**Proanthocyanidins isolated from lotus seed skin mitigate glycolipid metabolism disorder through p-38/Nrf2/NF-κB signaling pathway**

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Original Article

Proanthocyanidins isolated from lotus seed skin mitigate glycolipid metabolism disorder through p-38/Nrf2/NF-κB signaling pathway

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Running title: Proanthocyanidin B1 ameliorates glycolipid metabolism dysfunction

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Abstract

Lotus seed skin extract is rich in flavonoids, making it a promising candidate for developing health products. In a previous study, we found that proanthocyanidins from lotus seed skin, particularly proanthocyanidin B1 (PB1), can indirectly activate the Nrf2 signaling pathway, exerting an antioxidant effect. In this study, we isolate proanthocyanidins from lotus seed skin (PLS) using ethanol extraction and RP-HPLC identification, and investigate its effects on glycolipid metabolism both in vivo and in vitro. Our results demonstrate that PLS reduces body weight in high-fat diet (HFD) mice by decreasing food efficiency. PLS also normalizes serum glucose, insulin secretion, glycosylated hemoglobin (HbA1c), and intraperitoneal glucose tolerance (IPGTT). Furthermore, PLS significantly improves blood lipid parameters and inhibits the expression of six proinflammatory factors, including IL-1α, IL-1β, IL-3, IL-6, IFN-γ, and TNF-α in HFD mice. Additionally, analysis of fresh liver tissues reveals that PLS and PB1 induce the expression of antioxidant proteins such as HO-1 and NQO1 by activating the p38-Nrf2 signaling pathway and inhibiting the NF-κB signaling pathway.
In conclusion, proanthocyanidins from lotus seed skin regulate glycolipid metabolism disorders by targeting the p38/Nrf2/NF-κB signaling pathway. Our study offers a new approach for the high-value comprehensive utilization of lotus seed skin by-products and precise dietary intervention for metabolic syndrome.

**Keywords:** proanthocyanidins, lotus seed skin, p-38/Nrf2/NF-κB, glycolipid metabolism

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**Introduction**
The lotus plant, distributed in regions including Asia, Iran, and Egypt, has been widely used in traditional Chinese medicine. As a treasure, every part of the lotus plant is valuable: the underground stem known as lotus root, the seeds within the lotus pod, the leaves for medicinal purposes, and the flowers for their aesthetic value. Lotus seeds, serving as a vegetable, a food source, and a medicinal ingredient, are particularly rich in functional compounds such as flavonoids and alkaloids. These seeds exhibit various bioactive functions, including anti-obesity, antioxidant, and anti-tumor properties [1].

Proanthocyanidins, as polyphenols, exist widely in a variety of natural plants. Fruits such as grapes [2], apples [3] and blueberries [4] are the best sources of proanthocyanidins. It has been reported that proanthocyanidins not only possess strong free radical scavenging ability [5], but also can regulate the balance of lipid metabolism [6], and reduce blood glucose [7]. Proanthocyanidins C1 has anti-aging activity and can significantly prolong the healthy life and average life of mice [8]. Grape seed proanthocyanidins extract alleviates colitis caused by dextran sulphate sodium salt via regulating inflammatory cytokines and oxidative stress, maintaining intestinal barrier function, and improving microbial flora [9]. Rhodiola oligomeric proanthocyanidins extract significantly increased the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) in the serum, heart, liver, and brain of mice, and decreased the content of malondialdehyde (MDA) [10]. Oligo-proanthocyanidins inhibited ROS production and glutathione/oxidized glutathione ratio in streptococcal-induced diabetic rats [11].

The Nrf2 signaling pathway is recognized as the primary endogenous antioxidant signaling pathway [12]. Transient activation of Nrf2 leads to the expression of detoxifying enzymes and antioxidant proteins, which safeguard...
pancreatic β-cells from oxidative damage and enhance insulin sensitivity by inhibiting glucose absorption, boosting AMPK phosphorylation, and suppressing gluconeogenesis [13]. Experimental findings from diabetic model mice and Nrf2 gene knockout diabetic patients indicate that the Nrf2 signaling pathway plays a pivotal role in the pathogenesis of disorders related to glycolipid metabolic disorders [14]. Notably, research using diabetic glomeruli suggests that dietary or pharmacological activation of the Nrf2 signaling pathway holds significant promise in the nutritional management of diabetic nephropathy [15]. Furthermore, Nrf2 can modulate glucose and lipid metabolism by regulating other transcription factors. Nrf2 can increase PPARγ expression levels, and the downstream genes activated by the binding of PPARγ and RXRα to the PPRE fragment can promote adipocyte formation, differentiation, and storage, as well as glucose metabolism [16]. Nrf2 can also enhance C/EBPβ expression levels, and the genes induced by the binding of C/EBPβ and C/EBPα to the CCAAT fragment regulate early adipocyte differentiation [17]. The nuclear factor NF-κB pathway has long been recognized as a prototypical pro-inflammatory signaling pathway due to its role in pro-inflammatory gene expression. Inflammation is closely linked to abnormal glucose and lipid metabolism.

Our previous study [18] demonstrated that the oligomeric proanthocyanidins extract from lotus seed skin purified using the microporous resin AB-8, along with procyanidin B1, can exert antioxidant effects by activating the Nrf2 signaling pathway in HepG2 cells. In the present study, proanthocyanidins from lotus seed skin (PLS) were isolated and purified using ethanol extraction and rotary evaporation. Their chemical structures were identified using reversed-phase high-performance liquid chromatography (RP-HPLC). We further investigated the preventive effect of lotus seed skin extract on glycolipid metabolism disorders both in vitro and in vivo.

In this study, we used high-fat diet-induced obese mice to investigate the effects of PLS on the activation of the antioxidant signaling pathway Nrf2/HO-1, lipid levels, blood glucose levels, and the expression of inflammatory factors in high-fat diet-induced obese mice. Additionally, we used PB1, the main functional component of PLS, to verify its effect on protein kinases upstream of the Nrf2 signaling pathway in cells. Our aim was to assess the effects of PLS on glycolipid metabolism by targeting Nrf2, laying a theoretical foundation for the further application of proanthocyanidins from lotus seed skin in dietary nutrition.

Materials and Methods

Preparation of PLS

Lotus seed skin was freshly harvested by a local company, Hong Xinglong Co., Ltd., of Xiangtan County (Xiangtan, China). The PLS was prepared according to the following process. In brief, the lotus seed skin was ground into powder,
passed through a 60 mesh sieve and stored in a refrigerator at −20°C. The powder was weighed in certain quantities and extracted in 70% ethanol with a solid ratio of 1:10 (w/w, 50°C, 60 min). After rotating evaporation, the extract was freeze-dried directly to obtain the lotus seed skin extract rich in proanthocyanidins. Proanthocyanidin B1 (PB1) was purchased from Sigma–Aldrich (St Louis, USA), with purity > 98%.

**RP-HPLC analysis**

The dry purified proanthocyanidins extract of lotus seed skin was reconstituted in a 50% aqueous methanol solution (chromatographic grade; in water), filtered through an organic film (0.45 μm), and collected into a sample bottle for reversed-phase high-performance liquid chromatography (RP-HPLC) analysis. The proanthocyanidins were verified by RP-HPLC using a WAT054275 Symmetry C18 liquid chromatography column (4.6 mm × 250 mm, 5 μm; Agilent Technologies Inc. Santa Clara, USA). The injection volume was 10 μL, with the flow rate as 0.5 mL/min, and the column temperature at 35°C. The wavelength for detection was 280 nm. In this study, proanthocyanidins, especially proanthocyanidin B1, B2 and B4, were detected in lotus seed skin. The chromatographic diagram is shown in Figure 1.

**Animal care and treatment**

Male C57BL/6J mice aged 5 weeks were obtained from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). The mice were housed in standard cages in a room at 23±2°C with 55%–60% relative humidity and a 12/12 h light/dark cycle. The room was well ventilated, and food was provided in accordance with the feed formula and nutrition information. After one week of adaptive feeding, 30 mice were randomly divided into 5 groups according to body weight: STD (mice were given a standard diet); HFD (mice were given a high-fat diet); HFD + low-dose PLS (mice were given a high-fat diet and low-dose PLS, 1%); HFD + high-dose PLS (mice were given a high-fat diet and high-dose PLS, 2%); and STD + high-dose PLS (mice were given a standard diet and high-dose PLS, 2%). PLS was dissolved in corn oil and injected into mice through peritoneal injection. The activity and mental state of the mice were observed every day, and the body weight was measured twice a week. After 8 weeks of feeding (13 weeks of age), the mice were sacrificed after overnight fasting. Food efficiency was calculated based on food intake and weight gain. Food efficiency rate (%) = Total food intake (g)/Weight gain (g) × 100%.

**Glucose and lipid levels**

Mouse blood was extracted from the ocular veins and placed in a 2 mL centrifuge tube. Following a 30-min incubation period at room temperature, the sample was
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The sample was centrifuged at 3500 g for 5 min. The resulting supernatant is referred to as serum, which was carefully collected and stored at −80°C. Additionally, 100 mg of hepatic tissue was homogenized with 1 mL of ice-cold extract and subjected to centrifugation at 8000 g for 10 min to yield the supernatant fraction. The levels of glucose (GLU), insulin, total triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C) in the serum as well as TG and total cholesterol (TC) in the liver were determined using commercial kits (Nanjing Jiancheng, Institute of Biotechnology, Nanjing, China) in accordance with the manufacturer’s instructions. The intraperitoneal glucose tolerance test (IPGTT) was used to measure the blood glucose value at each time point (15, 30, 60, 90 and 120 min), and the area under the glucose curve was calculated.

Hematoxylin-eosin (H&E) staining

A portion of the left lobe of the liver was removed from each mouse and fixed in 4% paraformaldehyde. After 24 h, the liver samples were embedded in paraffin and cut into sections using a cryostat. These sections were then stained with hematoxylin-eosin (H&E) for histological analysis. Pathological imaging was subsequently conducted using the DFC420C pathological imaging system (Leica, Heidelberg, Germany).

Bio-Plex-based assays of inflammatory cytokines

RAW 264.7 cells were pre-cultured and starved to eliminate the effect of fetal bovine serum (FBS). Cells were treated with 10, 20 μM PLS for half an hour before exposure to 40 ng/mL LPS. The levels of 23 cytokines, including IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IP-10, TNF-α, IFN-γ, MCP-1, MIP-1α, MIP-1β, GM-CSF, RANTES, PDGF-BB, FGF basic, eotaxin, and VEGF, were detected using the Bio-Plex Pro Human Cytokine 27 Plex Panel Kit (Bio-Rad Hercules, USA) and the Bio-Plex Cytokine Assay System (Bio-Plex 200; Bio-Rad) according to the manufacturer's instructions, and the results were analyzed with Bio-Plex Manager software (version 4.0).

Cell culture and treatment

The specific method used was described previously [18,19]. Briefly, human hepatoblastoma HepG2 cells (ATCC, Rockville, USA) were cultured at 37°C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; HyClone, USA) supplemented with 10% FBS (Biological Industries, Kibbutz Beit Haemek, Israel). Murine macrophage RAW264.7 cells (ATCC) were cultured at 37°C with 5% CO₂ in DMEM containing 10% FBS. HepG2 and RAW 264.7 cells were seeded into 6 cm plates at a density of 10⁶ and preincubated at 37°C in 5% CO₂ for 24 h. The cells were treated with a series of concentrations of PLS (0, 25, 50
μg/mL) and PB1 (0, 10, 20 μg/mL) for 4‒12 h and then washed with cold PBS twice. The mice were treated with PB1 or PLS, and their effects on antioxidant proteins in the liver were detected.

In the validation experiment, combined fatty acid (CFA)-induced HepG2 cells were used. The stock solutions of CFA were prepared following the methods in a previous study [20]. In brief, 2 M oleic acid and 0.2 M palmitic acid were mixed into DMEM. Bovine serum albumin (BSA; 2%) without free fatty acids was added as a dissolved carrier. The proteins of cells were harvested using RIPA buffer (Beyotime, Shanghai, China) on ice for 20‒30 min and then centrifuged at 14,000 g for 10 min at 4°C. The supernatants were stored at –80°C for subsequent protein quantitative and western blot analysis. A bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China) was used to measure the concentration of the lysate protein.

**Western blot analysis**

Protein extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). Next, the membranes were placed in skim milk for 1 h at room temperature and then incubated with primary antibody at 4°C overnight. After being washed with TBST, the membranes were incubated with secondary antibody at room temperature for 1 h. The images were acquired and photographed with an Image Quant LAS 4000 mini (General Electric Medical System Co., Ltd., Boston, USA) after adding ECL (XinSaiMei, Suzhou, China). The primary antibodies used in this study included anti-Nrf2, anti-NQO1, anti-HO-1, anti-p-p38, anti-p38, anti-p-MEK, anti-MEK, anti-p-JNK, anti-JNK, anti-p-AKT, anti-AKT, anti-p-PKC, anti-PKC and anti-p65 and were purchased from Santa Cruz Biotechnology (Santa Cruz, USA); anti-IκB-α, anti-p-c-Jun, anti-c-Jun, anti-Lamin B, anti-α-tubulin, anti-β-actin, anti-PEPCK, and anti-CPT1A were purchased from Cell Signaling Technology (Beverly, USA). Inhibitors include SB202190 (p38 inhibitor), U0126 (MEK1/2 inhibitor), SP600125 (JNK inhibitor), LY294002 (AKT inhibitor) and R0318220 (PKC inhibitor) were purchased from Cell Signaling Technology (Beverly, USA), ML385 (Nrf2 inhibitor) and MG132 (NF-κB inhibitor) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). The secondary antibodies, anti-mouse and anti-rabbit, were purchased from Cell Signaling Technology (Beverly, USA).

**Statistical analysis**

ImageJ analysis software was used for quantitative analysis of each band. Every assay was repeated in triplicate, and the data are presented as the mean ± SD SPSS 20.0 (IBM) was utilized for one-way ANOVA to assess the mean
differences between groups, followed by the Duncan method for multiple comparisons. \( P<0.05 \) was considered statistical significance.

**Results**

**PLS reduced body weight in HFD mice**

After 8 weeks of high-fat diet feeding, the average body weight of the HFD group increased from 23.08 g to 40.65 g \( (P<0.05) \), an increase of 76.13%, indicating that the high-fat mouse model was successfully established (Figure 2A). When the visceral organs of the STD and STD+2% PLS groups were weighed, there was no significant difference in organ weight between the two groups. This finding suggested that the weight loss in mice after the addition of PLS to the diet was not caused by appetite suppression but was the result of reduced food efficiency (Figure 2B–D). According to Figure 2A,B, the daily food intake of the mice in each experimental group was basically the same (average 2.73 g/d); when the mice were fed with high-fat diet and PLS simultaneously, the body weight of the mice in the HFD+2% PLS group was effectively reduced by 51.10% \( (P<0.05) \) compared with that in the HFD group. However, the body weight of the mice in the HFD+1% PLS group decreased by only 16.95%, which was not significant.

**PLS reduced insulin resistance and enhances insulin sensitivity in HFD mice**

Previous study has shown that long-term high-fat intake not only increases body fat content; increases the levels of TC, TG and LDL-C [21] in serum; and causes disorders of glycolipid metabolism but also increases insulin resistance, reduces insulin sensitivity and increases blood glucose. To confirm the preventive effect of PLS on HFD-induced hyperglycemia and insulin resistance, we investigated the levels of serum glucose, serum insulin, glycosylated hemoglobin (HBALC) and intraperitoneal glucose tolerance test (IPGTT).

According to Figure 3A,B, compared with the STD group, the HFD group displayed a significant increase in serum glucose levels and insulin secretion after long-term high-fat feeding. When fed with 1% or 2% PLS, the fasting blood glucose levels of the mice in the two groups decreased 10.78% and 23.63%, respectively. The fasting blood glucose levels of the mice in the HFD + 2% PLS group were reduced to the normal level of those in the STD group. Accordingly, the content of insulin in serum of mice in the two groups also tended to be normal. Moreover, both 1% and 2% PLS significantly decreased HFD-induced HBALC (Figure 3C) and IPGTT (Figure 3D), and 2% PLS had a stronger inhibitory effect than 1% PLS. This suggests that PLS can reduce insulin resistance and enhance insulin sensitivity in high-fat diet-fed mice then the blood glucose content and insulin secretion return to normal, which indicated that PLS has a regulatory effect on glucose metabolism.
**PLS reduced lipid content in the livers of HFD mouse**

The liver is an important target organ for lipid metabolism and plays a prominent role in lipid metabolism balance. Accordingly, the detection of lipid levels in the liver after PLS intervention is helpful for comprehensively understanding the role of PLS in lipid metabolism regulation. As shown in Figure 4, the TG (Figure 4A) and TC (Figure 4B) levels in the liver of the HFD group were significantly greater than those in the STD group ($P<0.05$). However, when PLS was added to the high-fat diet, the levels of these two indicators decreased rapidly. Compared with those in the HFD group, the TG and TC levels in the HFD+1% PLS group decreased by 17.69% and 35.82%, respectively ($P<0.05$), and the TG and TC levels in the HFD+2% PLS group decreased by 38.80% and 60.27%, respectively ($P<0.01$). Figure 4G shows the histological morphology of the liver tissue through hematoxylin and eosin (H&E) staining. The livers of high-fat diet-fed mice exhibited noticeable cellular damage, which was notably alleviated by PLS. Furthermore, the higher concentration showed better efficacy than the lower concentration.

**PLS reduced the lipid content in the serum of HFD mouse**

High-fat diet leads to dyslipidemia in mice. After the intervention with 1% and 2% PLS, the level of TG decreased substantially to 14.29% and 32.80% lower than that in the HFD group, respectively (Figure 4C). Although the content of TC also decreased by 2.56% and 4.79%, respectively, the difference was not significant (Figure 4D). The HDL-C content increased (4.67%) but not significantly after the addition of 1% PLS, while the HDL-C content decreased after the addition of 2% PLS (Figure 4E). The content of LDL-C decreased by 10.45% after the addition of 1% PLS but was not significant, while increased after the addition of 2% PLS (Figure 4F). This demonstrated that PLS intervention can effectively reduce the TG level in the blood of high-fat mice. Although the content of TC decreased, the effect was small and not significant, and PLS had little effect on the levels of HDL-C and LDL-C.

**PLS inhibited the secretion of inflammatory cytokines**

Studies have shown that patients with hyperlipidemia also have increased levels of inflammatory factors [22]. Therefore, inflammatory factors in high-fat mice were examined. As shown in Figure 5, the six inflammatory factors with the most significant changes were listed, including IL-1α, IL-1β, IL-3, TNF-α, IL-6, and TNF-γ. The results showed that the presence and expression levels of inflammatory factors in the serum of mice were generally low, while the expression levels of inflammatory factors were significantly increased by long-term high-fat feeding. Both low and high concentrations of PLS downregulated inflammatory cytokines, which was significantly different from that of the high fat group.
PLS and PB1 induced the expression of the antioxidant protein

In the previous study [18], we found that PLS plays an antioxidant role in the mouse liver by activating the Nrf2 signaling pathway. We suspect that PB1 plays a role in PLS. Nrf2, HO-1 and NQO1 proteins in liver of mice injected with PB1 and PLS were significantly greater ($P<0.05$), and the protein expression level was significantly greater after treatment for 72 h than after 48 h (Figure 6).

PLS improves glycolipid metabolism through p38/Nrf2/NF-κB signaling pathway

To determine the upstream protein kinase regulating Nrf2 expression, we treated HepG2 cells with inhibitors using 20 μM PB1 for screening the three major signaling pathways of MAPKs, PI3K/AKT and PKC. As shown in Figure 7A, Nrf2 protein expression was significantly promoted after 20 μM PB1 treatment. p-38 and PKC inhibitors treatment significantly decreased the expression of Nrf2, suggesting that the upstream protein kinases p-38 and PKC may be involved in the increase in Nrf2 expression induced by PB1.

To investigate whether PB1-induced Nrf2 protein expression is linked to phosphorylation of upstream kinases P-38 and PKC, we studied the effects of PB1 at different concentrations (0-40 μM) on these kinases. Figure 7B shows that only p-38 was significantly phosphorylated in MAPKs after PB1 treatment ($P<0.05$ or $P<0.01$), increasing with PB1 concentration up to 20 μM. AKT and PKC phosphorylation remained unaffected by PB1. This suggests that PB1 regulates Nrf2 protein expression by stimulating p-38 phosphorylation, independent of PKC. Using LPS-induced RAW264.7 cells, we found PB1 inhibited NF-κB expression by increasing IκB-α levels, without affecting c-Jun phosphorylation (Figure 8). This indicates PB1 can inhibit NF-κB expression. Further validation with p-38 (SB202190), Nrf2 (ML385), and NF-κB (MG382) inhibitors confirmed PLS significantly inhibited gluconeogenesis and improved fatty acid oxidation, effects abolished by inhibitors (Figure 9), confirming PLS acts through the p-38/Nrf2/NF-κB signaling pathway.

Discussion

Due to aging, urbanization, and societal development, the prevalence of non-communicable chronic diseases like diabetes, metabolic syndrome, and cardiovascular disease has risen [23]. Glycolipid metabolism disorders are major contributors to these diseases [24]. In this study, proanthocyanidins were extracted, isolated, and identified from lotus seed skin, a byproduct of lotus seeds. Using a high-fat mouse model, we investigated the impact of lotus seed skin extract (PLS) containing proanthocyanidins on glycolipid metabolism disorders. Continuous high-fat diet for 8 weeks induced obesity and hyperlipidemia in mice. However, mice fed PLS showed weight loss, possibly due to reduced food efficiency. Despite their medicinal and nutritional value, lotus seed skin are often discarded. Yet, lotus seed skin is rich in proanthocyanidins.
with various bioactive functions. They can improve metabolic health in obese mice by modulating gut microbiota and attenuate LPS-induced inflammatory responses in RAW264.7 cells by regulating NF-κB, MAPK, and Nrf2/HO-1 pathways [25].

Long-term high-fat intake increases body fat content and serum TC, TG, and LDL-C levels, leading to dyslipidemia, insulin resistance, reduced insulin sensitivity, and elevated blood glucose levels [26–28]. The liver, crucial for lipid metabolism, plays a key role in maintaining lipid balance [29]. In this study, intervention with PLS significantly reduced serum and liver TG and TC levels in mice, and it can normalize blood glucose and insulin secretion. Proanthocyanidins have been shown to enhance insulin sensitivity and maintain glucose homeostasis [30,31], which is consistent with our experimental findings.

Hyperlipidemia is associated with elevated inflammatory factors [32]. PLS consumption reduced the expression of proinflammatory cytokines, protecting against damage from a high-fat diet. Development of glycolipid metabolism disorders is linked to oxidative stress, which damages lipids, proteins, and DNA [33,34]. Nrf2 coordinates cellular defense mechanisms against oxidative stress, controlling gene expression through antioxidant response elements [35]. PLS and PB1 induce antioxidant protein expression in mouse livers by activating the Nrf2 pathway, indicating significant antioxidant effects. Li et al. [36] reported that procyanidin A from litchi pericarp improves glucose homeostasis in diabetic ICR mice by regulating mTOR signaling and oxidative stress. Ding et al. [37], using a combination of low-dose streptozotocin and a high-carbohydrate/high-fat diet to induce diabetes in rats, discovered that grape seed proanthocyanidin extract may aid in the treatment of type 2 diabetes by reducing oxidative stress and endoplasmic reticulum stress in skeletal muscle. These findings suggest that proanthocyanidins may regulate glycolipid metabolism disorders by alleviating oxidative stress damage.

MAPK activation involves phosphorylation of p-38, MEK, and JNK, while PI3K/AKT and PKC activation phosphorylate AKT and PKC. Oxidative stress activates MAPK, PI3K/AKT, and PKC, influencing Nrf2 activation [38-40]. These pathways are critical in glucose homeostasis, often implicated in obesity and diabetes [41]. Using kinase inhibitors, we found PB1 may regulate Nrf2 via p38 phosphorylation. In grapes, p-38 activates Nrf2 through the PI3K/Akt pathway [42], while proanthocyanidin A2 activates Nrf2 via JNK and P38 phosphorylation [43].

Lipopolysaccharide (LPS) triggers inflammation in macrophages via Toll-like receptor 4, activating NF-κB and its upstream regulator [44, 45]. This inflammation can disrupt glucose and lipid metabolism [46]. Nrf2 activation, leading to increased HO-1 expression, inhibits NF-κB, reducing intestinal mucosal injury and enhancing tight junction function [47]. In our study, PB1 inhibited the expression of the downstream protein NF-κB in the Nrf2 signaling pathway, consistent with previous findings regarding the detection of proinflammatory factors. The results from treating CFA-induced HepG2 cells with inhibitors further supported the idea that PLS may improve abnormal glycolipid metabolism through the p-38/Nrf2/NF-κB signaling pathway.
In this study, proanthocyanidins were extracted, isolated, and identified from lotus seed skin, a byproduct of lotus seeds. Various parameters in the liver and serum of mice fed a high-fat diet (HFD) were measured, revealing significant regulatory effects of lotus seed skin proanthocyanidins on lipid and glucose metabolism. Molecular evidence indicates that proanthocyanidins can serve as redox balance regulators, ameliorating glycolipid metabolism disorders through the p38/Nrf2/NF-κB signaling pathway.

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**Conflicts of Interest**

The authors declare that they have no conflict of interest.
References


41. Schultze, S.M., et al., PI3K/AKT, MAPK and AMPK signalling: protein kinases


Figure Legends

Figure 1. Separation and identification of proanthocyanidins in lotus seed skin extract by reverse phase high-performance liquid chromatography (RP-HPLC)

Figure 2. Effect of PLS on physiological indexes of mouse body weight, including initial weight, final weight and weight gain. Compared with that in the STD group, the final weight in the HFD group was significantly greater; *P<0.05. (B) Food intake. There were no statistically significant differences in food intake among the five experimental groups. (C) Relative weight gain and relative intake. *P<0.05 vs STD mice, #P<0.05 vs HFD-fed mice. (D) Food efficiency. A high concentration of PLS significantly enhanced the food efficiency of the high-fat diet-fed mice, *P<0.05 vs HFD-fed mice.

Figure 3. Effect of PLS on the serum glucose metabolism index in mice. (A) Serum glucose; (B) serum insulin; (C) HbA1c; (D) IPGTT in mice. PLS significantly reduced the blood glucose-related parameters in high-fat diet-fed mice. *P<0.05; **P<0.01
vs HFD-fed mice.

**Figure 4. Effect of PLS on the lipid metabolism indexes of (A) liver TG, (B) liver TC, (C) serum TG, (D) serum TC, (E) serum HDL-C, and (F) serum LDL-C in mice**  
PLS significantly reduced the blood lipid-related parameters in high-fat diet-fed mice. *P < 0.05; **P < 0.01 vs HFD-fed mice.  
(G) Hematoxylin and eosin (H&E) staining of mouse liver tissue sections. Hematoxylin and eosin (H&E) staining revealed hepatocellular damage in high-fat diet-fed mice.

**Figure 5. Effects of PLS on inflammatory cytokines in the serum of mice**  
(A) IL-1α; (B) IL-1β; (C) IL-3; (D) IL-6; (E) IFN-γ; (F) TNF-α. PLS significantly decreased the levels of inflammatory factors in high-fat diet-fed mice. *P < 0.05; **P < 0.01 vs HFD-fed mice.

**Figure 6. Effects of PLS and PB1 on the expression of liver antioxidant proteins in time course**  
The proteins were detected by Western blotting. The fold change in the protein concentration was calculated as the intensity of the treatment relative to that of the control and was normalized to that of ACTIN by densitometry. *P < 0.05, **P < 0.01, PB1 (48 h), PB1 (72 h) vs Control; #P < 0.05, ##P < 0.01, PLS (48 h), PLS (72 h) vs Control.

**Figure 7. Effect of PB1 on protein kinases upstream of Nrf2 signaling pathway**  
(A) The regulatory effect of PLS on Nrf2 under different inhibitor conditions. *P < 0.05 vs Blank. (B) Effect of PB1 on the phosphorylation of the protein kinases MAPK, AKT and PKC. *P < 0.05, **P < 0.01 vs PB1 (0 µM). The proteins were detected by Western blotting. The fold change in the protein concentration was calculated as the intensity of the treatment relative to that of the control and was normalized to that of GAPDH or ACTIN by densitometry.

**Figure 8. Effect of PB1 on protein kinases downstream of Nrf2 signaling pathway**  
(A) N-p65; (B) IκB-α; (C) p-c-Jun. The proteins were detected by Western blotting. The housekeeping genes selected were lamin B, α-tubulin, and β-actin. *P < 0.05 vs PB1 (0 µM) + LPS (0 ng/mL); #P < 0.05 vs PB1 (0 µM) + LPS (40 ng/mL).

**Figure 9. Effects of different inhibitors on the expression of glycolipid metabolism in CFA-induced cells**  
(A) PEPCK; (B) CPT1A. The proteins were detected by Western blotting. The housekeeping gene selected was β-actin. *P < 0.05 vs PB1 (0 µM) + CFA; *P < 0.05, ##P < 0.01 vs PB1 (20 µM) + CFA.
**Highlights**

Lotus seed skin has been reported to contain abundant flavonoids, especially proanthocyanidin extract (PLS) with high content of proanthocyanidin B1 (PB1). Previously, we found that lotus seed skin extract exhibited potent antioxidant property and allevated metabolic dysfunction caused by hyperglycemia or hyperlipaemia, but the underlying mechanism remained unclear. Here, more investigations were performed to reveal the exact molecular mechanism caused by PB1 under the action of PLS on metabolic dysfunction regulation.

- PLS improves blood lipid parameters and inhibits six proinflammatory factors production.
- PLS and its major constitute PB1 exert their intervening effects by activating p38-Nrf2 pathway and inhibiting NF-κB signaling.
- We offer a new approach for the high-value comprehensive utilization of lotus seed skin and precise dietary intervention for metabolic syndrome.
70x43mm (300 x 300 DPI)
A  

![Graph A](image1.png)

B  

![Graph B](image2.png)

C  

![Graph C](image3.png)

D  

![Graph D](image4.png)

140x167mm (300 x 300 DPI)
160x112mm (300 x 300 DPI)
A

PB1 (20 μM) - + + + + +

Nrf2
GAPDH

B

Treatment time: 2h

PB1 (μM)  0  10  20  40

p-p38
p-MEK
p-JNK
p-AKT
p-PKC
β-actin

140x166mm (300 x 300 DPI)
Lotus seed skin
Extract
High fat diet (C57BL/6J)
Lotus seed skin extract
HPLC

Body weight ↓
Food efficiency ↑
Serum glucose, insulin ↓
HbA1c, IPGTT ↓
Serum TG ↓
Serum proinflammatory factors ↓
Liver TG, TC ↓
Antioxidant proteins (Nrf2, NQO1, HO-1) ↑

P38 inhibitor — Nrf2 ↓
PB1 — NF-κB ↓
p-38, nrf2, Nf-κB inhibitor — PEPCK ↑
CPT1A ↓

99x71mm (300 x 300 DPI)