotus leaves, High-fat diet, Anti-obesity, Lipid metabolism, Anti-inflammation, Liver injury

Introduction
Obesity is a metabolic disorder caused by excessive fat accumulation, which results in abnormal weight gain [1]. The obese population is growing rapidly, affecting younger populations and becoming more difficult to control [2], as well as a global epidemic. Obesity is closely related to the occurrence of various chronic metabolic diseases such as nonalcoholic fatty liver disease [3], cardiovascular disease [4], and type 2 diabetes [5], which seriously affect the quality of life of patients. Obesity also contributes to a variety of cancers in humans by damaging the immune system [6]. Although current obesity treatment drugs have certain weight loss effects, the adverse effects are relatively large [7]. Therefore, development of safe, efficient, natural anti-obesity medicines and functional foods has become a research focus in the health industry.

Lotus leaves are fresh or dried leaves of the perennial aquatic plant Nelumbo nucifera [8], which is both medici- nal and edible. Lotus leaves have a long history of being planted and used in China and are widely distributed and cultivated in drainage basins of the Yellow, Changjiang and Zhujiang Rivers in China [9]. The main active constituents in lotus leaves are flavonoids and alkaloids. Studies have shown that lotus leaf flavonoids mainly include quercetin, isoquercetin, kaempferol and other monomers or glycosides, and the principal alkaloids are...
monobenzyl isoquinoline, bisbenzyl isoquinoline, apomorphine, dehydroaporphine, oxidized apomorphine, and proaorphone [10]. The studies have shown that lotus leaf extracts have extensive pharmacological activities, including anti-obesity [11], antioxidation [12], anti-cardiovascular disease [13], and anti-cancer [14].

According to the researches on the anti-obesity effect of lotus leaves, it is found that most of the experiments in vitro introduced the extract or the isolated monomer compounds are useful for the suppression of obesity in adipocyte by inhibiting lipase activity, lipogenesis and adipocyte differentiation [15–18]. In vivo experiments, the anti-obesity effects were measured by body weight, blood lipids and liver function indicators in obese mice induced by high-fat diet [19–21]. However, obesity, as a chronic metabolic disease, affects systemic functions. It not only causes dyslipidemia, liver damage, but also leads to low-grade inflammation, which further promote the development of physiological disorders such as insulin resistance and metabolic syndrome [22]. In 2018, Kim et al. reported that the lotus leaf water extract improved the antioxidant capacity of obese mice and reduced the level of oxidative stress, but they did not clarify the mechanism of action [23]. Back in 2010, Wu et al. reported that lotus leaf water extracts reduced lipid accumulation by increasing the phosphorylation of AMP-activated protein kinase, and inhibiting the expression of acetyl-CoA carboxylase, fatty acid synthetase (FAS) and 3-hydroxy-3-methyl glutaryl coenzyme A reductase [24].

Lotus leaves have the advantages of being abundant and cheap, with various physiological activities. The present study focused on obesity as a systemic disease, and not only detected classic blood lipids and liver function indicators (TC, TG, LDL-C, HDL-C, ALT, AST, AKP). At the same time, the degree of chronic low-grade inflammation accompanied by obesity, were measured by inflammatory factors, were measured by antioxidant levels and inflammatory factors. In addition, unlike previous Wu’s reports that focused on the mechanism of lipid synthesis, we started from the PPAR pathway to detect the expression of related genes, and explored anti-obesity mechanism of lotus leaf extracts. The results may provide ideas for dietary therapy and interventions for the growing obese population.

Materials and methods
Preparation of lotus leaf extract
Lotus leaves (20 g; Anhui Provinces) were crushed, and 70% ethanol was added at a liquid/material ratio of 20:1. After heating at 60 °C for 3 h, the mixed liquid was cooled and filtered to obtain the crude extract, which was then purified via FL-3 macroporous absorbent column chromatography [25] (Shanghai Lanji Technology Development Co., Ltd., Shanghai, China). Ethanol (70%) was used as eluant to flush the column until the eluant became colorless. The eluted solution was collected and evaporated, and the residue was dried and ground to obtain the lotus leaf extract.

Determination of lotus leaf extract composition
Two milligrams each of hyperoside (Beijing Solibao Technology Co., Ltd., Beijing, China), kaempferitrin, astragalin, phlorizin, and quercetin (Beijing Putian Tongchuang Biological Technology Co., Ltd., Beijing, China) were accurately weighed, then dissolved in 2 mL of methanol (HPLC grade) to prepare the standard solution. Lotus leaf extract (10 mg) was dissolved in 1 mL of DMSO, then 1.5 mL methanol and 1.5 mL H2O were added. Samples were filtered through a 0.22-μm microporous membrane to obtain the test solution.

Separation chromatography was performed using an UltiMate3000 HPLC System (Thermo Fisher Scientific, Waltham, MA, USA) and a Thermo Accucore C18 column (150 × 1.6 mm, 2.6 μm). Mobile phase A was acetonitrile (HPLC grade); mobile phase B was 0.5% glacial acetic acid aqueous solution. The mobile phase gradient was: 0 min, 12% A; 0–30 min 40% A; 30–35 min 100% A; 35–40 min, 100% A. The flow rate was 0.5 mL/min; the column temperature was 35 °C; the detection wavelength was 360 nm; and the injection volume was 10 μL. Flavonoid types were determined according to retention time and analyzed using the chromatographic peak area of each component.

Animal models and treatment
Fifty healthy C57BL/6J mice (weighing 20 ± 2 g, 25 males and 25 females) were randomly divided into the normal, model, l-carnitine, low-concentration lotus leaf extract (LL-L), and high-concentration lotus leaf extract (LL-H) groups. The normal group received normal maintenance foods and drinking water; the other groups received a D1249251 high-fat diet (Chongqing Medical University, Chongqing, China) and 10% sugar water. After 2 weeks, without changing the diet, mice in the l-carnitine group were intragastrically administered 200 mg/kg l-carnitine daily, mice in the LL-L group were intragastrically administered 100 mg/kg lotus leaf extract daily, and mice in the LL-H group were intragastrically administered 200 mg/kg lotus leaf extract daily [26]. After 7 weeks, all mice were fasted for 12 h and humanely killed. Blood was collected from the orbits, and the epididymal fat (from the males) and livers were collected for further experiments. The liver and epididymal fat indexes were calculated as organ weight (g)/mouse weight (g) × 100.
Detection of serum AKP, AST, ALT, TC, TG, LDL-C and HDL-C levels
The plasma was centrifuged at 4000 rpm for 10 min, then the supernatant was collected. Serum alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (AKP), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDLC), and low-density lipoprotein cholesterol (LDL-C) levels were measured using kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Determination of serum cytokine TNF-α, IFN-γ, IL-6, IL-1β, IL-4, and IL-10 levels
The mouse blood was centrifuged at 4000 rpm for 10 min, then the supernatant was carefully collected. The serum cytokine levels of tumor necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ), and interleukin (IL)-1β, IL-6, IL-4, and IL-10 were measured according to each kit’s instructions (Beijing Chenglin Biotechnology Co., Ltd., Beijing, China).

Pathological examination of the liver and epididymal fat
Liver and epididymal fat (~0.5 cm²) were fixed in 10% formalin for 48 h, then processed, dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissue histology was examined under an optical microscope (BX43 microscope, Olympus, Tokyo, Japan).

Quantitative PCR (qPCR) assay
The mouse liver tissues were pulverized; total RNA (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was extracted with RNAzol reagent (Invitrogen, Carlsbad, CA, USA), and the concentration of the extracted total RNA was diluted to 1 μg/μL. Next, 5 μL of diluted total RNA solution was used for reverse transcription to synthesize the cDNA template as per the kit instructions (Tiangen Biotech Co., Ltd., Beijing, China). The cDNA template (1 μL) was mixed with 10 μL SYBR Green PCR Master Mix and 1 μL upstream and downstream primers (Table 1). The solution was reacted at 95 °C for 30 s, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 95 °C for 30 s, followed by 55 °C for 35 s. The relative gene expressions were calculated via the 2^(-ΔΔCt) method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal reference.

Statistical analysis
The data were analyzed using SPSS 17.0 and GraphPad Prism 7 statistical software. Experimental results are expressed as the mean ± standard deviation. One-way analysis of variance was used and a Tukey’s multiple comparisons test was used for between-group comparisons. P < 0.05 was considered statistically significant.

| Gene name | Sequence |
|------------|----------|
| LPL        | Forward: 5'-AGGGCTCTGCTGCTGAGTTGTA-3'  |
| CPT1       | Forward: 5'-AGCAACTCACAACCCTGCGACAGTA-3'  |
| PPAR-γ     | Forward: 5'-GCTCAGGTTTACACTACGAGGT-3'  |
| C/EBP-α    | Forward: 5'-TGCGAAATGTGCCGGCA-3'  |
| GAPDH      | Forward: 5'-ACCAGGAAAGCAGTGGATG-3'  |
| CYPTA1     | Forward: 5'-AGCAACTAACAACCTGCGATCTA-3'  |
|            | Reverse: 5'-GCGGCTGATTAAGGATG-3'  |

LPL lipoprotein lipase, CPT1 carnitine palmitoyltransferase 1, PPAR-γ peroxisome proliferator-activated receptor gamma, C/EBP-α CCAAT/enhances binding protein alpha, CYPTA1 cholesterol 7 alpha hydroxylase, GAPDH glyceraldehyde-3-phosphate dehydrogenase

Results

Compositional analysis of the lotus leaf extract
Figure 1 shows the activated chemical compounds in the lotus leaf extract via HPLC. Based on the data, the corresponding regression equations were established, with the content of standards as abscissa and the peak area as the ordinate (Table 2). Components in lotus leaf extract were calculated by peak area.

Effect of the lotus leaf extract on mouse body weight
At the beginning of the experiment, there was no difference in mouse body weight between the groups (P > 0.05). After feeding a high-fat diet for 7 weeks, the mouse weights in the model group increased faster than those of the normal group (Fig. 2). After intervention, the mice in treatment group gained weight slower than did the model group (P < 0.05). And no significant difference was observed in LL-H group and l-carnitine group (P > 0.05).

Liver and epididymal fat indexes
Obesity is often accompanied by an increase of white fat, which is manifested by the weight increase of white adipose tissue, and epididymal fat tissue is typical white adipose tissue. After the mice were sacrificed, their epididymal white adipose tissue is weighed and compared with body weight to obtain epididymal fat indexes.
which could be used to explain the degree of obesity in mice. Obesity is often accompanied by disorder of lipid metabolism in the liver, which manifests as liver enlargement due to lipid accumulation, leading to an increase in liver weight.

The liver and epididymal fat indexes of the model group were highest, indicating fat accumulation in the liver and

Fig. 1 Flavonoids constituents of lotus leaf extract. Standard chromatograms (top); lotus leaf extract flavonoids chromatograms (bottom): 1. kaempferitrin; 2. hyperoside; 3. astragalin; 4. phlorizin; 5. quercetin
epididymal tissues (Table 3). Compared with the model group, the liver and epididymal fat indexes of the l-carnitine and LL groups were reduced to different degrees (P < 0.05).

**Histopathological examination of the liver and epididymal fat**

In the normal group, the hepatocytes were arranged neatly, with complete structure and fewer fat vacuoles (Fig. 3a). Hepatocytes in the model group were arranged irregularly with many fat vacuoles of different sizes. The hepatic cords radiating around the central vein were invisible. In the l-carnitine and LL groups after treatment, the radial hepatic cords around the central vein were clearly visible, few fat vacuoles were observed, and the hepatocytes were neatly arranged and structurally intact.

In the normal group, the adipocytes in the epididymal fat tissues were smallest, with a uniform shape (Fig. 3b). In the model group, the adipocytes were largest and uneven. The epididymal adipocytes were smaller in the l-carnitine and LL groups than in the model group, indicating effectively less fat cell hypertrophy.

**Serum levels of the biochemical indicators ALT, AST and AKP in mice**

The ALT, AST, and AKP levels in the model mouse group were significantly higher than those in the normal group (P < 0.05; Fig. 4), indicating that obesity caused liver damage. After treatment, the serum ALT, AST, and AKP levels were significantly decreased compared with those of the control group (P < 0.05). The high-concentration lotus leaf extract was more effective than was the low-concentration extract.

**Serum levels of TC, TG, LDL-C and HDL-C in mice**

The serum TC, TG and LDL-C levels were increased in the high-fat-diet-induced hyperlipidemic mice, but the HDL-C level was decreased compared with that in the normal mice (P < 0.05; Fig. 5). The lotus leaf extract decreased the TC, TG and LDL-C levels and increased the HDL-C level in the hyperlipidemic mice, and the results for both the high-concentration lotus leaf extract and l-carnitine groups were comparable.

**Serum levels of TNF-α, IFN-γ, IL-1β, IL-6, IL-4, and IL-10 in mice**

In all experimental groups, TNF-α, IFN-γ, IL-1β, and IL-6 levels were highest, and the IL-4 and IL-10 levels were lowest in the model mice (Fig. 6). After intervention, serum TNF-α, IFN-γ, IL-1β, and IL-6 contents were decreased in the LL-L, LL-H, and l-carnitine groups, IL-4 and IL-10 was increased compared with that in the model group (P < 0.05).

**PPAR-γ, C/EBP-α, PPAR-α, LPL, CPT1 and CYP7A1 mRNA expressions in the liver tissue**

To investigate the changes in gene expressions regulated by lotus leaf extracts in mice, we analyzed the lipid

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### Table 2 Flavonoid content in lotus leaf extract

| Compound     | Regression equation | R²   | Content in lotus leaf extract (mg/g) |
|--------------|---------------------|------|-------------------------------------|
| Kaempferitin | y = 8.1347x + 3.7415 | 0.9844 | 75.79 ± 0.02                        |
| Hyperoside   | y = 3.1862x + 9.5029 | 0.9897 | 31.11 ± 0.02                        |
| Astragalin   | y = 11.338x + 0.8515 | 0.9994 | 9.53 ± 0.01                         |
| Phloretin    | y = 0.9844x + 0.3059 | 0.9843 | 0.26 ± 0.01                         |
| Quercetin    | y = 10.386x − 3.0995 | 0.9990 | 0.096 ± 0.05                        |

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### Table 3 Liver and epididymal fat indexes of mice in each group

| Group     | Normal                  | Model                  | l-Carnitine           | LL-L       | LL-H       |
|-----------|-------------------------|------------------------|-----------------------|------------|------------|
| Liver     | 3.81 ± 0.30             | 4.17 ± 0.22            | 3.86 ± 0.19           | 4.02 ± 0.16 | 3.93 ± 0.21 |
| Index     | 1.01 ± 0.23             | 1.55 ± 0.28            | 1.02 ± 0.14           | 1.18 ± 0.15 | 1.14 ± 0.21 |

Values presented are the mean ± standard deviation (liver Index: n = 10/group, epididymal fat index: n = 5/group)

*a–c Means with different superscript letters are significantly different (P < 0.05) according to Tukey's multiple comparisons test. l-carnitine, mice treated with 200 mg/kg l-carnitine; LL-L, mice treated with 100 mg/kg lotus leaf extract; LL-H, mice treated with 200 mg/kg lotus leaf extract.*
metabolism-related genes (Fig. 7). *PPAR-α*, *LPL*, *CPT1*, and *CYP7A1* mRNA expression levels were lowest in the livers of the model mice, and *PPAR-γ* and *C/EBP-α* expressions were highest. These results were opposite those of the normal group (*P < 0.05*). Lotus leaf extract and L-carnitine suppressed the *PPAR-γ* and *C/EBP-α* mRNA expressions.
expressions and enhanced the PPAR-α, LPL, CPT1, and CYP7A1 mRNA expressions. The high-concentration lotus leaf extract had a more pronounced effect.

**Discussion**

High-energy diets and low-exercise lifestyles are becoming increasingly common; thus, the incidence of obesity increases annually. Long-term high-fat diets are the main cause of lipid metabolism disorders. Compared with healthy people, those with dyslipidemia present marked increases in TG, TC, LDL-C and free fatty acid levels as well as decreased HDL-C [27]. As the main organ for lipid metabolism, the liver is involved in β-oxidation of fatty acids, adipogenesis, and lipoprotein uptake and secretion, playing an important role in lipid metabolism [28]. In obese people, dyslipidemia leads to fat accumulation in hepatocytes, followed by the entire liver, with the result of enlarged due to lipid accumulation and a certain increase in liver weight. The fat vacuole areas and fat infiltration are increased, that further cause liver injury [29, 30]. Obesity is significantly positively correlated with various abnormalities in liver function indicators, such as the serum enzyme indicators ALT, AST, and AKP, which are significantly increased [31]. Also, the obesity is often
accompanied by an increase in the weight of white fat tissue, and epididymal fat tissue is typical of white fat tissue. Lotus leaf extract inhibited the increased body weight, liver quality, and epididymal fat mass, reduced the TG, TC, LDL-C levels, and significantly increased the HDL-C levels in obese mice. The extract also alleviated hepatic fat vacuoles and excessive fat accumulation and decreased the serum ALT, AST, and AKP levels to prevent early liver injury.

Obesity is a state of systemic chronic low-grade inflammation [32], mainly resulting from the interaction between adipose tissue and macrophages. In addition to large amounts of free fatty acids, adipose tissue also secretes TNF-α, IL-6, IL-1, and the macrophage...
migration inhibitor, monocyte chemoattractant protein-1, which causes an imbalance between the pro-inflammatory and anti-inflammatory cytokines, leading to chronic persistent inflammation [33, 34]. Lotus leaf extracts significantly inhibit the release of inflammatory cytokines IL-1β, IL-6, TNF-α, and IFN-γ and promote the release of anti-inflammatory IL-4 and IL-10 to alleviate chronic low-level inflammation accompanied by obesity.

When preadipocytes differentiate into adipocytes, transcription factors regulate adipocyte formation by regulating the expression of differentiation-related proteins. PPAR-γ [35, 36] and C/EBP-α [37] are core transcription factors involved in adipocyte differentiation, and positive feedback occurs between them to induce differentiation, cloning and proliferation of adipocytes [38]. The PPAR family regulates lipid metabolism and participates in energy metabolism and lipid deposition by regulating the transcription of multiple target genes. PPAR-α is a subtype of PPAR and plays an important role in lipid metabolism in the liver [39]. PPAR-α regulates the expression of genes related to lipid and lipoprotein metabolism, which promotes β-oxidation of fatty acids, ketogenesis, metabolism and TG storage and reduces lipid accumulation, thereby modulating lipid metabolism disorders [40]. PPAR-γ plays the opposite role by promoting lipid synthesis and fat accumulation by regulating lipid-related genes.

CPT-1 is a rate-limiting enzyme for fatty acid oxidation and promotes β-oxidation of fatty acids [41]. Lipoprotein lipase (LPL) catalyzes the decomposition of TG from chylomicron (CM) and very low-density lipoprotein (VLDL) into fatty acids and monoglycerides for tissue oxidation and energy storage [42]. LPL also participates in converting apolipoproteins and phospholipids between VLDL and HDL, thus increasing HDL-C levels [42]. These two lipases are target genes regulated by PPAR-α and PPAR-γ [43–45]. CYP7A1 is a rate-limiting enzyme that catalyzes bile acid synthesis by cholesterol, which is important for maintaining the internal balance of bile acids [46]. Lotus leaf extracts reduce adipocyte differentiation by down-regulating PPAR-γ and C/EBP-α mRNA expressions and upregulating PPAR-α, CPT-1, LPL and CYP7A1 mRNA expressions, thus reducing fat accumulation and accelerating fat oxidation to modulate lipid metabolism disorders.

In our experiment, flavonoid glycosides were the main compounds isolated from lotus leaf, which had good physiological activities. Kaempferitin could down-regulate the mRNA expression of lipid synthesis genes and pro-inflammatory genes TLR4, TNF-α, NLRP3, Caspase-1, IL-18, IL-1β, SRRBP-1c, PPAR-γ, and elevate PPAR-α mRNA expression in obese Wistar rats [47]. In human RA-FLS MH7A cell line test, kaempferitin exerted anti-inflammatory activity by reducing IL-1β, IL-6, TNF-α, matrix metalloproteinase (MMP)-1 and MMP-3 levels [48]. Hyperoside, which has good antioxidant capacity, protected the liver from damage by increasing antioxidant enzyme activities, inhibiting oxidative stress and lipid peroxidation damage [49, 50]. And anti-inflammatory effect of hyperoside was achieved by inhibiting NF-κB (nuclear factor kappa-B) activation and IκB (inhibitor of NF-κB)-α degradation [51]. Astragalin have a good inhibition of lipogenesis and fat accumulation in 3T3-L1 adipocytes, which showed anti-obesity effect by down-regulating the mRNA expression of PPAR-γ, C/EBP-α, FAS, and leptin [52]. At the same time, other physiological activity studies have found that astragalin have the ability to improve anti-oxidant and anti-inflammatory activities [53, 54]. Flavonoids regulate signal transduction and transcription factor expression and inhibit oxidative stress to suppress inflammation [55]. Flavonoids in lotus leaf cooperated with each other to fight against obesity. Therefore, lotus leaf extracts regulated lipid-related genes, including PPAR-γ, C/EBP-α, PPAR-α, LPL, CPT1, and CYP7A1, to reduce adipocyte differentiation and fat accumulation and promote oxidation of fat for energy supplies. Lotus leaves may prevent and treat obesity and have a high value in the medicinal and food industries.

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Authors’ contributions
YW performed the majority of the experiments and wrote the manuscript; FT, TY, BLX and LXR contributed to the data analysis; XZ designed and supervised the study, and checked the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Datasets used and/or analysed during the current study that are not included in the manuscript are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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