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ORIGINAL ARTICLE

Dehulled Adlay (*Coix lachryma-jobi L.*) ameliorates hepatic gluconeogenesis and steatosis in streptozotocin/high-fat diet-induced diabetic rats

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Abstract

Adlay (*Coix lachryma-jobi L.*) is a traditional Chinese herbal medicine with various biological activities. We investigated the anti-diabetic effects of different parts of adlay seeds, including polished adlay (PA), adlay bran (AB) and dehulled adlay (DA) in a streptozotocin (STZ)/high fat diet (HFD) diabetic rat model (DM). DM rats supplemented with or without PA (43%), AB (3%), or DA (46%) diet for 8 weeks. The plasma glucose and insulin levels and the insulin resistance index (HOMA-IR) were increased in DM group; among the three adlay diets, DA has the best effects attenuating all of these alterations in DM rats. Both AB and DA alleviated diabetes-impaired glucose tolerance. The increased hepatic phosphoenolpyruvate carboxykinase protein expression in DM group was improved by all of the three adlay diets. The increased ratio of glucose-6-phosphatase to glucokinase in DM group was suppressed by DA supplementation, further suggesting DA diet is most effective among the three diets. Both AB and DA diets had beneficial effects against hepatic steatosis, with better effects observed in DA group. These results suggest that the DA diet, composed of both polished adlay and adlay bran, possesses the best potential to improve glucose homeostasis, at least in part, by alleviating hepatic glucose metabolism and steatosis.

Keywords: Dehulled adlay, Diabetes mellitus, Gluconeogenesis, Hepatic steatosis

1. Introduction

The prevalence of both obesity and diabetes mellitus (DM) has been increasing rapidly across the world, considering them as major public health concerns and economic burden on health care systems [1,2]. Excess body adiposity, assessed by a high body-mass index (BMI), is the single strongest predictor for the risk of type 2 DM (T2DM) [3]. Obesity can lead to insulin resistance and hyperglycemia, which are the main characteristics of DM. In T2DM, insulin resistance contributes to the increase of hepatic glucose production (HGP) and the decrease of glucose uptake in liver, muscle and adipose tissues. Additionally, β-cell dysfunction in the pancreas leads to the reduction of insulin release, which is then insufficient to maintain the normal glucose levels [4,5].

According to the World Health Organization, over 1.9 billion adults worldwide are overweight or obese in 2016 [6]. In 2019, the International Diabetes Federation estimated that DM affects approximately 463 million populations [7]. T2DM accounts for more than 95% of all cases of DM in 2023 [8]. The prevention and management of obesity and diabetes require a multifaceted approach that involves more than just medical treatment; encouraging healthy lifestyles, such as healthy eating habits and regular physical activities, is essential in tackling these global health challenges [4,9]. In recent years,

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supplementation of herbal products in daily life to aid in the management of obesity and DM has become an increasingly popular trend [10].

Adlay (*Coix lacryma-jobi L.*), also named Jobs tears or Chinese pearl barley, is a traditional and widely-cultivated crop used for both medicinal and dietary purposes in Asia [11]. A number of studies have demonstrated that adlay seed possesses a variety of health benefits, including anti-cancer potential [12], anti-inflammatory properties [13], and antioxidant capabilities [14]. It has also been shown to have lipid-lowering abilities, insulin-sensitizing activity and hypoglycemic effects [14–22]. The adlay seed can be divided into three parts, namely adlay hull, adlay testa, and dehulled adlay (DA) sequencing from outside to inside. The DA can be further classified into adlay bran (AB) and polished adlay (PA) [12].

Although the beneficial effects of adlay seeds on lipid and glucose metabolism have been established, which part of adlay seeds is most effective remains inconclusive. The present study aimed to investigate the anti-diabetic effects of three different parts of the adlay seed, referred to as PA, AB, and DA. We used low-dose streptozotocin (STZ) combined with high fat diet (HFD) to induce T2DM, which is a commonly-used T2DM model with typical symptoms of insulin resistance, hyperglycemia and altered lipid profile, and is suitable for evaluating the antidiabetic potential of test compound [23,24]. As Such, these three parts of adlay seeds were incorporated into the HFD and administered to diabetic rats for a duration of 8 weeks to assess their effects on hyperglycemia, dyslipidemia, hepatic glucose metabolism and hepatic steatosis.

2. Materials and methods

2.1. Preparation of experimental diets

The Taichung Shuenyu No. 4 (TCS4) species of adlay (*Coix lachryma-jobi L.* var. ma-yuen Stapf), kindly provided by Dr. Wenchang Chiang (Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan), was used in the present study. The adlay powders were prepared as mentioned previously [25]. In brief, adlay seeds were air-dried at 40 °C and dehulled in a grinder to obtain dehulled adlay (DA), which were further separated to obtain adlay bran (AB) and polished adlay (PA). These acquired materials were then grounded into powders and sieved through a 20-mesh screen with the aperture of 0.94 mm. Table 1 listed the composition of experimental diets. The DA is composed of PA and AB. The PA, AB, and DA diet contained 43% PA, 3% AB, and 46% DA, respectively, to make those experimental diets with equal calories of carbohydrate (58.5% kcal), protein (19% kcal) and fat (22.5% kcal). The normal control (NC) diet had calories of 394.2 kcal/100 g, and the other experiments diets, considered as high fat diets (HFD), contained 0.5% cholesterol and had 420.9 kcal/100 g. According to the analysis by Huang and Chiang (1999) on compositions of different fractions of adlay seed, the amounts of moisture, crude protein, crude fat, ash, total carbohydrate, total dietary fiber, and coixenolide in DA, AB, and PA were [mean (SD)] 9.1 (0.4), 10.0 (0.2), and 10.6 (0.4)% (moisture), 21.3 (0.2), 18.5 (0.2), and 21.9 (0.2)% (protein), 8.3 (0.3), 24.4 (0.1), and 2.9 (0.2)% (fat), 2.1 (0.1), 7.0 (0.0), and 1.9 (0.1)% (ash), 68.3 (0.2), 50.1 (0.1), and 72.9 (0.3)% (carbohydrate), 10.6 (0.7), 15.3 (3.8), and 5.8 (1.0)% (dietary fiber), and 146 (4), 473 (10), and 30 (2) ppm (coixenolide), respectively [25].

Table 1. The composition of experimental diets.

Ingredient (%)	NC	DM	PA	AB	DA
Corn starch	64.8	59.1	28.8	58	30.1
Casein	20	20	10.9	19	11.0
Cellulose	5	5	2.6	4.6	0.5
Mineral[a]	4	4	4	4	4
Vitamin[b]	1	1	1	1	1
Lard	3	8	6.8	7.3	4.5
Soybean oil	2	2	2	2	2
Cholesterol	–	0.5	0.5	0.5	0.5
Cholic acid	–	0.2	0.2	0.2	0.2
Choline chloride	0.2	0.2	0.2	0.2	0.2
Polished adlay	–	–	43	–	–
Adlay bran	–	–	–	3	–
Dehulled adlay	–	–	–	–	46
Total calories (kcal/100 g)	394.2	420.9	420.9	420.9	420.9
Carbohydrate (%)	68.29	58.54	58.54	58.54	58.54
Protein (%)	20.29	19.01	19.01	19.01	19.01
Fat (%)	11.42	22.45	22.45	22.45	22.45
Total (% kcal)	100	100	100	100	100

NC: Normal control diet; DM: diabetic rats on high fat diet; PA: diabetic rats on high fat diet with polished adlay; AB: diabetic rats on high fat diet with adlay bran; DA: diabetic rats on high fat diet with dehulled adlay.

[a] AIN-93 vitamin mixture.

[b] AIN-93 mineral mixture.

2.2. Animals and experimental design

All experiments were approved by the Animal House Management Committee of the National Taiwan Ocean University (protocol number: 110020), and all animal procedures were carried out in accordance with the ARRIVE guidelines for care and use of laboratory animals. Wistar rats at age of

6 weeks were purchased from BioLASCO Taiwan Corporation, and housed in a temperature-controlled room at 22 ± 2 °C, with relative humidity of 40%−60%, and a 12 h light/dark cycle. Food (LabDiet 5001, PMI Nutrition International, St. Louis, MO, USA) and water were provided ad libitum. After 2-week acclimatization, rats were subcutaneously given streptozotocin (STZ) at a dose of 50 mg/kg BW dissolved in 0.1 M citrate buffer (pH 4.5). In order to increase the successful rate of the induction of diabetes, second dose of STZ was given one week after the first injection. Blood glucose levels were measured one week after the second dose of STZ to ensure diabetes was successfully induced. Each animal with a blood glucose greater than 200 mg/dL was considered to be diabetic. These diabetic rats were randomly assigned into 4 groups with 7 rats in each group, including diabetic rats fed with HFD (DM), PA diet (PA), AB diet (AB), and DA diet (DA). The control group was Wistar rats received vehicle subcutaneously twice and fed with normal control diet (NC). The body weight was recorded weekly, while food consumption and water intake were monitored 3 times per week on alternate weeks. The feed efficiency ratio was calculated by applying the following equation: Feed efficiency (%) = [body weight gain (g)/food intake (g/day)] ×100%. Three days before sacrifice, rats were placed in metabolic cages to collect urine. After 8-week diet intervention, rats were fasted for 12 h and all the animals were euthanized under carbon dioxide (CO_2: O_2 = 7:3). The blood samples were withdrawn from the abdominal aorta into heparin-coated tubes and centrifuged to obtain plasma, which was stored at −80 °C for use. The livers were harvested, weighted, and stored in 4% paraformaldehyde for histological staining or at −80 °C for further experiments.

2.3. Determination of plasma glucose and oral glucose tolerance test (OGTT)

Blood sampling from retro-orbital sinus was used for measuring the fasting glucose levels, which were detected by using an Accu-Chek® blood glucose meter (Roche Diagnostics, Rotkreuz, Switzerland). The OGTT was carried out by oral gavage of a glucose load of 1.5 g/kg BW to the animals after 7-week diet intervention. Plasma glucose levels were measured before (baseline) and after the glucose administration (30, 60, 120, and 180 min). The area under curve (AUC) of OGTT was then calculated using Prism 6 (GraphPad Software, La Jolla, CA, USA) as a measure of glucose tolerance.

2.4. Measurement of plasma insulin and the homeostasis model assessment of insulin resistance (HOMA-IR) index

The determination of fasting plasma insulin was using rat insulin ELISA kit (Mercodia AB Inc., Uppsala, Sweden). The HOMA-IR index was calculated using the concentrations of fasting insulin and glucose, per the following formula: HOMA-IR = [fasting insulin (μU/mL) × fasting glucose (mmol/L)]/22.5.

2.5. Biochemical analysis

The plasma contents of triglyceride (TG) and total cholesterol (TC) were determined by enzymatic colorimetric methods (Audit Diagnostics, Cork, Ireland). The plasma levels of AST and ALT were analyzed using AST and ALT assay kit, respectively (Randox, Antrium, UK). All the tests were conducted following the instructions of the manufacturers.

2.6. Measurement of hepatic enzyme activities

The liver lysate was prepared and the enzyme activities were conducted as previously described [26]. Briefly, liver tissues were homogenized in N-acetyl-cysteine buffer (pH 7.0) under an ice bath, and then centrifuged to obtain the cytosol fraction. To measure the glucokinase (GK) activity, the liver cytosol extract was added to an assay solution containing of 0.25 M glycylglycine buffer (pH 7.5), 0.75 M $MgSO_4$, 7.5 mM NADP, 0.75 M glucose, 1 M KCl, 0.75 M ATP, and 7 U/mL glucose-6-phosphate dehydrogenase. The reaction converted glucose to glucose-6-phosphate (G6P) and reduced NADP to NADPH. Absorbance was determined at 340 nm and the GK activity was expressed as nmol NADPH/min/mg protein.

The glucocse-6-phosphatase (G6Pase) activity was indirectly measured the production of inorganic phosphorus (Pi), a byproduct of the reaction converting G6P to glucose. The liver cytosol extract was combined with a solution comprised of 0.05 M Tris-maleate buffer (pH 7.5) and 0.1 M G6P. After reaction, the generated Pi was detected using a micro-colorimetric method as previously described [27]. The resulting reaction solution was mixed with 5% trichloroacetic acid, followed by centrifugation to obtain the supernatants. These supernatants were then incubated with 5 N H_2SO_4, 2.5% ammonium molybdate, and 10% ascorbic acid for further reaction. Subsequently, absorbance was analyzed at 660 nm by a microplate reader. Ultimately, the

G6Pase activity was presented as nmol Pi/min/mg protein.

2.7. Western blotting

Protein was extracted from liver samples using radioimmunoprecipitation lysis buffer. The lysates (30 μg total protein) were electrophoresed on 10% Tris-Glycine sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% skim milk in 1x Tris-buffered saline with Tween (TBST) at room temperature for 1 h, and then incubated at 4 °C overnight with primary antibodies against phosphoenolpyruvate carboxykinase (PEPCK, 1:1000 v/v dilution, sc-32879) and glyceraldehude-3-phosphate dehydrogenase (GAPDH, 1:5000 v/v dilution, sc-47724, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 v/v dilution, Cell signaling Technology) at room temperature for 1 h, and then probed with enhanced chemiluminescence detection reagent (BioRad Laboratories Inc., Redmond, WA). The signal was detected using X-ray films (Fujifilm Co., Tokyo, Japan). Densitometric analysis was performed using ImageJ software (National Institutes of Health, USA) on scanned films from at least three independent experiments. The intensity of all protein bands was normalized to their GAPDH and expressed as arbitrary units.

2.8. Determination of hepatic lipid content

Extraction and isolation of triglyceride (TG) and total cholesterol (TC) from livers were determined as previously described [28]. Briefly, liver tissues were homogenized in 2:1 v/v chloroform-methanol, and then centrifuged to obtain the total lipids. The lipids were then mixed with Triton X-100, and treated by vacuum concentration (SC 110, Savant Instruments Inc., Woonsocket, USA) to remove solvents. Hepatic TG and TC content were then determined by using enzymatic colorimetric kits, respectively (Audit Diagnostics, Cork, Ireland).

2.9. Histological analysis of liver

Fresh harvested liver tissues were fixed in 10% formalin, embedded in paraffin, and sliced into 4 μm thick sections, which were used for hematoxylin and eosin (H&E) staining. Histopathological alterations in liver tissues were assessed in 5 random microscopic fields at × 400 magnification using a light microscope (U-LH100HG, BX53, Olympus Corporation, Tokyo, Japan), and quantified by using Image-Pro Plus 6.0 (Media Cybernetics, Inc. Rockville, MD, USA).

2.10. Statistical analysis

All values were presented as means ± SD. The statistical analysis was performed by one-way analysis of variance (ANOVA) to compare among groups through Duncan's multiple-range test (SPSS statistical software package, version 17.0, SPSS, Chicago, IL, USA). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. The effects of three adlay diets on body weight, food intake, and water consumption in STZ/HFD-induced diabetic rats

As expected, STZ-injected diabetic rats fed with HFD had increased body weight gradually, but the increase was less than that in NC rats (Table 2).

Table 2. Effects of adlay diets on the body weight, food intake, water intake, and urine volume in rats fed with different experimental diets for 8 weeks.

Diet	NC	DM	PA	AB	DA
Initial BW (g)	379 ± 14	366.2 ± 25.5	373.9 ± 26.5	373.4 ± 14.4	379.5 ± 14.8
Final BW (g)	549.1 ± 19.0 ^{a}	425.4 ± 17.6 ^{b}	433.6 ± 64.9 ^{b}	435.5 ± 15.7 ^{b}	458.0 ± 80.2 ^{b}
BW gain (g)	170.1 ± 19.6 ^{a}	59.2 ± 28.5 ^{b}	59.7 ± 78.0 ^{b}	62.1 ± 27.2 ^{b}	78.5 ± 85.3 ^{b}
Food intake (g/day)	27.0 ± 2.1 ^{c}	45.3 ± 2.1 ^{a}	41.1 ± 4.5 ^{ab}	44.1 ± 3.6 ^{ab}	40.1 ± 7.1 ^{b}
Energy intake (Kcal/day)	106.4 ± 8.2 ^{a}	190.8 ± 9.0 ^{b}	172.8 ± 18.9 ^{b}	185.8 ± 15.0 ^{b}	168.8 ± 29.9 ^{b}
Feed efficiency (%)	6.31 ± 0.71 ^{a}	1.31 ± 0.64 ^{b}	1.40 ± 1.79 ^{b}	1.43 ± 0.66 ^{b}	2.32 ± 3.25 ^{b}
Water intake (mL/day)	49.2 ± 18.4 ^{b}	250 ± 14 ^{a}	237.2 ± 51.8 ^{a}	244.0 ± 15.8 ^{a}	212.8 ± 86.0 ^{a}
Urine volume (mL/day)	28.6 ± 16.6 ^{b}	191.7 ± 17.6 ^{a}	178.5 ± 37.3 ^{a}	184.0 ± 14.1 ^{a}	161.1 ± 67.4 ^{a}

Results are expressed as mean ± S.D. for each group (n = 7). Significant difference was determined by one-way ANOVA followed by Duncan's multiple range test. Values with different superscript letters mean significant differences with each other ($p < 0.05$). NC: Normal control diet; DM: diabetic rats on high fat diet; PA: diabetic rats on high fat diet with polished adlay; AB: diabetic rats on high fat diet with adlay bran; DA: diabetic rats on high fat diet with dehulled adlay: BW, body weight.

After 8-week diet intervention, the body weight gain was less in all diabetic rats fed with or without adlay diets, compared to NC rats fed with NC diet (170.1 ± 19.6 g in NC group v.s. 59.2 ± 28.5 g in DM, 59.7 ± 78.0 g in PA, 59.7 ± 78.0 g in AB and 78.5 ± 85.3 g in DA group; Table 2). The diabetic rats had greater food intake when compared to NC rats (45.3 ± 2.1 g/day in DM v.s. 27.0 ± 2.1 g/day in NC). Only DA diet showed slight but significantly inhibitory effect on food intake (40.1 ± 7.1 g/day). However, there was no significant difference in feed efficiency between the DM and DA group (1.31 ± 0.64% v.s. 2.32 ± 3.25%, Table 2). Nevertheless, the increased volume of drinking water and urine in DM group reflected the typical phenotype of diabetes, with no notable effects of adlay diets on these two parameters (Table 2).

3.2. The effects of three adlay diets on blood glucose, insulin, and lipid profile in STZ/HFD-induced diabetic rats

Additionally, the DM group had greater fasting glucose and insulin levels and HOMA-IR value than the NC group, indicating a successful induction of diabetes (Table 3). Diabetic rats fed with PA and AB diets showed slight but not significant effects on fasting glucose and insulin levels, whereas the DA group had significant lower fasting glucoses and insulin levels compared to the DM group (Table 3). The values of HOMO-IR, calculated from fasting glucose and insulin levels, showed a significant decrease in all three adlay diet groups, suggesting the insulin resistance might be attenuated by supplementation of the three different parts of adlay (Table 3). In order to further evaluating the ability of the various adlay diets on insulin secretion and action after a meal, the OGTT was performed. During the 180 min of test period, the glucose levels of DM group stayed at the plateau, revealing a characteristic of insulin resistance (Fig. 1A). The area under the curve (AUC) calculated form OGTT showed that supplementation of AB and DA diet is capable of attenuating the increase of AUG, whereas PA supplementation had no effect (Fig. 1B). Moreover, the DM rats exhibited significantly higher blood TC levels compared with rats in the NC group, but the levels were no difference among DM rats in the presence or absence of the three adlay diets (Table 3). The blood TG content was significantly decreased in AB group and DA group compared with NC group and DM group, respectively; although there was no significant difference between NC and DM groups (Table 3).

3.3. The effects of three adlay diets on hepatic glucose metabolism in STZ/HFD-induced diabetic rats

Given that liver plays a pivotal role on regulating glucose homeostasis, we next investigate whether the three different adlay diets has different effects on hepatic glucose metabolism. Glucokinase (GK) plays a crucial role in hepatic glucose oxidation (glycolysis), and glucose-6-phostatase (G6Pase) is a key enzyme of gluconeogenesis [29]. Therefore, the ratio of G6Pase to GK roughly reflects the hepatic glucose production (HGP) [30]. First of all, we observed that the GK activity was decreased by 26% in DM group, but the decrease was not statistically significant (Table 4). The three adlay diets had no effects on GK activity. G6Pase was significantly activated in DM group, which was suppressed by supplementation of AB and DA, but not PA. The ratio of G6Pase to GK was greater in DM group, and only DA supplementation had the inhibitory effect (0.42 ± 0.20 in DM group v.s. 0.13 ± 0.06 in DA group), suggesting that DA diet is the most effective, among the three adlay diets, to attenuate the HGP.

Phosphoenolpyruvate carboxykinase (PEPCK) is also the rate-limiting enzymes for hepatic gluconeogenesis, in addition to G6Pase [29]. We then determined the protein expression of hepatic PEPCK. The DM rats exhibited increased protein expression levels of PEPCK as compared with NC

Table 3. Plasma levels of glucose, insulin and lipids, as well as insulin resistance index (HOMA-IR) in rats fed with different experimental diets for 8 weeks.

Diet	NC	DM	PA	AB	DA
Glucose (mg/dl)	194.4 ± 22.4 ^{c}	344.7 ± 20.2 ^{a}	341.6 ± 42.8 ^{a}	309.2 ± 33.7 ^{ab}	295.6 ± 59.7 ^{b}
Insulin (μg/L)	0.48 ± 0.17 ^{b}	1.36 ± 0.61 ^{a}	0.89 ± 0.80 ^{ab}	0.89 ± 0.27 ^{ab}	0.67 ± 0.19 ^{b}
HOMA-IR	5.9 ± 2.5 ^{c}	29.0 ± 12.7 ^{a}	17.1 ± 11.5 ^{b}	16.7 ± 4.4 ^{b}	11.9 ± 3.2 ^{bc}
TG (mg/dl)	127.7 ± 29.7 ^{a}	95.4 ± 27.7 ^{ab}	93.8 ± 27.7 ^{ab}	64.8 ± 15.9 ^{c}	65.2 ± 11.4 ^{c}
TC (mg/dl)	81.2 ± 9.8 ^{b}	221.6 ± 70.0 ^{a}	257.6 ± 49.2 ^{a}	218.9 ± 82.1 ^{a}	228.5 ± 77.2 ^{a}

Results are expressed as mean ± S.D. for each group (n = 7). Significant difference was determined by one-way ANOVA followed by Duncan's multiple range test. Values with different superscript letters mean statistical significance with each other ($p < 0.05$). NC: Normal control diet; DM: diabetic rats on high fat diet; PA: diabetic rats on high fat diet with polished adlay; AB: diabetic rats on high fat diet with adlay bran; DA: diabetic rats on high fat diet with dehulled adlay: TG, triglyceride; TC, total cholesterol.

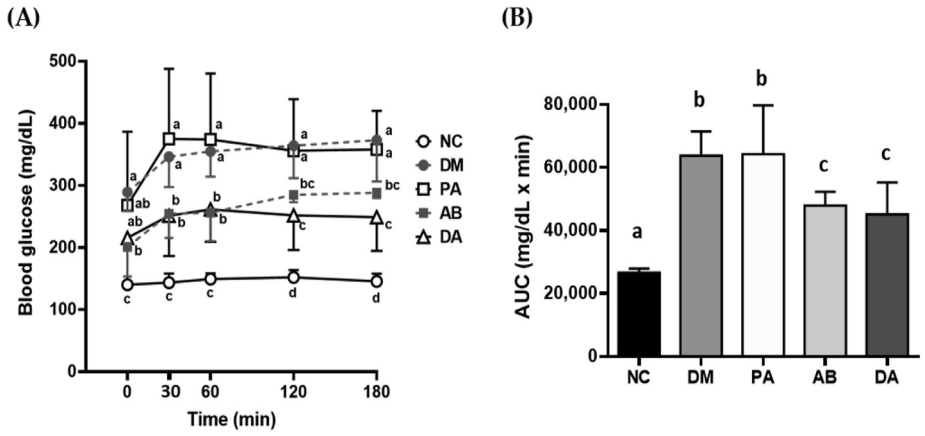

Fig. 1. Effects of different adlay diets on blood glucose levels and area under curve (AUC) during OGTT. Wistar rats received the normal diet for 7 weeks were control group (NC). STZ-induced diabetic rats received the high fat diet, and high fat diet blended with polished adlay, adlay bran and dehulled adlay powders were group DM, PA, AB and DA, respectively. After fasting for 12 h, rats were subjected to the oral glucose tolerance test (OGTT). Blood was sampled and examined at 0 (baseline), 30, 60, 120, and 180 min for glucose levels. (A). AUC was then calculated (B). Results are expressed as the mean ± S.D., n = 7. Values with different superscript letters mean significantly differences (p < 0.05) by one-way ANOVA post hoc Duncan's multiple range test. NC: Normal control diet; DM: diabetic rats on high fat diet; PA: diabetic rats on high fat diet with polished adlay; AB: diabetic rats on high fat diet with adlay bran; DA: diabetic rats on high fat diet with dehulled adlay.

Table 4. The hepatic carbohydrate enzymes in rats fed with different experimental diets for 8 weeks.

Diet	NC	DM	PA	AB	DA
GK (nmol NADPH/min/mg protein)	5.20 ± 2.07 ^{a}	3.84 ± 0.80 ^{a}	2.85 ± 1.72 ^{a}	4.30 ± 1.89 ^{a}	5.00 ± 1.35 ^{a}
G6Pase (nmol Pi/min/mg protein)	0.43 ± 0.11 ^{b}	1.56 ± 0.60 ^{a}	1.05 ± 0.24 ^{ac}	0.94 ± 0.26 ^{bc}	0.64 ± 0.29 ^{bc}
G6Pase/GK ratio	0.09 ± 0.04 ^{b}	0.42 ± 0.20 ^{a}	0.31 ± 0.10 ^{ac}	0.25 ± 0.12 ^{ab}	0.13 ± 0.06 ^{bc}

Results are expressed as mean ± S.D. for each group (n = 7). Significant difference was determined by one-way ANOVA followed by Duncan's multiple range test. Values with different superscript letters mean statistical significance with each other ($p < 0.05$). NC: Normal control diet; DM: diabetic rats on high fat diet; PA: diabetic rats on high fat diet with polished adlay; AB: diabetic rats on high fat diet with adlay bran; DA: diabetic rats on high fat diet with dehulled adlay; GK, glucokinase; G6Pase; glucose-6-phosphatase; Pi, inorganic phosphate.

rats; whereas the increase was reduced in the presence of all three adlay diets (Fig. 2). Notably, the PEPCK protein expression was even less in the AB and DA group than in the NC group.

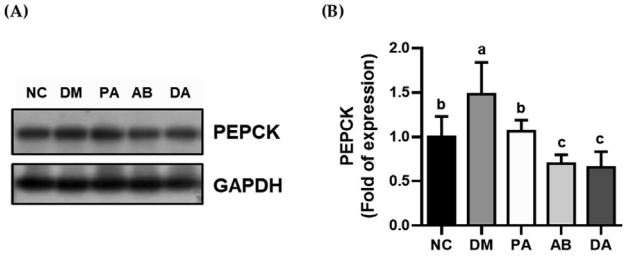

Fig. 2. The change of hepatic PEPCK protein expression in rats fed with different experimental diets for 8 weeks. Protein expression of PEPCK was measured by Western blotting. Densitometric analysis for protein levels corrected to each internal control was shown. Results are expressed as mean ± S.D. for each group (n = 5). Mean values with different superscript letters are significantly different from one-way ANOVA followed by Duncan's multiple range test (p < 0.05). NC: Normal control diet; DM: diabetic rats on high fat diet; PA: diabetic rats on high fat diet with polished adlay; AB: diabetic rats on high fat diet with adlay bran; DA: diabetic rats on high fat diet with dehulled adlay.

3.4. The effects of three adlay diets on hepatic steatosis in STZ/HFD-induced diabetic rats

It's known that hepatic fat accumulation is associated with the impairment of insulin-mediated suppression of HGP and the decline of hepatic insulin clearance [31], we then explore the effects of the three different adlay diets on hepatic lipid profiles. As shown in Table 5, the concentrations of TG and TC in the livers were significantly increased in DM group (TG: 22.6 ± 5.87 mg/g liver in DM group v.s. 4.23 ± 2.29 mg/g liver in NC group; TC: 44.9 ± 8.91 mg/g liver in DM group v.s. 2.52 ± 0.99 mg/g liver in NC group). Supplementation with PA had inhibitory effects on the increased TG content, but had no effect on TC content. Both AB and DA supplementation were capable of reversing the increased TG content and partially suppressing the increased TC content induced by diabetes. Notably, the liver weight and relative liver weight were significantly increased in DM group as well. AB supplementation only inhibited the relative

Table 5. The liver weight and lipid profile in rats fed with different experimental diets for 8 weeks.

Diet	NC	DM	PA	AB	DA
Liver weight (g)	15.5 ± 1.1 ^{d}	32.4 ± 25.5 ^{ab}	33.9 ± 1.7 ^{a}	29.7 ± 3.9 ^{bc}	29.0 ± 2.5 ^{c}
Relative liver weight (g/100 g B.W.)	2.8 ± 0.2 ^{c}	7.6 ± 0.4 ^{a}	7.7 ± 0.5 ^{a}	6.8 ± 0.9 ^{b}	6.4 ± 0.8 ^{b}
TG (mg/g liver)	4.2 ± 2.3 ^{c}	22.6 ± 5.9 ^{a}	15.0 ± 8.8 ^{b}	6.6 ± 2.9 ^{c}	6.4 ± 1.7 ^{c}
TC (mg/g liver)	2.5 ± 1 ^{d}	44.9 ± 8.9 ^{a}	38.2 ± 12.2 ^{a}	26.8 ± 3.0 ^{bc}	20.4 ± 7.6 ^{c}

Results are expressed as mean ± S.D. for each group (n = 7). Significant difference was determined by one-way ANOVA followed by Duncan's multiple range test. Values with different superscript letters mean statistical significance with each other ($p < 0.05$). NC: Normal control diet; DM: diabetic rats on high fat diet; PA: diabetic rats on high fat diet with polished adlay; AB: diabetic rats on high fat diet with adlay bran; DA: diabetic rats on high fat diet with dehulled adlay; TG, triglyceride; TC, total cholesterol.

liver weight, while DA supplementation suppressed both the absolute and relative liver weight increased by diabetes (Table 5).

To further investigate the impacts of adlay diets on diabetes-induced hepatic fat accumulation, histologic analysis was performed. As expected, the liver of DM group exhibited significantly increased fat vacuoles with no infiltration of inflammatory cells, indicating hepatic steatosis (Fig. 3A). However, the hepatic steatosis induced by diabetes was mitigated by both AB and DA supplementation, with better effects observed in the DA group (Fig. 3A and B). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT), the two indicators of hepatic injury, were then examined. AST level was found to be slightly but significantly higher in the DM group than those in the NC group, while ALT remained unaffected compared with the NC group, suggesting a very mild liver dysfunction and indicating the diabetes-induced steatosis did not progress into nonalcoholic steatohepatitis, an aggressive form of fatty liver disease (Fig. 3C and D). All three adlay diets had no effect on the increased AST levels.

4. Discussion

Our results in the present study indicated that supplementation of adlay exerts anti-diabetic potential in the STZ/HFD-induced diabetic rat model.

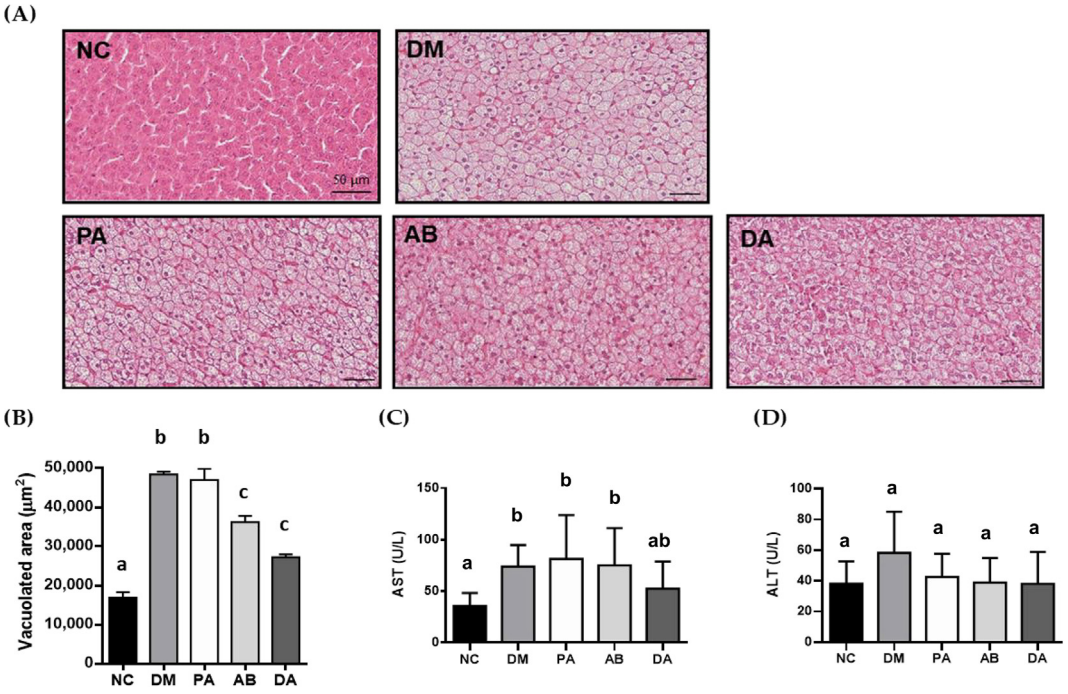

Fig. 3. The change of hepatic morphology and plasma AST and ALT levels in rats fed with different adlay diets after 8 weeks. Representative hematoxylin and eosin stained images of fat vacuoles in liver tissues were shown in (A) and quantified in (B). Scale bar = 50 micrometer. Plasma AST and ALT levels were presented in (C) and (D). Results are expressed as mean ± S.D. for each group (n = 5). Mean values with different superscript letters are significantly different, $p < 0.05$ by one-way ANOVA followed by Duncan's multiple range test. NC: Normal control diet; DM: diabetic rats on high fat diet; PA: diabetic rats on high fat diet with polished adlay; AB: diabetic rats on high fat diet with adlay bran; DA: diabetic rats on high fat diet with dehulled adlay.

Supplementation of DA reduced fasting blood glucose and insulin levels and HOMA-IR values in DM rats; whereas PA or AB only had effects on lowering HOMA-IR index. In OGTT test, supplementation of AB or DA reduced AUC compared with the DM group. These findings suggest that DA diet is the most effective in terms of anti-hyperglycemia among the three compositions of adlay diets. Moreover, we found that supplementation of AB or DA resulted in a significant decrease of hepatic PEPCK protein expression and G6Pase activity in diabetic rats, indicating that the upregulated hepatic gluconeogenesis is suppressed by AB and DA diet supplementation. Notably, the increased G6Pase/GK ratio in diabetic rats was only mitigated by DA diet, further suggesting that the DA diet is the most potent diet to improve hepatic glucose metabolism among the three diets. Thus, it is reasonable to assume that the hypoglycemic effects of DA or AB diet supplementation was mediated by regulating hepatic gluconeogenesis, leading to inhibition of HGP and the subsequent glucose secretion into the bloodstream. Nevertheless, the hepatic lipid accumulation was observed in diabetic rats, and supplementation with all three diets had inhibitory effects on hepatic TG content. The increased hepatic TC content and the relative liver weight were reduced by either AB or DA supplementation. Furthermore, the absolute liver weight was reduced by DA supplementation. These findings also support the notion that the DA diet, among the three adlay diets, has the most effective impact on improving hepatic lipid disorder. Additionally, histological analysis confirmed that both AB and DA diets ameliorated the hepatic steatosis induced by diabetes, with the DA diet being more effective.

The DA diet is considered a high whole-grain diet as it contains 46% w/w dehulled adlay. Accumulating evidence has demonstrated that higher consumption of whole-grain foods is significantly associated with a lower risk of T2DM and improve glycemic control and blood lipids in diabetic patients [9,32]. It has been known that whole grains affect glucose and insulin responses after a meal, partly due to their high amount of dietary fiber to slow the rate of food digestion [33]. Furthermore, whole grains consist of endosperm, bran, and germ. In general, the endosperm is highly rich in starch, the bran is particularly rich in fiber, protein, B-vitamins, minerals, flavonoids and tocopherols [34]. It's known that dehulled adlay retains the bran layer, which is rich in various bioactive compounds such as dietary fiber, phenolics, flavonoids, phytosterols and fatty acids that possess numerous health benefits [13,16,35]. Therefore, these nutrients and phytochemicals from adlay bran may contribute to the positive effects of dehulled adlay in the regulation of lipid and glucose metabolism.

Increasing evidence suggests that adlay bran has hypoglycemic activity. Dietary fiber from adlay bran has also been shown to play a role in reducing blood glucose levels [36]. Adlay bran oil (ABO), derived from pressing the bran, has demonstrated its ability to reduce leptin, adipose tissue, and blood LDL contents in HFD-fed rats [16]. Tseng et al. showed that supplementation of ABO for 4 weeks exhibited improvement in hyperlipidemia and hyperglycemia, as well as hepatic gluconeogenesis in STZ/HFD-induced T2DM rats [20]. Furthermore, various flavonoids and phenolic compounds extracted from adlay brans have been demonstrated to have anti-inflammatory and anti-oxidative potential [37–39], which may be involved in its anti-diabetic effects, as inflammation, and oxidative stress are major contributors to diabetes and obesity [40,41]. Additionally, it is worth noting that nutrients from endosperm of adaly seeds may also be involved in the anti-diabetic effects. Polysaccharides extracted from adlay seeds have exhibited a significant decrease in blood glucose and lipid levels in STZ/HFD-induced diabetic mice, partially through their impact on gut microbiota, mainly by facilitating the growth of bacteria that produce short-chain fatty acid [42,43]. Furthermore, three glycans isolated from adlay seeds, known as Coixans A, B, and C, elicited remarkable hypoglycemic action in alloxan-induced diabetic mice (a type 1 DM model) [17]. Collectively, these findings support the notion that the anti-diabetic effects of DA diet may be attributed to both its bran layer and endosperm.

Indeed, dehulled adlay has been reported to have beneficial impacts on obesity and diabetes. Yeh et al. showed that dehulled alday displayed hypolipidemic and hypoglycemic properties in STZ/HFD-induced diabetic rats [19]. Additionally, polysaccharide derived from dehulled adlay, acted as prebiotics, promoted a healthy gut microbiota and helped maintain intestinal barrier function [44]. Furthermore, dehulled adlay fermented by Bacillus was found to improve lipid profiles, antioxidant status, and gut microflora in HFD-fed hamster [45]. These benefits of dehulled adlay have been observed in humans as well. A study found that daily intake of 60 g of dehulled adlay powder for 6 weeks had a positive effect on blood lipids and chronic inflammation in overweight or obese individuals [46]. Another recent study demonstrated that consuming a meal containing 40–55 g of dehulled adlay powder and resistant starch for 12 weeks improved hyperlipidemia in subjects with

mild to moderate dyslipidemia [47]. Overall, these studies suggest that incorporating dehulled adlay into the daily diet may have benefits for treating obesity and diabetes through multiple regulatory pathways, including suppressing oxidative stress, dampening inflammatory responses, and modulating the gut health. In our current study, we suggest that the hypoglycemic effects of dehulled adlay are through alleviating gluconeogenesis and lipid accumulation in the liver.

Hepatic glucose production (HGP) is responsible for approximately 90% of endogenous glucose production, and is crucial for maintaining systemic glucose homeostasis [48]. During fasting, the liver produces glucose through gluconeogenesis and transports it into the bloodstream. Conversely, after a meal, the liver uptakes, processes, and stores glucose in response to high plasma insulin levels [49]. However, these hepatic functions are dysregulated in T2DM, leading to hyperglycemia and hyperinsulinemia [50]. Net HGP is the combined results of glucose fluxes through various pathways including glycogenesis, glycogenolysis, glycolysis and gluconeogenesis [51]. In this regard, we found that gluconeogenesis was attenuated by dehulled adlay by evidence of decreasing the PEPCK protein expression and lowering the ratio of G6Pase/GK, both of which are induced by diabetes. Additionally, dehulled adlay may improve glycolysis by restoring the GK activity. These positive effects of dehulled adlay on glucose metabolism resulted in a net decrease in HGP and a reduction in hyperglycemia. Notably, the impairment of insulin-mediated suppression of HGP is generally considered to represent hepatic insulin resistance [52]. Therefore, our findings suggest that dehulled adlay may improve hepatic insulin resistance, as it reduced diabetes-induced HGP. A similar finding was observed by Tseng et al. and they demonstrated that ABO supplementation decreased PEPCK mRNA and protein expression and increased GK mRNA and protein expression in STZ/HFD-induced T2DM rats [20]. The present study did not investigate the other related pathways such as glycogenolysis and glycogenesis, which should be explored in further research.

HGP is also indirectly regulated by products of lipolysis in adipose tissues [49]. In T2DM, increase of insulin resistance and lipolysis in white adipose tissues lead to the continued delivery of nonesterified fatty acids (NEFA) and glycerol to the liver despite the high plasma insulin levels, which contributes to lipid accumulation and insulin resistance in the liver. These changes promote synthesis of intrahepatocellular TG and development of nonalcoholic fatty liver disease (NAFLD), which is considered a leading cause of liver disorder, and can progress into NASH, cirrhosis, liver failure, and hepatocellular carcinoma [53]. Furthermore, the presence of NAFLD can be a consequence and cause of T2DM [54]. NAFLD contributes to the development of T2DM by worsening hepatic and peripheral insulin resistance, resulting in increased HGP and hepatic lipid accumulation. Conversely, T2DM is recognized as a risk factor for the progression into NASH [55,56]. In our study, dehulled adlay supplementation was found to improve hepatic TG content and reduce lipid accumulation in the liver, suggesting that the hypoglycemic effects of dehulled adlay may be through alleviating hepatic steatosis. This finding is consistent with a study conducted by Yeh et al. which demonstrated that dehulled adlay ameliorated hepatic steatosis, inflammation, and insulin resistance in a high fructose/HFD-induced NAFLD rat model [57]. Moreover, in the HFD-fed mice, supplementation with ethanolic or water extracts of adlay seed improved insulin resistance, hyperglycemia, and hepatic steatosis and inflammation [58]. However, further investigations are required to elucidate the active components of dehulled adlay and understand the mechanism underlying their benefits on hepatic steatosis.

Although the exact active components were unidentified, several compounds derived from adlay bran have shown the anti-steatosis potential. Campestanol and β-sistosterol, the phytosterols from adlay bran oil, have been reported to inhibit gut absorption of cholesterol [59,60], and lower blood LDL-C levels [61]. Our study indicated that the hepatic TC content in DM rats was reduced by AB or DA diet, but no improvement was observed in the blood TC content with any of these three adlay diets. This suggests that further examination of different types of cholesterol might be necessary. Besides, it would be intriguing to investigate the potential involvement of campestanol or β-sistosterol in the reduction of hepatic steatosis mediated by dehulled adlay. Moreover, adlay bran contains various phenolic compounds such as quercetin, p-coumaric acid, and sinapic acid, which have shown potential in improving glucose and lipid disorders [38,39]. Quercetin, a flavonoid present in adlay bran, has been extensively studied and has demonstrated numerous benefits in improving metabolic syndrome. It exhibits anti-hyperglycemia, anti-hyperlipidemia, anti-obesity, and anti-NAFLD effects through multiple mechanisms like enhancing insulin secretion and sensitivity, increasing adiponectin, decreasing leptin, and providing antioxidant and

anti-inflammatory activities [62,63]. p-Coumaric acid, a phenolic acid derived from adlay bran, is known to possess a wide spectrum of biological properties including antioxidant, anti-inflammatory, antimicrobial activities, and have beneficial effects against diabetes, obesity, hyperlipidemia, and NAFLD [64,65]. Similarly, sinapic acid, another phenolic acid from adlay bran, exhibits potent antioxidant, anti-inflammatory, anti-diabetic, and anti-NAFLD activities [66,67]. Given that numerous phenolic compounds have been identified in adlay bran [39], it would be worthwhile to further investigate their roles in dehulled adlay-mediated attenuation of diabetes and NAFLD.

Currently, the molecular mechanisms responsible for the DA-mediated improvement of NAFLD are under investigation and remain unclear. In a rat model of high fructose and HFD, DA was shown to improve steatosis through possibly increasing plasma and fat adiponectin, decreasing fat leptin, reducing hepatic inflammation and increasing hepatic PPARγ expression [57]. Adiponectin, an anti-inflammatory adipokine, is known to modulates glucose metabolism and fatty acid oxidation, and plays a role in preventing insulin resistance and the development of NAFLD [68]. In contrast, leptin, a proinflammatory adipokine, is involved in food intake, energy homeostasis, glucose and lipid metabolism, as well as NAFLD progression [69]. The increase of adiponectin and decrease of leptin levels in adipose tissue, mediated by DA, suggest that DA produces a less inflammatory state in adipose tissue, which in turn modulates the crosstalk between the adipose tissue and the liver, leading to the reduced hepatic inflammation. In the liver, PPARγ, a transcriptional factor involved in lipid and glucose metabolism, ameliorates NAFLD by various effects including promoting lipogenesis and adipogenesis, improving insulin resistance, relieving ER stress, reducing oxidative stress and inflammation, and attenuating fibrosis [70]. Therefore, it's plausible to assume that DA alleviates the pathogenesis of NAFLD by activating hepatic PPARγ signaling. Considering that enhanced de novo hepatic lipogenesis contributes significantly to the development of NAFLD, it would be valuable to study the molecules regulating this process, such as two key enzymes acetyl-CoA carboxylase (ACC) and fatty-acid synthase (FAS), as well as two key transcriptional factors sterol regulatory element binding protein 1c (SREBP-1c) and carbohydrate regulatory element binding protein (ChREBP) [71]. In response to lipid overload, fatty acid oxidation mediated by PPARα, which mainly occurs in the mitochondria, is enhanced to increase lipid catabolism and provide a source of energy. However, prolonged and uncontrolled activation of this process can induce oxidative stress and exhaust antioxidant competences, followed by further diminishing mitochondria function, exacerbating oxidative stress and potentially promoting inflammation and disease progression. Notably, Chiang et al. reported that both ethanolic and water extracts of adlay seeds can improve hepatic steatosis and inflammation in HFD-fed mice by downregulating the mRNA expression of FAS and SREBP-1c, while upregulating PPARα expression [58]. In addition, both extracts of adlay seeds were found to inactivate ACC *in vitro* via phosphorylation by AMPK activation, an energy-sensing enzyme involved in regulating hepatic energy metabolism [72]. Therefore, it's worthwhile to investigate the role of AMPK in the beneficial effects of DA on diabetes and NAFLD in further studies.

In the present study, we discovered that lipid disorder induced by DM was ameliorated by the three adlay diets. All the three adlay diets showed significant improvements in reducing liver TG and TC content. Additionally, the blood TG content in DM rats was decreased below the baseline by AB or DA supplementation. It's of note that the blood TG levels in DM rats were slightly lower compared to the NC rats, although the difference was not statistically significant. This phenomenon of increased hepatic TG levels but decreased blood TG levels in response to HFD was consistent with our previous observation [73]. The liver plays a crucial role in the uptake, synthesis, storage, secretion, and catabolism of fatty acid and TG [74]. Lipogenesis, the process of fatty acid and TG synthesis, can be stimulated by glucose itself and insulin signals in response to feeding [75]. Importantly, insulin resistance and diabetes can boost lipogenesis, decrease TG clearance, and enhance hepatic VLDL secretion [76,77]. However, lipogenesis is highly responsive to diet changes. It has been reported that HFD increases liver lipogenesis but decrease VLDL secretion, thereby promoting the fat accumulation in the liver [78]. Hence, in our experiment, we observed the combined effect of increased hepatic TG levels and slightly decreased blood TG levels, which can be attributed to the stimulation of lipogenesis by HFD while simultaneously reducing VLDL secretion.

The limitations of this study are (1) the active ingredients in these three parts of adlay seeds for ameliorating hepatic gluconeogenesis and steatosis in diabetic rats are unclear, (2) the PEPCK activity is not examined, although the protein expression level is tested, and (3) the molecular mechanism of action such as the role of hepatic AMPK activity is unclear.

These issues deserve further investigation in the future.

5. Conclusion

To summarize, the dehulled adlay diet, composed of bran and endosperm, is the most effective compared to the diets solely composed of adlay bran or polished adlay on the improvement of hepatic glucose metabolism and steatosis in a STZ/HFD diabetic rat model. This suggests that combination of the two components of bran and endosperm provides additive anti-diabetic effects. Dehulled adlay is a good source of nutrients, dietary fiber, minerals, and phytochemicals. Therefore, relatively high consumption of dehulled adlay as a replacement for refined grain could potentially aid in regulating glucose and lipid metabolism. According to the data obtained in this and previous studies, we conclude that dehulled adlay displays hypoglycemic and hypolipidemic properties through various regulatory pathways, including reducing oxidative stress and inflammation, modulating gut health, and ameliorating hepatic gluconeogenesis and steatosis. Overall, these findings provide further evidence supporting the current recommendations of increasing whole grain consumption as part of a healthy diet for the prevention of T2DM.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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