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Anti-obesity effects of *Hovenia dulcis* branches in mouse adipocytes, 3T3-L1 cells, and high-fat diet obese mice

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Hovenia dulcis has been reported to have various pharmacological activities, but most studies were done with its fruits. However, from an economic point of view, the use of discarded leaves and branches as by-products is very valuable. In this study, the *in vitro* and *in vivo* anti-obesity activities of *Hovenia dulcis* branch extract (HDB) were investigated to evaluate the applicability of HDB as an anti-obesity agent. In differentiated 3T3-L1 cells, HDB inhibited lipid droplet accumulation. And HDB downregulated CEBPα, PPARγ, and perilipin-1, and upregulated ATGL, p-HSL, HSL, p-AMPK, UCP-1, PGC-1α, PRDM16, LC3-II, and p62/SQSTM1. In addition, HDB increased free glycerol content. In HFD-induced obese mice, HDB reduced body weight and total fat weight. In addition, HDB decreased blood LDL-cholesterol, blood total cholesterol, and blood triglyceride. These results indicate that HDB has anti-obesity activity and HDB can be used as a healthy functional food agent for weight reduction.

1. Introduction

Obesity is characterized by excessive lipid accumulation in adipose tissue due to an imbalance between energy intake and consumption (Chang and Hung 2022). Obesity has emerged as an important public health problem worldwide because it can lead to the development of diseases such as diabetes, cardiovascular disease, nonalcoholic fatty liver, infertility, renal disorders, and cancer (Chang and Hung 2022; Zhou et al. 2022). Globally, the prevalence of obesity increased rapidly from 3.2 to 10.8% for adult males and from 6.4 to 14.9% for adult females from 1975 to 2014 (Chang and Hung 2022). Although many anti-obesity drugs such as Orlistat, Liraglutide, Semaglutide, and Setmelanotide have been developed to treat obesity, their use has been limited due to side effects (Chang and Hung 2022). Therefore, attention is focused on the development of anti-obesity agents without side effects using natural plants.

Hovenia dulcis, a deciduous tree belonging to the Rhamnaceae family, has been used as an herbal medicine for detoxification, diuresis, thirst-quenching, and liver protection (Na et al. 2013; Yang et al. 2022). In addition, *H. dulcis* has been used as a raw material for functional foods and beverages for alleviating alcohol toxicity in East Asia (Hyun et al. 2010; Choi et al. 2014). In view of the anti-obesity activity of *H. dulcis*, it was reported that the fruits of *H. dulcis* inhibited excessive lipid droplet formation in 3T3-L1 cells through AMPK signaling (Kim et al. 2014). In addition, the anti-obesity activity of *H. dulcis* fruit was also demonstrated in an obese animal model (Song et al. 2020). Although *H. dulcis* has been widely cultivated for commercial purposes for the development of functional foods, only the peduncle of *H. dulcis* is used and the leaves and branches of *H. dulcis* are discarded as by-products (Je et al. 2021). So, from an economic point of view, the use of leaves and branches is considered to be of high value (Je et al. 2021). Therefore, in this study, we analyzed the inhibitory effect of *H. dulcis* branches (HDB) against the excessive accumulation of lipid droplets to verify their anti-obesity activity in mouse adipocytes, 3T3-L1 cells.

2. Investigations and results

2.1. Effect of HDB on the accumulation of lipid droplets in 3T3-L1 cells

We first compared the inhibitory activity of HDB on lipid droplet accumulation according to the extraction temperature (4~80 °C). HDB was treated for D0-D8 under the differentiation of 3T3-L1 cells with DMI and insulin (Fig. 1A). The inhibitory activity of HDB on lipid droplet accumulation was found to be high in the order of 80 °C < 4 °C < 20 °C < 40 °C = 60 °C (Fig. 1B). Thus, HDB extracted for 12 h at 60 °C was used for further study. HDB decreased the accumulation of lipid droplets in a concentration-dependent manner (Fig. 1C).

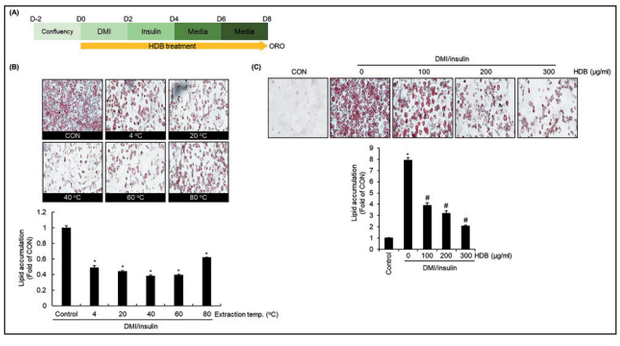


Fig. 1: Effect of HDB on lipid droplet accumulation in 3T3-L1 cells. (A) Experimental design. (B) HDB extracted with distilled water at 4 °C~80 °C for 12 h was treated with 3T3-L1 cells. (C) HDB extracted with distilled water at 60 °C for 12 h was treated with 3T3-L1 cells. Lipid droplet accumulation was measured using ORO staining assay.

2.2. *Effect of HDB on the protein levels associated with the accumulation of lipid droplets in 3T3-L1 cells*

We performed Western blot analysis to investigate whether HDB regulates the expression of various proteins related to lipid droplet accumulation. HDB was treated for D0-D8 under the differentiation of 3T3-L1 cells with DMI and insulin (Fig. 2A). As shown in Fig. 2B, the expression of PPARγ, CEBPα, and perilipin-1 proteins was decreased in HDB-treated 3T3-L1 cells in the presence of DMI/insulin. In addition, HDB significantly increased protein levels of ATGL, p-HSL, HSL, p-AMPK, AMPK, UCP-1, PGC-1α, PRDM16, LC3-II, and p62/SQSTM1.

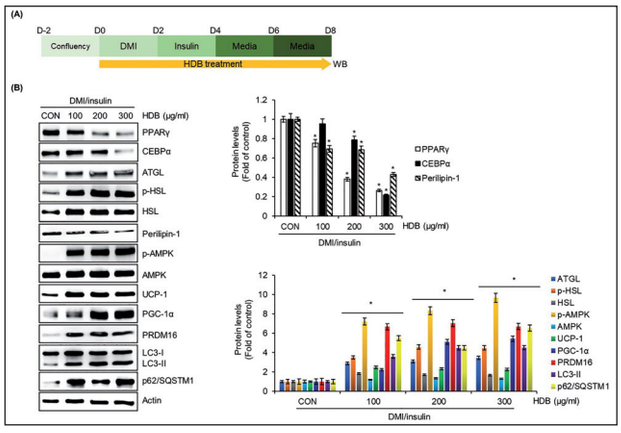


Fig. 2: Effect of HDB on the protein levels related to lipid droplet accumulation in 3T3-L1 cells. (A) Experimental design. (B) HDB extracted with distilled water at 60 °C for 12 h was treated with 3T3-L1 cells. Protein levels were measured using Western blot analysis.

2.3. *Effect of HDB on the accumulation of lipid droplets in early, intermediate, and late phase of adipocyte maturation in 3T3-L1 cells*

We investigated whether HDB inhibits lipid accumulation in the early, middle, and late phases of adipocyte maturation. HDB was treated for D0-D8, D0-D2, D2-D6, or D6-D8 under the differentiation of 3T3-L1 cells with DMI and insulin (Fig. 3A). As shown in Fig. 3B, HDB treatment at the early phase (D0-D2) of adipocyte maturation significantly inhibited lipid droplet accumulation. Although the inhibitory activity against lipid droplet accumulation was lower than that of HDB treatment in the early phase, HDB treatment in the intermediate (D2-D6) and late phases (D6-D8) also showed remarkable inhibition of lipid droplet accumulation. In addition, downregulation of PPARγ and upregulation of ATLG, p-HSL, p-AMPK, PRDM16, and LC3-II were observed in the treatment of HDM in early (D0-D2), intermediate (D2-D6), and late phase (D6-D8) (Fig. 3C)

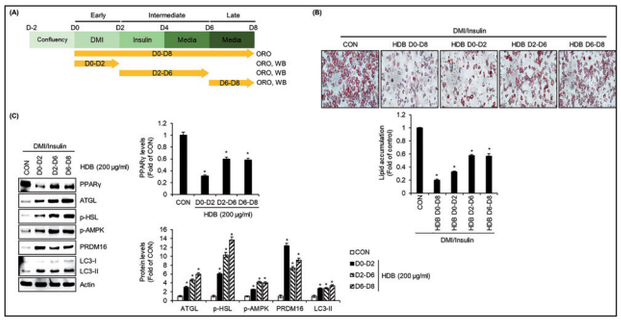


Fig. 3: Effect of HDB on the adipogenesis phases in 3T3-L1 cells. (A) Experimental design. (B and C) HDB extracted with distilled water at 60 °C for 12 h was treated with 3T3-L1 cells. Lipid droplet accumulation and protein levels were measured using ORO staining assay and Western blot analysis, respectively.

2.4. *Effect of HDB on lipolysis in 3T3-L1 cells*

Since HDB treatment in the late phase of the maturation phase of adipocytes inhibited lipid droplet accumulation in 3T3-L1 cells, the effect of HDB on lipolysis was investigated. 3T3-L1 cells were differentiated with DMI and insulin for D0-D6 and then treated with HDB for D6-D8 or D6-D10 (Fig. 4A). Lipid accumulation was induced in 3T3-L1 cells without HDB treatment, and HDB was then treated until D6-D8 and D6-D10, and then lipid droplet accumulation and glycerol content were measured. As shown in Fig. 4B and 4C, HDB treatment on D6-D8 and D6-D10 significantly inhibited lipid droplet accumulation and increased free glycerol contents in 3T3-L1 cells.

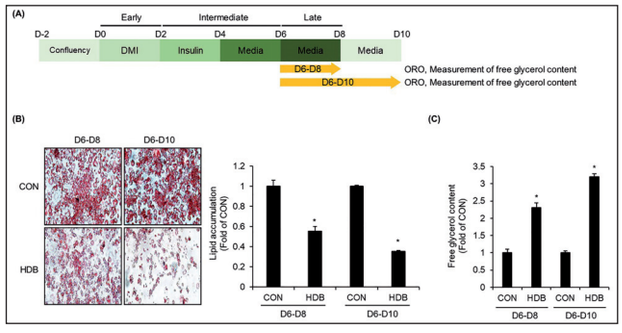


Fig. 4: Effect of HDB on lipolysis in 3T3-L1 cells. (A) Experimental design. (B and C) HDB extracted with distilled water at 60 °C for 12 h was treated with 3T3-L1 cells. Lipid droplet accumulation and free glycerol content were measured using ORO staining assay and ELISA kit, respectively.

2.5. *In vivo effects of HDB in high-fat diet-induced obese mice*

To verify the anti-obesity activity of HDB in a high-fat diet-induced obese mouse model, we administered a high-fat diet and HDB to the mice for 8 weeks, and analyzed body weight, total fat weight, and blood levels of LDL-cholesterol, total cholesterol, and triglycerides. As shown in Fig. 5A, weight gain was observed

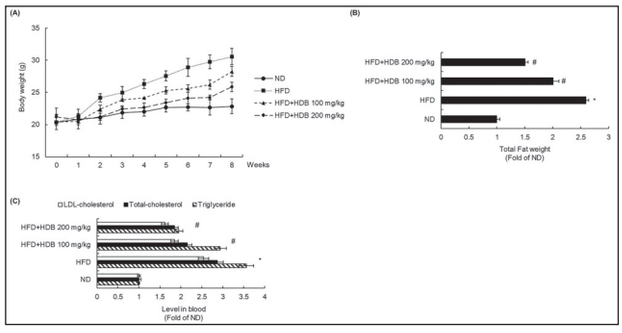


Fig. 5: *In vivo* anti-obesity effect of HDB in HFD-induced obese mice. (A) Body weight was assessed once a week. (B) Total fat weight was measured after the mice were sacrificed after 8 weeks of treatment. (C) Serum analysis was performed using ELISA kit after the mice were sacrificed after 8 weeks of treatment.

over time in all groups. In the high-fat diet (HFD) group, body weight increased 1.34-fold compared to the normal diet (ND) group. However, in the HDB groups (100 mg/kg and 200 mg/kg), the body weight increased 1.24-fold and 1.13-fold, respectively, compared to the ND group. In addition, the total fat weight of the HFD group, 100 mg/kg of HDB group, and 200 mg/kg of HDB group increased 2.59-fold, 2.01-fold, and 1.51-fold, respectively, compared to the ND group (Fig. 5B). As shown in Fig. 5C, the content of LDL-cholesterol, total cholesterol, and triglyceride in the blood of HFD group animals increased 3.56-fold, 2.87-fold, and 2.54-fold, respectively, compared to the ND group. The content of LDL-cholesterol, total cholesterol, and triglyceride in

the blood of HDB (100 mg/kg) group mice increased 2.94-fold, 2.15-fold, and 1.85-fold, respectively, compared to the ND group. The content of LDL-cholesterol, total cholesterol, and triglyceride in the blood of HDB (200 mg/kg) group mice increased 1.95-fold, 1.85-fold, and 1.62-fold, respectively, compared to the ND group.

3. Discussion

Lipids, one of three macronutrients necessary for human life, play a positive role in energy generation, hormone regulation, and organ protection. However, it is known that excessive accumulation of lipids in the human body leads to obesity, which causes obesity-related diseases (Krahmer et al. 2013). In this study, HDB was observed to inhibit the excessive accumulation of lipid droplets in 3T3-L1 cells, which indicates that HDB may exert anti-obesity activity.

The process of differentiation of preadipocytes into mature adipocytes is called adipogenesis. Through this process, excess lipid droplets are accumulated in mature adipocytes, which is characteristic of obesity (Lefterova and Lazar 2009; Rosen and MacDougald 2006; Tang and Lane, 2012). Therefore, inhibition of adipogenesis has been used as an important indicator to evaluate the anti-obesity activity of drugs (Yang et al. 2022). PPARγ and CEBPα are representative factors that promote adipogenesis (Yang et al. 2022). It was reported that there was no increase in body weight in an obese mouse model in which PPARγ and CEBPα were suppressed (Guo et al. 2020; Millward et al. 2007). In this study, it was observed that HDB downregulated the protein levels of PPARγ and CEBPα. This finding suggests that HDB may inhibit adipogenesis, which may contribute to the inhibition of lipid droplet accumulation in 3T3-L1 cells.

Excessive triacylglycerol (TAG) in lipid droplets in adipose tissue is observed in obesity (Lass et al. 2011). Excessive accumulation of triglycerides in obesity is due to decreased lipolysis (Langin 2006). Therefore, weight loss can be expected by inducing the decomposition of triglycerides (Langin 2006). As the fatty acids (FA) are separated from the triglycerides one by one, TAG is sequentially decomposed into diacylglycerol (DAG), monoacylglycerol (MAG), and finally three fatty acids and one glycerol (Duncan et al. 2007). During lipolysis, ATGL induces hydrolysis of TAG to DAG, and HSL induces hydrolysis of TAG to DAG and subsequently to MAG (Lass et al. 2011). In adipose tissue deficient in ATGL, excessive lipid accumulation is observed due to reduced lipolysis (Lass et al. 2011). In addition, perilipin-1 surrounding lipid droplets in adipose tissue is known to inhibit lipolysis (Duncan et al. 2007). Indeed, perilipin-1 deficiency promoted lipolysis, and its overexpression inhibited lipolysis (Duncan et al. 2007). Summarizing these previous reports, it is believed that promoting the expression of ATGL and HSL and inhibiting the expression of perilipin-1 can induce lipolysis. In this study, we observed that the levels of ATGL, p-HSL and HSL were increased, and the level of perilipin-1 was decreased in HDB-treated 3T3-L1 cells. These findings indicate that HDB may decrease the accumulation of lipid droplets through lipolysis.

The conversion of white adipose tissue (WAT) to brown adipose tissue (BAT) through browning causes the energy stored in WAT to be consumed as heat (Lo and Sun 2013). Therefore, it is known that the browning of WAT can prevent excessive weight gain (Kuryłowicz and Puzianowska-Kuźnicka 2020). AMPK induces browning of WAT and promotes the β-oxidation of fatty acids (van der Vaart et al. 2021). UCP-1 is mainly expressed in BAT and plays a role in releasing stored energy as heat (Cannon and Nedergaard, 2004). PGC-1α induces thermogenesis by converting WAT to BAT (Lo and Sun, 2013). PRDM16 promotes the browning of WAT, and PRDM16 is also known as a representative factor determining the development of brown fat (Seale et al. 2007). Indeed, there is a report that an increase in energy expenditure was observed in mice transfected with PRDM16 (Seale et al. 2007). Therefore, these reports suggest that activation of AMPK and increased expression of UCP-1, PGC-1α, and PRDM16 indicate the browning of WAT. In this study, phosphorylation of AMPK and increased expression of UCP-1, PGC-1α, and PRDM16 were observed in HDB-treated 3T3-L1 cells. These results indicate that the browning of WAT by HDB is thought to contribute to suppressing the accumulation of lipid droplets by HDB.

Autophagy is a process in which cytoplasmic proteins or organelles are degraded by autophagosomes, and it has been reported that activation of autophagy in adipocytes can reduce lipid accumulation (Singh et al. 2009). In addition, it has been reported that the inhibition of lipid droplet accumulation by autophagy is achieved through a lipolytic mechanism (Zhang et al. 2020). These reports suggest that the induction of autophagy in adipocytes may be an effective strategy to inhibit the accumulation of lipid droplets. In this study, we observed that HDB increased the level of microtubule-associated protein 1A/1B-light chain 3 (LC3)-II and p62/Sequestosome 1 (SQSTM1) as autophagic markers in 3T3-L1 cells. These results indicate that the activation of adipocyte autophagy by HDB may contribute to HDB-mediated inhibition of lipid droplet accumulation in 3T3-L1 cells.

Since an HFD-induced obese mouse model is often used to test the *in vivo* anti-obesity activity (Park et al. 2019), we administered HDB to HFD-induced obese mice to evaluate the *in vivo* anti-obesity activity of HDB. As a result, compared to HFD-induced obese mice, body weight and fat weight were reduced in HDB-administered mice, and LDL-cholesterol, total cholesterol, and triglyceride contents in the blood were decreased. These results show that a HDB enriched diet can inhibit weight gain associated with a high-fat diet.

Although *Hovenia dulcis* branches contain various bioactive compounds, vanillic acid and ferulic acid were recently found in distilled water extracts of *Hovenia dulcis* branches (Choi et al. 2018). Vanillic acid has been reported to exhibit anti-obesity effects through the activation of AMPK signaling and thermogenic factors *in vivo* and *in vitro* (Jung et al. 2018). And it has also been reported that ferulic acid inhibits obesity and obesity-related metabolic diseases in an HFD-induced obese mouse model (Wang et al. 2018). Since HDB used in this study is also a water extract, it is expected that HDB may contain vanillic acid and ferulic acid, and it is thought that vanillic acid and ferulic acid may contribute to the anti-obesity activity of HDB. However, accurate component analysis related to the anti-obesity activity of HDB will be needed.

To summarize the results obtained in this study, HDB suppressed lipid accumulation by inhibiting adipogenesis, inducing lipolysis, inducing the browning of adipocytes, and inducing autophagy of adipocytes in 3T3-L1 cells. In addition, HDB decreased body weight, fat weight, blood LDL-cholesterol, blood total cholesterol, and blood triglyceride in HFD-induced obese mice. These results show that HDB can be used as a healthy functional food agent for weight loss.

4. Experimental

4.1. Chemical reagents

Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), insulin, and Oil Red O staining solution were purchased from Sigma Aldrich (St. Louis, MO, USA). The primary antibodies against peroxisome proliferator-activated receptor γ (PPARγ, #2430), CCAAT enhancer-binding protein α (CEBPα, #8178), adipose triacylglycerol lipase (ATGL, #2138), hormone-sensitive lipase (HSL, #4107), p-HSL (#4137), perilipin-1 (#9349), AMP-activated protein kinase (AMPK, #5831), p-AMPK (#2535), uncoupling protein 1 (UCP-1, #14670), microtubule-associated protein 1A/1B-light chain 3 (LC3, #2775), p62/Sequestosome 1 (p62/SQSTM1, #39749), and β-actin (#5125), and secondary antibodies such as anti-rabbit IgG, HRP-linked Antibody (#7074) and anti-mouse IgG, HRP-linked Antibody (#7076) were purchased from Cell Signaling (Beverly, MA, USA). The primary antibodies against peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α, sc-518025) and PR domain containing 16 (PRDM16, ab106410) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Abcam (Cambridge, United Kingdom), respectively.

4.2. HDB Preparation

H. dulcis branches (Voucher number: FMC_220302A1) were provided by the Forest Medicinal Resources Research Center, Korea. 10 g of *H. dulcis* branches was immersed in 200 ml of distilled water and left at 4~80 °C for 12 h. After extraction under temperature conditions, the extracts were centrifuged at 15,000 rpm at 4 °C for 10 min, and then the clear supernatant was freeze-dried. The freeze-dried extracts from *H. dulcis* branches (HDB) were dissolved in distilled water for treatment of the cells and stored at -80 °C.

4.3. Cell culture

As mouse adipocytes, 3T3-L1 cells were used in this study because it is mainly used to verify *in vitro* anti-obesity activity. 3T3-L1 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM/F-12 medium containing 10% bovine calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin at 37 °C under a humidified atmosphere of 5% CO_2.

4.4. Differentiation of 3T3-L1 cells

Two days (D0) after reaching 100% confluence, 3T3-L1 cells were cultured in DMI medium (DMEM/F-12 medium, 10% fetal bovine serum, 1 µM dexamethasone, 0.5 mM IBMX, and 10 µg/ml insulin) for 2 days (D2). Then, the cells were cultured in an insulin medium (DMEM/F-12 medium, 10% fetal bovine serum, and 10 µg/ml insulin) for two days (D4). Then, the culture medium (DMEM/F-12 containing 10% fetal bovine serum) was changed every two days (D6-D8). All experiments were analyzed by harvesting cells on D8 after HDB treatment.

4.5. Oil Red O (ORO) staining

3T3-L1 cells were fixed with 10% formalin for 1 h at room temperature. Then, 3T3-L1 cells were dehydrated with 60% isopropanol for 5 min and subsequently stained with Oil Red O solution (60% isopropanol and 40% water) for 20 min. After washing 3T3-L1 with distilled water, the stained lipid droplets of 3T3-L1 cells were photographed with a light microscope (Olympus, Tokyo, Japan), After that, the stained lipid droplets of the cells were eluted with 100% isopropanol for quantitative analysis. The absorbance of the eluted solution was measured at 500 nm with a microplate reader (Human Cop., Xma-3000PC, Seoul, Korea).

4.6. Measurement of free glycerol content

Free glycerol was analyzed using a cell-based glycerol assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, 25 µl of cell culture supernatants and 100 µl of reconstituted free glycerol assay reagent were mixed and then incubated for 15 min at room temperature. After 15 min, the absorbance of the eluted solution was measured at 540 nm with a microplate reader (Human Cop., Xma-3000PC, Seoul, Korea).

4.7. Western blot analysis

Protein changes in HDB-treated 3T3-L1 cells were analyzed by Western blot analysis. The total protein extracted from 3T3-L1 cells was quantified using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA USA). The protein (30 µg) was separated on 12% or 8% SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Thermo Fisher Scientific). After blocking at room temperature for 1 h, the membranes were incubated with the primary antibodies (1:1,000) at 4 °C overnight. After that, the membranes were incubated with the secondary antibodies (1:1000) at room temperature for 1 h. After treating ECL Western blotting substrate on the membrane, the protein band was visualized using LI-COR C-DiGit Blot Scanner. Quantitative analysis of visualized protein bands was performed using the software UN-SCAN-IT gel version 5.1 (Silk Scientific Inc. Orem, UT, USA).

4.8. In vivo anti-obesity activity of HDB

All animal procedures were conducted in accordance with the guidelines for the care and use of laboratory animals issued by Andong National University. Five-week-old male C57BL/6N mice (20±2 g) were purchased from Central Lab. Animal Inc. (Seoul, Korea). All mice were housed at 25±2 °C and 50±10% humidity under a 12-h light/dark cycle. Before treatment, mice were acclimated to the experimental environment for one week. After one week, mice were randomly distributed into groups of 5 mice: the normal diet group (ND), high-fat diet group (HFD), 100 mg/kg of HDB group, and 200 mg/kg of HDB group. The mice were given a free approach to the normal diet, high-fat diet, and water. HDB was administered orally to mice daily. Mice were observed for 8 weeks until the experiment was terminated. Mice's body weight was measured once a week for 8 weeks, and the total fat weight of the mice was measured after the sacrifice of the mice after 8 weeks of treatment. The contents of LDL-cholesterol, total cholesterol, and triglyceride in the blood were measured using an LDL-cholesterol assay Kit (Crystal Chem, IL, USA), Cholesterol Quantification assay Kit (Sigma-Aldrich), and Triglyceride assay kit (Abcam, Cambridge, United Kingdom) respectively.

4.9. Statistical analysis

All the data are shown as mean±SD (standard deviation). Statistical significance was determined by Student's *t*-test. Differences with $^{*}P < 0.05$ and $^{\#}P < 0.05$ were considered statistically significant.

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Conflict of interest: None declared.

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