

# Walnut Polyphenols Inhibit Pancreatic Lipase Activity in Vitro and Have Hypolipidemic Effect on High-Fat Diet-Induced Obese Mice

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**Abstract** This study was aimed at the chemical composition and inhibitory effects of walnut polyphenols (WP) in vitro lipase activity and on obesity in obese mice models. More than 20 individual phenolics such as gallotannins, ellagitannins, flavonoids and phenolic acids were identified in WP. Studying the interactions between porcine pancreatic lipase (PL) and WP were based on fluorescence quenching and an enzymatic assay. The addition of WP to lipase caused a reduction of protein fluorescence intensity at 310 K. In addition, we found that the effect of WP on PL was dependent on reaction medium and substrate used and the half maximal inhibitory concentration of WP was determined to be 163 µg/mL. At the beginning of the experiment, mice were divided into 3 groups, one of them served as normal control group (NCG), the second as hyperlipidemia control group (HCG), the last as walnut polyphenol-treated group (WTG). After 8 weeks of treatment, we investigated the effects of WP on weight gain, food intake and biochemical indexes in obese mice. The results showed that WP could significantly decrease body weight in obese mice (-13.52%,  $P < 0.05$ ). However, there was no remarkable difference in food intake among three groups (1760.2–1823.3 g). In addition, WP could significantly decrease the TG, TC and LDL-cholesterol (LDL-C) concentrations and increase the HDL-cholesterol (HDL-C) concentration when compared to the HCG (-36.12%, -31.27%, -73.3%, and +59.72%, respectively,  $P < 0.01$ ). The levels of hepatic TG and TC were significantly decrease in WTG when compared to the HCG (-27.72% and -48.43%, respectively,  $P < 0.01$ ). In conclusion, these results suggest that WP could be a potentially therapeutic alternative in the treatment of obesity caused by a high-fat diet.

**Keywords:** walnut polyphenols, lipase, fluorescence quenching, obese mice

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## 1. Introduction

Obesity is a major health problem worldwide and is now classically characterized by a cluster of several metabolic disorders [1]. Obesity is caused by the results of an imbalance between energy intake and expenditure [2]. A recent study has found that suppression of food intake, stimulation of energy expenditure and lipase inhibition are the main therapies for obesity [3].

Lipases are important enzymes for lipid absorption and secreted by the pancreas, play a vital role in the efficient digestion of triglycerides [4]. PL is a key lipase, responsible for the hydrolysis of 50-70% total dietary fats [5]. PL inhibition is one of the most widely researched mechanisms used to determine the potential effect of natural products as antiobesity drugs [6,7,8]. Orlistat, one of two clinically approved drugs for obesity treatment, is used for inhibiting PL [9]. Although it has good effect on treating obesity, long-term use may cause some side

effects such as oily stools, oily spotting and so on. At present, the potential of natural products for the treatment of obesity has become a hot research topic. Some results have been reported that polyphenol or some flavonoids are pancreatic lipase inhibitors [10,11,12].

Walnuts are one of the most popular nuts in the world and contain large amounts of bioactive substances such as polyphenol, polysaccharide, protein and so on. Polyphenols are well-known antioxidants with protective effects against cardiovascular disease, cancer, and other degenerative conditions associated with inflammation [13]. In addition, it has been reported that polyphenols can inhibit pancreatic lipase and thereby influence fat digestion and energy intake [14]. Although the inhibitory effects of some polyphenols on PL have been investigated [15,16,17], this is the first report on the inhibitory activity of WP against PL. So, this paper aimed to quantify the inhibition of PL in vitro by WP, as well as to determine the preventive effects of WP on the development of obesity in mice fed a high-fat diet.

## 2. Materials and Methods

### 2.1. Materials

Walnut samples were collected at Kunming and porcine pancreatic lipase was from Sigma Chemical (St. Louis, MO). A working solution was prepared with 100 mM Tris-HCl (pH 8.2). Triton X-100 and triolein were purchased from Sangon Biotech. p-nitrophenyl laurate (pNP laurate) was purchased from Aladdin Reagent (Shanghai). All the reagents were analytical or of chromatographic purity. Water was ultrapure water.

### 2.2. Extraction of Walnut and Determination of Total Polyphenol Content

Walnut polyphenols were prepared as follows: dried walnut testa was powdered and extracted at 60°C for 1 h with 50% (v/v) ethanol (solid-liquid ratio, 1:10), the solvent was subsequently evaporated. Then, the concentrates were formulated as 20 mg/g solution.

The total polyphenol content in walnut extracts was determined by Folin-Ciocalteu colorimetric method according to Ainsworth and Gillespie [18] with minor modifications. Briefly, extract (1.0 mL) was mixed with 5.0 mL of the Folin-Ciocalteu reagent (1:10) and 4.0 mL of Na<sub>2</sub>CO<sub>3</sub> (7.5% aqueous solution). The absorption was measured at 765 nm in an Ultrospec 2100 spectrophotometer (UV-Vis, Amersham Biosciences) after 1 h of incubation at room temperature and in the dark. A standard curve was generated with gallic acid standard substance (concentration ranged from 10 to 70 µg/mL). The calibration curve equation was  $y=0.1062x + 0.0144$  and had a correlation coefficient of  $R^2 = 0.9991$ . WP were evaluated at a final concentration of 420 mg/g. The total phenolic contents were expressed as percentages (%) or milligrams per gram (gallic acid equivalents).

### 2.3. LC-MS Analysis

Quantitative analysis of walnut extracts were carried out in an Accela HPLC system from Thermo Fisher Scientific (San Jose, CA, USA) with a G1314A UV detector and a reversed-phase column Atlantis T3 C18 (100 × 2.1 mm, 3 µm). Mobile phase A and B were respectively, 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The extract (10 µL) was separated at 20°C at a flow rate of 350 µL/min using the following eluents with the linear gradient mode: The 33 min gradient method began with 100% A in 1 min, 100-92% in 3 min, 92-80% A in 20 min, 80-70% A over 2 min, 70-0% A over 2 min and finally 100% B for 5 min.

Mass spectra were obtained from Thermo Fisher Scientific (San Jose, CA, USA) with an electrospray ionization source and ion trap detector operated in negative ion mode. Instrument control and data acquisition were run on Xcalibur Data Analysis 2.0.7 software [19].

### 2.4. Fluorescence Spectra

Fluorescence measurements were conducted on a spectrofluorimeter model LS-50 (PerkinElmer, USA) spectrometer. Briefly, appropriate quantities of WP solution were transferred to a 10 ml tube, then 1.0 ml of 0.5 mg/ml lipase solution was added, and the solution was

diluted to 10 ml with Tris buffer and incubated at 37°C for 30 min, then, the mixtures were centrifuged at 7000 rpm for 10 min and the supernate was injected in 1.0 cm quartz cells. Scanning parameters for all measurements were optimized with slit width 10.0 nm for excitation and emission. The excitation wavelength was 295 nm, and the emission spectra was recorded in the wavelength range of 310-450 nm at a scan rate of 300 nm/min and the resolution was 1.0 nm [6,20].

### 2.5. Lipase Activity Assay

Lipase activity was evaluated by the hydrolysis of pNP laurate spectrophotometrically recorded at 400 nm. Lipase from porcine pancreas was dissolved in ultrapure water at 10 mg/ml and the supernate was used after centrifugation at 8000 rpm for 10 min. The assay buffer was 100 mM Tris-HCl (pH 8.2) and pNP laurate was used as the substrate. The substrate solution was prepared through dissolving pNP laurate (0.08% w/v) in 5 mM sodium acetate buffer (pH 5.0) containing 1% Triton X-100 and was heated in boiling water for 1 min to aid dissolution, then cooled to room temperature. The assay was carried out in a total volume of 1000 µl containing 300 µl Tris-HCl buffer, 450 µl substrate solution, 100 µl of WP with different concentrations and 150 µl lipase. The blank assay contained 400 µl assay buffer, 450 µl substrate solution and 150 µl lipase. After mixing with WP, lipase and substrate solution, the mixtures were incubated at 37°C for 30 min and heated in a boiling water bath for 10 min to stop the reaction. Then the mixtures were centrifuged at 8000 rpm for 10 min and read at 400 nm against the blank in a UV-visible spectrophotometer [21].

### 2.6. Preparation of High Fat Diet (HFD)

The HFD was prepared according to the following method: it includes 83.25% standard diet [22], 10% lard, 1.5% cholesterol, 0.2% NaTDC, 5% Sugar and 0.05% Propylthiouracil [23]. The compositions of the experimental diets are shown in Table 1.

Table 1. Composition of the experimental diets

| Components         | Standard diet |         | High-fat diet |         |
|--------------------|---------------|---------|---------------|---------|
|                    | g/kg          | kcal/kg | g/kg          | kcal/kg |
| Cornstarch         | 400           | 1600    | 333           | 1332    |
| Sucrose            | 100           | 400     | 133           | 532     |
| Dextrinated starch | 140           | 560     | 116.5         | 466     |
| Lard               | -             | -       | 100           | 900     |
| Cholesterol        | -             | -       | 15            | 450     |
| Casein             | 200           | 800     | 167           | 668     |
| Soy oil            | 70            | 630     | 58            | 522     |
| NaTDC              | -             | -       | 2             | -       |
| Propylthiouracil   | -             | -       | 0.5           | -       |
| Mineral mix        | 30            | -       | 25            | -       |
| Vitamin mix        | 10            | -       | 8             | -       |
| Cellulose          | 50            | -       | 42            | -       |
| Total              | 1000          | 3990    | 1000          | 4870    |

“-”: it represents absence.

### 2.7. Animals and Treatments

This study was conducted according to Kirin Pharmaceutical's guidelines for ethical animal care, handling and termination. Animal experiments were

carried out in compliance with the standards for use of laboratory animals. After a 2-week adaptation period, animals were randomly divided into 3 groups. One of them served as normal control group (NCG) [24,25]: kept on standard diet and daily gavaged with normal saline. The second as hypercholesterolemia control group (HCG): received HFD and daily gavaged with normal saline. The third as WP-treated group (WTG): received HFD and daily gavaged with WP at a dose of 200  $\mu\text{g/g}$  body weight for 8 weeks. The average food intake by each group and the body weight by each mouse were measured once weekly.

## 2.8. Biochemical Analysis

The serum was obtained by centrifugation of the blood at 3000 rpm for 15 min and the total cholesterol (TC), total triglycerides (TG), and HDL-C concentrations were immediately determined using the corresponding diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, PR China). The LDL-C was calculated by the Friedwald formula [26]:  $\text{LDL-C} = \text{TC} - [\text{HDL-C} + (\text{TG}/5)]$ . To determine liver total cholesterol (TC) and triglycerides (TG), one gram of liver from each mouse was homogenized in 10 mL isopropanol. The homogenate was allowed to stand for 48 h at 4°C. The mixture was

centrifuged 10 min at 2500 g and the supernate was used for lipid analysis. TC and TG were determined using reagent kits as described above.

## 2.9. Statistical Analysis

Statistical analysis was performed with the use of SPSS software (version 19.0, Chicago, IL, USA). Results are expressed as mean  $\pm$  SD. The statistical significance of experimental observations was determined by one-way ANOVA. Statistical significance was set at  $P < 0.05$ .

## 3. Results

### 3.1. Identification of Phenolic Compounds in WP

WP were found to contain a highly complex mixture of gallotannins, ellagitannins, flavonoids and phenolic acids; more than 20 individual phenolics were identified. Corresponding molecular formulas, their retention time (tR) and their molecular mass were shown in Table 2. In addition, many of identified phenolic compounds were found along the chromatogram (Figure 1).

Table 2. Phenolic compounds were identified in WP

| Number | tR (min) | Compound identification        | Formula   | Measured mass |
|--------|----------|--------------------------------|-----------|---------------|
| 1      | 2.66     | HHDP-glucose isomer            | C20H18O14 | 482.07        |
| 2      | 2.84     | Vanillic acid                  | C8H8O4    | 168.15        |
| 3      | 3.16     | HHDP-glucose isomer            | C20H18O14 | 482.07        |
| 4      | 3.25     | Pyrogalllic acid               | C6H6O3    | 126.11        |
| 5      | 3.26     | Gallic acid                    | C7H6O5    | 170.02        |
| 6      | 4.47     | HHDP-glucose isomer            | C20H18O14 | 482.07        |
| 7      | 5.92     | Neochlorogenic acid            | C16H18O9  | 354.31        |
| 8      | 7.07     | Pedunculagin/casuarinin Isomer | C34H24O22 | 784.07        |
| 9      | 8.22     | Chlorogenic acid               | C16H18O9  | 354.31        |
| 10     | 9.72     | Pedunculagin/casuarinin Isomer | C34H24O22 | 784.07        |
| 11     | 13.62    | Salidroside                    | C14H20O7  | 300.30        |
| 12     | 15.25    | (-)-epicatechin                | C15H14O6  | 290.08        |
| 13     | 20.14    | Ferulic acid                   | C10H10O4  | 194.19        |
| 14     | 20.57    | (-)-epicatechin-3-O-gallate    | C22H18O10 | 442.09        |
| 15     | 22.24    | 3-p-coumaroylquinic acid       | C16H18O8  | 338.10        |
| 16     | 23.47    | 4-p-coumaroylquinic acid       | C16H18O8  | 338.10        |
| 17     | 23.47    | Ellagic acid hexoside isomer   | C21H20O12 | 464.09        |
| 18     | 25.02    | Ellagic acid pentoside isomer  | C20H18O11 | 434.08        |
| 19     | 26.11    | Ellagic acid                   | C14H6O8   | 302.01        |
| 20     | 28.61    | Caffeic acid                   | C9H8O4    | 180.15        |
| 21     | 28.91    | Coumaric acid hexoside isomer  | C15H18O8  | 326.01        |
| 22     | 29.52    | Ellagic acid hexoside isomer   | C21H20O12 | 464.09        |

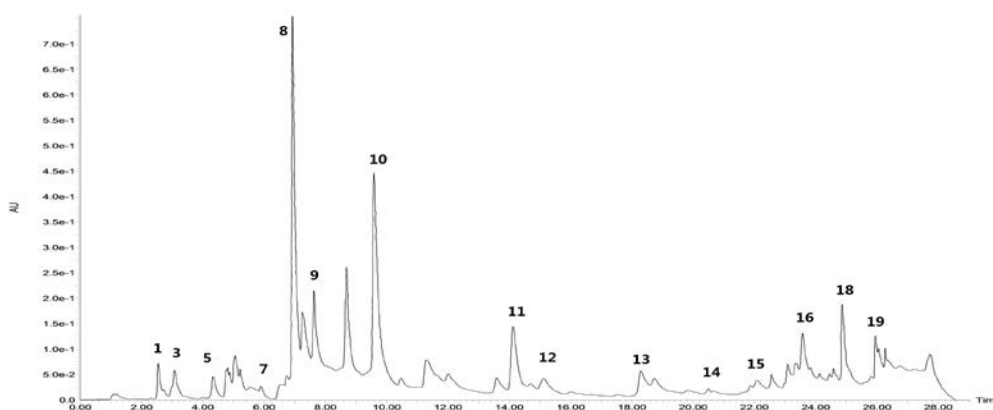
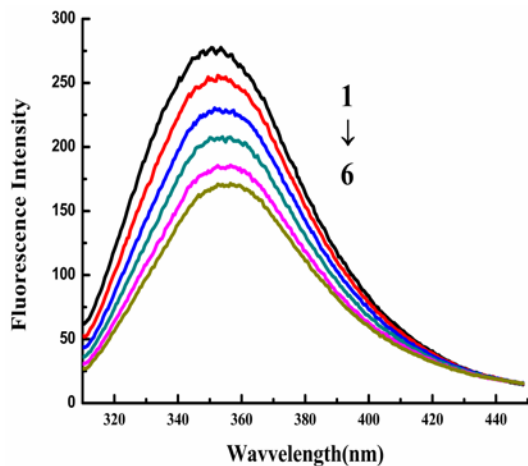


Figure 1. Chromatographic profile obtained at 280 nm for WP. Peak number identities are displayed in Table 2

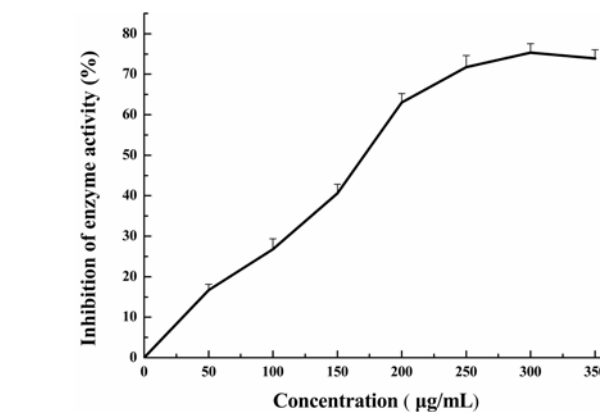
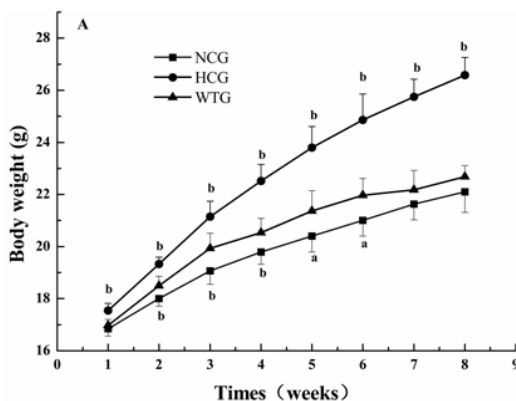
### 3.2. Fluorescence Quenching

The fluorescence intensity of lipase decreased at 350 nm for WP-PL systems (Figure 2). Thus, walnut polyphenols bound with lipase caused microenvironment changes. The observed  $\lambda_{em}$  for lipase ( $\lambda_{em} = 350$  nm) is higher than the  $\lambda_{em}$  for lipase for the isolate indole group of Trp ( $\lambda_{em} = 356.5$  nm).



**Figure 2.** The quenching effect of WP on lipase fluorescence intensity at 37°C and pH 8.2.  $\lambda_{ex} = 295$  nm. (1-6) with  $1.0 \times 10^{-5}$  M lipase. 1→6: WP concentration increased from 10 µg/mL to 60 µg/mL

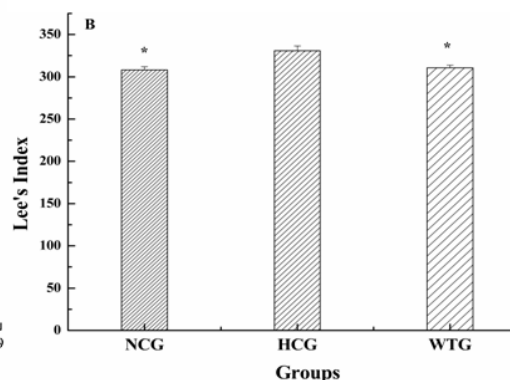
### 3.3. Inhibition of Lipase Activity by WP



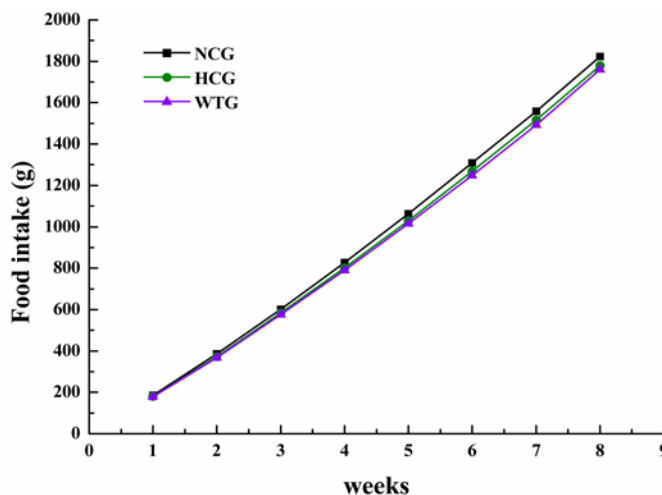
**Figure 3.** Effect of WP on porcine pancreatic lipase activity in vitro. WP concentration increased from 0 µg/mL to 350 µg/mL. The data are given as mean±SD (n = 3)

The inhibition effects of WP against porcine pancreatic lipase were determined by using different concentrations of WP (50-350 µg/mL) (Figure 3). WP inhibited the enzyme activity in a dose-dependent way and its suppressive effects against lipase reached maximum at 300 µg/mL (75.36% inhibition). The half maximal inhibitory concentration of WP was determined to be 163 µg/mL.

### 3.4. Effects of WP on Weight Gain, Food Intake in Obese Mice



**Figure 4.** Effects of WP on body weight gain and Lee's index in mice fed high-fat diet for 8 weeks. (A) Body weights (g) of mice (mean±SD) consuming the indicated diets for the 8-week intervention period. Significant difference between groups for each week is signified by letter a and b, a was represented as \* $p < 0.05$ , b was represented as \*\* $p < 0.01$ . (B) Lee's index of mice on indicated diets during the intervention period. Results are expressed as mean±SD of nine animals per group, \* $p < 0.05$



**Figure 5.** Effects of WP on food intake in mice fed high-fat diet for 8 weeks. Results are expressed as mean±SD of nine animals per group



The body weight gain in mice fed a high-fat diet containing WP was reduced by 13.52% compared to mice fed a HFD as shown in Figure 4A. In the case of HFD, the body weight gain was increased by 20.18% compared to mice fed a standard diet. As shown in Figure 4B, there

were also significant changes in Lee's index (308.03–330.76) among these groups. On the other hand, there was no remarkably difference in food intake (1760.2–1823.3 g) in Figure 5.

**Table 3. Effects of WP on serum parameters and liver lipids**

| Groups | Serum lipids (mmol/L) |             |             |             | Liver lipids (mg/g) |              |
|--------|-----------------------|-------------|-------------|-------------|---------------------|--------------|
|        | TG                    | TC          | HDL-C       | LDL-C       | TG                  | TC           |
| NCG    | 0.86±0.04             | 1.72±0.11   | 1.3±0.06    | 0.38±0.07   | 17.27±1.45          | 4.88±0.42    |
| HCG    | 1.55±0.1**            | 2.75±0.11** | 0.72±0.08** | 1.76±0.13** | 29.08±1.71**        | 10.24±0.67** |
| WTG    | 0.99±0.08             | 1.89±0.04   | 1.15±0.04   | 0.47±0.09   | 21.02±1.19          | 5.28±0.37    |

Values were measured after 8 wk for feeding. Data are means±SD for 9 mice. \*\* p < 0.01 versus HCG

### 3.5. Effects of WP on serum parameters and liver lipids

The serum lipid profile was significantly increase in HCG with respect to NCG. As shown in Table 3, TG and TC levels in HCG were significantly higher than that in NCG after 8-week treatment (+80.23% and +59.88%, respectively,  $P < 0.01$ ). the LDL-C level was significantly decrease in NCG (-78.41%,  $P < 0.01$ ) and the HDL-C level was significantly increase in NCG with respect to HCG (+80.56%,  $P < 0.01$ ). In addition, the levels of hepatic TG and TC were significantly decrease in NCG when compared to the HCG (-40.61% and -52.25%, respectively,  $P < 0.01$ ).

As shown in Table 3, WP significantly decreased the TG, TC and LDL-C concentrations and increased the HDL-C concentration when compared to the HCG (-36.12%, -31.27%, -73.3%, and +59.72%, respectively,  $P < 0.01$ ). In addition, the levels of hepatic TG and TC were significantly decrease in WTG when compared to the HCG (-27.72% and -48.43%, respectively,  $P < 0.01$ ).

## 4. Discussion

In this study, a total of 22 phenolic compounds, including gallotannins, ellagitannins, flavonoids and phenolic acids, were identified in walnut polyphenols. The LC/MS spectra were compared with those of candidate compounds found in previous report, especially when the presence of the compound was reported in walnut polyphenols [19,27].

Porcine lipase is the most important enzyme for the efficient digestion of triglycerides. Dietary triglycerides are hydrolyzed by pancreatic lipase to monoglycerides, free fatty acids and other small molecules, which will resynthesize triglycerides in the intestine for the person of food intake leading to obesity. So, inhibiting lipase in the digestive organs is an effective way to prevent the development of obesity [28]. PL has 7 tryptophan (Trp) residues, and the fluorescence of lipase is mainly dominated by Trp emission. When some molecules interact with PL, Trp fluorescence may change depending on the impact of the interaction on the protein conformation [29,30]. In this paper, the addition of WP to lipase caused a reduction of protein fluorescence intensity known as quenching effect, it meant that the interaction between PL and WP changed the microenvironment of Trp residues. The emission band of free lipase at 350 nm shifted toward 356.5 nm, which was related to more exposure of Trp residues and an unfolding protein structure [31,32,33].

Polyphenols, especially hydroxycinnamic acid derivatives, are widely distributed in plants [34,35]. These polyphenols are powerful antioxidants and have been reported to demonstrate antibacterial and anti-inflammatory [36]. A number of reports are shown that polyphenol-rich extracts from certain berries and tea are effective inhibitors of PL in vitro [21,37]. In addition, some of the studies show that ellagitannins, chlorogenic acid and caffeic acid have a good effect on lipase inhibition [38,39]. In this study, the effective inhibition caused by WP may be due to these polyphenol, which are present in polyphenol extracts.

As expected, the present study has confirmed that HFD can induce a great gain of body weight [40]. In this paper, the effect of WP on obesity was tested using mice fed a high-fat diet containing 200 µg/g of WP. WP prevented the HFD-induced increase in body weight. This effect did not depend on decreased food because there were no significant differences among three groups. Evidence from pre-clinical and some clinical studies indicated that administration of green tea containing caffeine [41] and vegetable such as rutgers scarlet lettuce with chlorogenic acid [42] resulted in lower body weight. In addition, some researchers have found a possible mechanism that protective effect against HFD-induced obesity in mice through an enhanced expression of uncoupling proteins and elevated AMPK phosphorylation in the visceral adipose tissue [43]. However, In this experiment, the data showed that supplement of WP can effectively inhibit weight gain, but the specific impact of WP on the development of obesity is still unknown and further investigation is needed.

These results suggest that WP have an antiobesity function: it prevents the hydrolysis of dietary fat in the small intestine and decreases the subsequent intestinal absorption of dietary fat. The hypolipidemic effects of WP showed in this study were similar with previous reports on mice fed a HFD [44,45]. Administration of WP reduced lipid contents in mice fed a HFD for 8 weeks. These results suggested that inhibition of intestinal lipid absorption was involved in the decrease of lipid contents, particularly triglyceride content.

## 5. conclusion

This experiment demonstrated that WP efficiently inhibited in vitro porcine PL activity. Additionally, it also showed that the treatment had powerful antiobesity activity on HFD-induced obese mice. Collectively, our results provided an important basis on the prevention and treatment of obesity.

## Acknowledgments

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## Statement of Competing Interests

None.

## List of Abbreviations

WP: walnut polyphenols, HFD: high fat diet, PL: pancreatic lipase, TC: total cholesterol, TG: total triglycerides, LDL-C: LDL-cholesterol, HDL-C: HDL-cholesterol, NCG: normol control group, HCG: hypercholesterolemia control group, WTG: walnut polyphenol-treated group, pNP laurate: p-nitrophenyl laurate.

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