Hawthorn leaf flavonoids alleviate nonalcoholic fatty liver disease by enhancing the adiponectin/AMPK pathway

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Abstract: Hawthorn (Crataeguspinnatifida) belongs to the genus Rosaceae family of plants. The hawthorn leaf, Crataeguspinnatifida Bunge, is used for both condiment and medicinal purposes to prevent and treat metabolic dysfunctions, such as hyperlipidemia, hypertension, and cardiovascular disease in traditional Chinese medicine. However, its effects on nonalcoholic fatty liver disease (NAFLD) remain obscure. The purpose of the present study was to investigate the protective effect of hawthorn leaf flavonoids (HLF), the dominant bioactive extracts of hawthorn leaves, on high fat diet (HFD)-induced hepatic steatosis and to elucidate its underlying mechanisms. HLF supplementation significantly lowered body weight, liver weight, liver/body weight ratio, improved serum parameters and liver dysfunction and markedly decreased hepatic lipid accumulation in HFD-fed rats. In addition, HLF intervention dramatically increased circulating adiponectin levels and up-regulated the expression of adiponectin receptors, particularly adiponectin receptor 2 (AdipoR2) in the liver. Moreover, adenosine monophosphate (AMP)-activated protein kinase (AMPK) was also activated, as well as AMPK-mediated alteration of sterol regulatory element binding protein-1c (SREBP-1c), peroxisome proliferator-activated receptor α (PPARα) and their downstream targets. Taken together, our data suggest that HLF ameliorates hepatic steatosis by enhancing the adiponectin/AMPK pathway in the liver of HFD-induced NAFLD rats.

Keywords: Hawthorn leaf flavonoids, nonalcoholic fatty liver disease, adiponectin, adenosine monophosphate-activated protein kinase

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common and frequent cause of chronic liver disease worldwide, resulting from the increasing prevalence of obesity [1]. It is considered as the hepatic manifestation and the pace setter of metabolic syndrome, which has become a growing public health concern[2]. NAFLD is characterized by excess liver lipid accumulation, which can ultimately evolve into hepatic steatosis and a progressive worsening status [3].

Importantly, a variety of pharmacological strategies are available to correct NAFLD; however, side effects, such as liver toxicity cannot be ignored. In contrast, dietary therapy has become more attractive and popular worldwide. In traditional Chinese medicine, many functional foods have also been prescribed in formulations for obesity and metabolic syndrome, and thus, they are referred to as food-medicine dual plants [4, 5]. Hawthorn (Crataeguspinnatifida), a genus of the Rosaceae family, is commonly distributed in Asia, Europe, North and Central America. It is commonly used as a delicious daily food source, and its leaf can be used in drinking tea in China. The hawthorn leaf has been prescribed in herbal formulae to treat various diseases, such as hyperlipidemia, atherosclerosis and dyspepsia [6-8]. To date, various chemical constituents have been identified in...
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The hawthorn leaf, including flavonoids, triterpenoids, steroids, lignans, organic acids, and nitrogen-containing compounds, among which, flavonoids are the most abundant chemical components [9]. Hawthorn leaf flavonoids (HLF) have demonstrated a potential effect in lipid regulation, and supplementation of HLF has been shown to significantly regulate serum total cholesterol (TC) and triglyceride (TG) [8]. In another study, HLF was reported to mediate lipoprotein lipase expression in mice with tissue-specific differences [6]. However, to date, few studies have examined the role of HLF on NAFLD and its underlying mechanisms.

Adipocytokines, including adiponectin, leptin, and resistin, among other factors, are secreted by adipocytes [10]. In recent years, the abnormal secretion of adipocytokines has been reported to contribute to the pathogenesis of NAFLD [11]. In addition, reduced adiponectin expression has been detected in ob/ob mice [12]. In contrast, adiponectin administration significantly decreased circulating concentrations of TG and free fatty acid (FFA) [13], suggesting that adiponectin might potentially exhibit protective activities against NAFLD. The beneficial effects of adiponectin have been partially attributed to increasing fatty acid oxidation and decreasing lipid synthesis in peripheral tissues, such as the liver and skeletal muscle [14]. Recent studies have also suggested that adiponectin exerts its regulatory effect on fat metabolism by activating adenosine monophosphate-activated protein kinase (AMPK) and its downstream transcription factors [15]. Here, we showed that HLF may prevent the development of NAFLD in HFD-fed rats and may enhance the adiponectin/AMPK signaling pathway.

Materials and methods

Animals and diets

All animal study protocols were approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (Approval Number: 2013045). Male Sprague Dawley rats, weighing 150-180 g, were obtained from the Shanghai SLAC Laboratory Animal Technology Company. The rats were housed in a temperature and humidity-controlled room, maintained on a 12 h light/dark cycle, with food and water provided ad libitum. All animal procedures were performed according to the guidelines for the care and use of laboratory animals.

HFD (16.4% lard, 17.2% sucrose, 10.4% casein, 1% cholesterol, 3% maltodextrin, 2% premix, and 50% standard chow; providing 40% of energy as fat, 19% as protein, and 41% as carbohydrates) and normal chow diet (NCD) were also purchased from Shanghai SLAC Laboratory Animal Technology Company.

Drug and chemicals

HLF (≥93.5% pure total flavonoids extracted from hawthorn leaves) were obtained from LinyiAikang Pharmaceutical Co., Ltd. (Linyi, China). The contents of HLF were determined using HPLC and UV-Spectrophotometry, and the compounds were recognized as quercetin, vitexin-4’-O-glucoside, vitexin-2’-O-rhamnoside, hyperoside.

Experimental design

After one week of adaptation, the rats were randomly divided into four groups (n=10 animals per group): NCD, HFD, HFD supplemented with 50 mg/kg/d HLF (HLF(L)), and HFD supplemented with 100 mg/kg/d (HLF(H)). HLF was administered orally between 9:00-10:00 am daily for 12 weeks. The NCD and HFD rats received an equivalent volume of normal saline vehicle. Their body weight was recorded every other day. At the end of the twelfth week, the rats were anesthetized with urethane after overnight fasting (12 h). Blood was withdrawn from the abdominal aorta into a vacuum tube, and serum samples were separated for further investigation. Livers were weighed and snap-frozen in liquid nitrogen and then stored at -80°C for the following experiments or fixed in 10% formalin for histopathological studies.

Serum biochemistry analysis

Serum TG, TC, high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) levels were measured using an automatic analyzer (Express Plus; Chiron Diagnostics, East Walpole, MA, USA). The levels of glucose, free fatty acid (FFA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were measured using a biochemical analyzer (Architect c8000, USA) according to the manufacturer’s instructions. Serum leptin, resistin, and adiponectin concentrations were measured by enzyme-linked
Table 1. Primer sequences used in RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>β-actin</td>
<td>AGGGAAATCTGTCGTTGAC</td>
<td>CGCTCATGGCCGAGATAGTG</td>
</tr>
<tr>
<td>AdipoR1</td>
<td>GCTGAAATGGAGAAGGAGATGTC</td>
<td>GAGGGAATGAGTATTATGGCC</td>
</tr>
<tr>
<td>AdipoR2</td>
<td>GGCAACATCTGAGACACATC</td>
<td>CTGGGAACCCCCTCTCAG</td>
</tr>
<tr>
<td>LXRx</td>
<td>TCAAGGGAGACGGCTACTGCT</td>
<td>CCGAATTCTTGCGCCCTCAG</td>
</tr>
<tr>
<td>PPARα</td>
<td>GCCAATGGACAGACATCAGG</td>
<td>GCCGAATTTTGGCCGGAAG</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>GGTACCTGCGGACAGCTTGA</td>
<td>GCCTGAGCTGAGCCTGAGTGTG</td>
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<tr>
<td>ChREBP</td>
<td>CGAGGCTCTGGATGCTGTGA</td>
<td>GAAGATTGGAGATATGTTGGGCT</td>
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<tr>
<td>CPT1</td>
<td>AGGTCCGGAAGCCATGTTGA</td>
<td>GCCTCATGGCCGTTGAAAGTGC</td>
</tr>
<tr>
<td>ACO</td>
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<td>TCACCGAGGATACACAGATTGAG</td>
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<tr>
<td>ACOX1</td>
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<td>CCAGGCCACCACTTAATGGAA</td>
</tr>
<tr>
<td>ACC</td>
<td>CGTCCCTGCTCACATGGAGA</td>
<td>GCCTAGCCAAGCGGATGGAGA</td>
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<tr>
<td>SCD-1</td>
<td>ACATGTCTGAGCTGAAAGCTGAGA</td>
<td>ACGAACAGGCTGAGGAGAAGGA</td>
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<tr>
<td>FAS</td>
<td>GCCTTGGCGACTCTTCCAGTTA</td>
<td>GCCTGATAGACACCAGGCGACTA</td>
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<tr>
<td>CD36</td>
<td>GCTTGAAGTGCAGCACCAT</td>
<td>GCCTTGCTGAGGCCAGAAC</td>
</tr>
</tbody>
</table>

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Changes in gene expression in liver tissues were confirmed using quantitative RT-PCR. Total RNA was extracted from the liver with Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcription of total RNA to cDNA was performed using reverse transcription kits (Promega, Madison, WI, USA). The primers (Takara, Tokyo, Japan) used in the experiment are shown in Table 1. Quantitative RT-PCR was performed using a SYBR Green PCR Master Mix kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s instructions. β-actin was used as an internal control to normalize the mRNA levels of all genes. All results were obtained from three independent experiments.

Western blotting analysis

For western blotting analyses, 100 mg of total protein was separated on 10% SDS-PAGE gels and blotted onto Immobilon-P PVDF membranes (Millipore, Billerica, MA, USA). After the membranes were blocked with 5% milk, the membranes were incubated with primary antibodies at 4 °C overnight. Antibodies against adiponectin receptors (AdipoR1, AdipoR2), AMPKα, and P-AMPKα were purchased from Abcam (Cambridge, MA, USA). Antibodies against sterol regulatory element binding protein-1c (SREBP-1c) and peroxisome proliferator-activated receptor α (PPARα) were obtained from Life Technology (Cambridge, CA, USA). β-actin was obtained from Hua’an Biology Technology (Hangzhou, China). Next, the membranes were incubated in HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies obtained from Thermo Scientific (Rockford, IL, USA) for 1 h. Signals were visualized using the GBOX Chemi XT4 System (Syngene, Cambridge, UK) with chemiluminescence HRP substrate (Millipore, Billerica, MA, USA). GeneTools software (Syngene) was used for quantification.

Immunosorbent assay (ELISA) using commercially available kits (Linco Research Inc., St. Charles, MO, USA).

Morphological analysis of hepatic steatosis

Liver sections were fixed in 10% formalin, dehydrated, and embedded in paraffin. The samples were cross-cut into slices of 4 μm and stained with hematoxylin and eosin (H&E). Liver morphology was visualized using light microscopy (×200) with the Olympus image analysis software system (Olympus America, Melville, NY, USA).

For Oil Red O staining, a stock solution of Oil Red O (0.5 g/100 ml) in isopropanol was prepared and protected from light. After fixation with 4% paraformaldehyde for 30 min, frozen liver sections of 10 μm thickness were stained with Oil Red O for 30 min. Non-specific staining was removed with 75% ethanol. The stained sections were visualized and imaged under a microscope (Olympus IX 71, Tokyo, Japan).

Liver lipid content analysis

Two milliliters of ethanol-acetone (1:1) was added to the hepatic tissues (100 mg), which were then homogenized and mixed thoroughly at 4 °C overnight. After centrifugation at 3000 rpm and 4 °C for 20 min, the supernatant was transferred into a new tube, and the TG and TC concentrations were measured using commercial test kits (Furui Institute of Biotechnology, Beijing, China).
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Statistical analysis

All values are expressed as the mean ± standard deviation (SD). All statistical analysis was performed using the SPSS package (Version 16, SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) or Student's t-test was used when applicable. *P*<0.05 was accepted as statistically significant.

Results

Effect of HLF on body weight and liver weight changes

Along with the feeding, all of the rats exhibited similar body weights regardless of their diet until 6th week (Figure 1A). On the 8th and 12th-week, HFD-fed rats showed a greater body weight increase compared to NCD rats (Figure 1A and 1B), and both 50 mg/kg/d and 100 mg/kg/d HLF supplementation could significantly ameliorate the HFD-induced body weight increase (Figure 1B). HFD also resulted in hepatomegaly, with nearly a 1.5-fold liver weight and liver/body weight ratio increase compared to those in NCD rats, whereas 12-week HLF supplementation reduced the liver-body ratio and liver weight (Figure 1C and 1D).

Effect of HLF on serum lipid and glucose parameters

Twelve-week HFD feeding significantly induced dyslipidemia and abnormal glucose metabolism, as shown by the increased serum TG (Figure 2A), TC (Figure 2B), LDL-c (Figure 2D), FFA (Figure 2E), and glucose (Figure 2F) levels compared with the NCD group (*P*<0.05). HLF intervention significantly decreased all of these

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Figure 1. Effect of HLF on body weight and liver weight. Male Sprague-Dawley rats fed with normal chow diet (NCD), high fat diet (HFD), HFD supplement with 50 mg/kg/d (low dose) or 100 mg/kg/d HLF (high dose) for 12 weeks. (A) Dynamic body weight was recorded every two weeks. Body weight (B), liver weight (C) and liver/body weight ratio (D) were recorded and calculated at the end of the experiment. (***) *P*<0.01 compared with NCD; (#) *P*<0.05 compared with HFD. HLF(L)--low dose hawthorn leaf flavonoids; HLF(H)--high dose hawthorn leaf flavonoids.
Figure 2. Effect of HLF on serum lipid and glucose parameters. Blood obtained from the rats were collected, and their serum was separated at the end of the experiment; lipid profiles for (A) serum TG, (B) TC, (C) HDL-c, (D) LDL-c, and (E) FFA were analyzed and (F) glucose levels were tested. (**) $P<0.01$ compared with NCD; (#) $P<0.05$, (##) $P<0.01$ compared with HFD.
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Figure 3. HLF ameliorated lipid accumulation in the liver. Liver samples were fixed, dehydrated and embedded in paraffin, and 4-μm sections were subsequently obtained and stained with hematoxylin and eosin (A). Frozen liver samples were embedded in OCT, and 10-μm sections were obtained and stained with Oil Red O (B). Liver TG (C) and TC (D) contents were determined using commercial kits. (**) P<0.01 compared with NCD; (#) P<0.05 compared with HFD.
parameters ($P<0.05$) except for serum TG (Figure 2A). Serum HDL-c did not show any difference between the HFD group and the NCD group, which was unexpected (Figure 2B).

**HLF ameliorated lipid accumulation in the liver**

As previously described, HFD induced hepatomegaly. Further H&E staining revealed enlarged hepatocytes with severe microvesicular steatosis in the HFD rats (Figure 3A), and Oil Red O staining confirmed hepatosteatosis and increased lipid deposition (Figure 3B). Increases in liver TG and TC content paralleled the histological changes obtained in the HFD rats (Figure 3C and 3D). However, HLF supplementation significantly alleviated hepatosteatosis and hepatic lipid accumulation in a dose-dependent manner. Liver sections in the HLF groups showed only moderate steatosis (Figure 3A) and less lipid-loaded hepatocytes (Figure 3B). Lipid quantification tests revealed decreased TG and TC content in the liver (Figure 3C and 3D).

**HLF attenuated liver dysfunction**

Because hepatomegaly and hepatosteatosis are often associated with liver injury, we performed an analysis of several liver enzymes. Elevated serum levels of ALT ($P<0.001$), AST, ALP ($P<0.001$) and LDH ($P<0.05$) were observed in the HFD rat group (Figure 4), while supplementation with HLF markedly reduced serum ALP ($P<0.001$) and LDH ($P<0.05$) levels (Figure 4C and 4D), indicating the hepatic protective effect of these agents (Figure 4).

**HLF regulates serum adipocytokines**

Adipocytokines play a fundamental role in obesity-related NAFLD; therefore, we analyzed representative adipocytokines. Both serum leptin and resistin of the animals were increased at 12-week HFD feeding, although the increase in
Figure 5. HLF regulates serum adipocytokines. Rat blood was collected, and serum was separated at the end of the experiment. Adipocytokines in circulation; (A) serum leptin, (B) resistin, and (C) adiponectin were analyzed. (*) $P<0.05$, (**) $P<0.01$ compared with NCD; (#) $P<0.05$ compared with HFD.

Figure 6. HLF regulates hepatic adiponectin receptors. Liver samples were collected after 12 weeks of feeding. Next, the mRNA expression of (A) AdipoR1 and (A) AdipoR2 was determined, and the protein expression of (B) AdipoR1 and (C) AdipoR2 was analyzed. Data (mean ± SD, n = 3) are presented as relative levels compared to NCD. (*) $P<0.05$ compared with NCD; (#) $P<0.05$ compared with HFD.
resistin did not exhibit a statistical difference (Figure 5A and 5B). Serum adiponectin, the most abundant adipose-specific adipocytokines, was decreased in HFD rats (Figure 5C). Although HLF had no obvious effect on serum leptin (Figure 5A) and resistin (Figure 5B), HLF showed a great reversed effect on serum adiponectin levels (Figure 5C), indicating that adiponectin is a HLF-sensitive parameter which is closely associated with NAFLD.

**HLF regulates hepatic adiponectin receptors**

Currently, two adiponectin receptors have been identified, with AdipoR2 as the liver-dominant isoform. In our study, we found that both AdipoR1 (Figure 6A) and AdipoR2 (Figure 6A) mRNA expression were significantly decreased in HFD rats compared with NCD rats and that HLF supplementation could markedly restore this decrease. These alterations were further confirmed by protein analysis (Figure 6B and 6C).

**HLF enhances adiponectin/AMPK signaling pathway molecules**

Because adiponectin can regulate lipid metabolism by stimulating the phosphorylation and activation of AMPKα in the liver, we examined AMPKα phosphorylation and its target genes. Several lipid metabolism-related and AMPKα targeted transcription factors, such as carbohydrate response element binding protein (ChREBP), SREBP-1c, and liver X receptor α (LXRα) mRNA expression in the liver was increased, while PPARα mRNA expression was decreased upon HFD feeding (Figure 7A). In addition, with HLF supplementation, only hepatic SREBP-1c and PPARα (Figure 7A) mRNA expression was...
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partially restored, demonstrating that the HLF effect might be specific to lipogenic and oxidative molecules. To confirm an HLF effect on lipogenic molecules, we determined the mRNA expression of CD36, acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS) and stearoyl CoA desaturase 1 (SCD1) and confirmed the lipogenesis-inhibiting effect of HLF, as observed with the down-regulation of CD36, FAS, and SCD1 mRNA expression compared with the untreated group (Figure 7B). Gene expression of the PPARα downstream target genes carnitine palmitoyltransferase-1 (CPT1), acyl-CoA oxidase (ACO) and acyl-CoA oxidase 1 (ACOX1) was reduced in HFD rats compared to NCD rats, and HLF showed an up-regulating effect on all of these genes (Figure 7C) in a dose-dependent manner.

Phosphorylation of AMPKα was significantly reduced in rats fed a HFD, and HLF showed an obvious restoration on AMPKα phosphorylation (Figure 7D and 7E), which might contribute to the aforementioned effect of HLF. Indeed, the effect of HLF on both SREBP-1c and PPARα was determined using western blot analyses. Upon HFD feeding, hepatic SREBP-1c protein accumulation (Figure 7D and 7F) was increased while PPARα (Figure 7D and 7G) was decreased, and the density of the bands was nearly restored to normal levels with 12-week HLF intervention.

Discussion

As described in various pharmacopoeias, hawthorn is one of the oldest pharmaceutical plants, and both its fruit and leaf have medicinal value with favorable therapeutic effects for digestive diseases, lipid metabolic disorders, cardiovascular diseases and other disorders [16-18]. Although it has been used in clinics for more than 2000 years in China, a limited number of studies have examined its pharmacological mechanisms. The hawthorn leaf has been reported to contain flavonoids, flavone-C-glycosides, catechins, amines, triterpene saponins, and oligomeric procyanidins, among which, flavonoids are the main bioactive compounds [19]. The lipid metabolism regulatory properties of HLF have recently received increasing attention [18, 20]. In the present study, we provided experimental evidence that HLF can prevent lipid metabolic dysfunction, particularly ameliorating hepatic steatosis in HFD-induced NAFLD rats, indicating the potential benefit of HLF on NAFLD.

In our study, rat body weight and liver weight increases were compromised with HLF supplementation, as well as hepatic steatosis and lipid accumulation, indicating that HLF has a more specific effect on NAFLD in these animals. Our results also showed that HLF lowered serum glucose, FFA, TC, and LDL-c, suggesting that HLF improved hyperglycemia and hyperlipidemia. However, the decrease in serum TG was not significant, which might be correlated with abnormal lipid transportation. The hepatoprotective function of HLF was further supported by the decrease in serum indicators, including ALP and LDH.

In this study, hyperleptinemia and hypoadiponectinemia in HFD rats indicated the imbalanced production of adipokines secreted from adipose tissue, which was associated with obesity and NAFLD [21, 22]. Furthermore, hypoadiponectinemia is a key etiological factor contributing to nearly all major pathological conditions associated with obesity [23]. Previous studies have shown that the activation of adiponectin signaling can ameliorate lipid metabolic disorders [15, 24]. HLF showed a large restorative effect on serum adiponectin in NAFLD rats, which makes adiponectin an interesting target of HLF.

The biological function of adiponectin is initiated by its binding to two specific seven-transmembrane domain-containing receptors: AdipoR1 and AdipoR2 [25, 26], where AdipoR2 is mainly distributed in the liver [27]. Adiponectin receptor knockout mice exhibit increased fat accumulation and insulin resistance [25, 26, 28, 29]. Our data showed that HLF significantly increased the expression of adiponectin receptors, particularly hepatic AdipoR2, indicating increased adiponectin activity in the liver. By binding to its membrane receptors, adiponectin can activate downstream molecules involved in regulating lipogenesis and lipid oxidation [30].

Typically, the biological effects of adiponectin are abrogated by the expression of a dominant negative version of AMPK, providing a necessary role in mediating the function of adiponectin [30]. We found that HLF markedly increased AMPKα phosphorylation, a finding that was
consistent with those obtained from a previous study reporting that adiponectin exerts its regulatory effect on fat metabolism via fat metabolism-related transcription factors, such as PPARα, SREBP-1c and ChREBP [33]. PPARα target genes involved in β-oxidation, such as CPT1, ACO, ACOX1, and other factors [34, 35]. CPT1 regulates long-chain fatty acid entry into mitochondria, while ACO and ACOX1 are enzymes that participate in the first step of the β-oxidation cycle [36-38]. SREBP-1c and ChREBP regulate fatty acid synthesis in the liver, thereby controlling lipogenesis-related transcription factors such as FAS, SCD-1 and ACC [39, 40].

Thus, we tested the expression of these transcription factors and their downstream targets in the liver. Our results indicated that HLF down-regulated the expression of SREBP-1c and up-regulated the expression of PPARα as well as their target genes. In general, the lipogenesis-related genes were down-regulated, while genes related to lipid oxidation were up-regulated.

In summary, our study provided evidence that HLF plays a role in HFD-induced NAFLD, potentially via the regulation of adiponectin/AMPK pathway-related molecules and their target genes. Taken together, these findings highlight that HLF may be an alternative as a safe food-medicine dual plant in the management of fatty liver and obesity-relative disorders. However, further studies are required to identify the precise mechanisms by which each ingredient protects against NAFLD and whether other mechanisms exist.

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Disclosure of conflict of interest

None.

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