

Anthocyanin Extract of Purple Corn Improves Hyperglycemia and Insulin Resistance of Rats Fed High Fat and Fructose Diet via GLP1 and GLP1R Mechanism

Ichda Chayati^{1,2,*}, Sunarti³, Yustinus Marsono⁴, Mary Astuti⁴

¹Department of Food and Fashion Engineering Education, Faculty of Engineering, Yogyakarta State University, Yogyakarta, Indonesia 55281

²Doctoral Program of Food Science, Department of Food and Agricultural Product Technology, Gadjah Mada University, Yogyakarta, Indonesia 55281

³Department of Biochemistry, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University, Yogyakarta, Indonesia 55281

⁴Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Gadjah Mada University, Yogyakarta, Indonesia 55281

*Corresponding author: ichda_chayati@uny.ac.id

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Abstract Hyperglycemia and insulin resistance are caused by high fat, fructose, or fat and fructose diet. The aim of this study was to investigate the effect of anthocyanin extract of purple corn (AEPC) on insulin resistance of rats fed high fat and fructose diet (HFFD). Insulin resistance was induced by feeding HFFD for 4 weeks and Wistar male rats were orally supplemented with the following treatments for the next six weeks: pioglitazone 1.35 mg kg⁻¹ body weight (bw) as a positive control group (PC), AEPC 6 mg kg⁻¹ bw (LA), AEPC 12.5 mg kg⁻¹ bw (MA), and AEPC 25 mg kg⁻¹ bw (HA), or no treatment as a negative control group (NC). A normal control group was fed a normal diet (NLC) for along 10 weeks. Insulin resistance was indicated by Homeostasis Model Assessments for Insulin Resistance (HOMA-IR) scores. The results showed that HFFD feeding increased HOMA-IR score and blood glucose levels, and decreased levels of plasma glucagon-like peptide 1 (GLP1) and pancreatic glucagon-like peptide 1 receptor (GLP1R). Administration of AEPC reduced HOMA-IR scores and blood glucose levels, increased HOMA-β and HOMA-IS scores, plasma GLP1 and pancreatic GLP1R levels, and improved pancreatic morphology. Our finding suggests that AEPC 12.5 mg kg⁻¹ bw gave the best conditions to improve insulin resistance as well as standard drug administration pioglitazone 1.35 mg kg⁻¹ bw via GLP1 and GLP1R mechanism.

Keywords: anthocyanin, purple corn, hyperglycemia, insulin resistance, GLP1, GLP1R

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1. Introduction

The global prevalence of diabetes was estimated to be 9% in 2014 [1]. According to Basic Health Research 2018, in Indonesia, prevalence of diabetes in 2018 was 10.9% of over 15 year-old population based on diabetes diagnose criteria by Indonesian Endocrinology Association 2015 [2]. One of the most responsible cause of diabetes is insulin resistance [3]. High-fat diets cause obesity-induced metabolic syndrome [4], oxidative stress [5,6,7], and trigger insulin resistance [8,9,10]. Moreover, overconsumption of fructose plays a role in insulin resistance [11,12], obesity [13], and oxidative stress [14,15]. Therefore, treatment with antioxidants can prevent hyperglycemia and insulin resistance caused by high-fat and high-fructose diets

(HFFD). One type of antioxidant is anthocyanin which is a group of flavonoid compounds that give the colors orange, red, purple, and bright blue, that is found naturally in fruits and vegetables. One source of anthocyanin is purple corn [16]. In Indonesia, purple corn is being cultivated and one of its kind is dark purple corn from Malang, East Java, Indonesia.

Several studies on purple corn anthocyanin extract demonstrate the ability to inhibit prostate carcinogenesis [17], improve oxidative stress conditions [18], inhibit carcinogenesis of mammas [19], inhibit colon cancer [16], and improve conditions in diabetic nephropathy [20]. However, there has been no study of anthocyanin extract of purple corn (AEPC) on hyperglycemia and insulin resistance in HFFD rats, therefore this investigation was important. Several studies resulted that anthocyanin acted as antihyperglycemia by delaying carbohydrate digestion

and inhibiting the rate of glucose absorption across the intestine [21-25]. This research focuses on the mechanism of antihyperglycemia and insulin resistance amelioration via GLP1 and GLP1R levels improvement.

2. Materials and Methods

2.1. Extract Preparation

Purple corn (*Zea mays*) seed kindly given by Dr. Arifin Noor Sugiharto was cultivated in Gadjah Mada University land. After 3 months, it was cropped and sun-dried (before 12 pm) to a water content of ca. 12%. Furthermore, it was ground and sieved using a 60-mesh size screen. The unsieved part powder was then extracted using ethanol-3% citric acid (1:10, w/v), macerated at 1000 rpm using magnetic stirrer at ambient temperature for 3 hours. The extract was centrifuged at 3000 rpm at ambient temperature for 20 minutes. The supernatant was evaporated to dryness at 40°C with rotary evaporator Buchi R-3000 (Buchi Labortechnik AG, Flawil, Switzerland). The thick extract was sealed and stored in dark condition at -20°C until use. The characteristic of AEPC was 2.55 mg/ml of total anthocyanin content and 0.229 μmol Trolox Equivalent (TE) mL^{-1} of total antioxidant capacity. Anthocyanin components of AEPC were cyanidin-3-glucoside 27.8 ppm and peonidin-3-glucoside 10.4 ppm (data not published).

2.2. Animals

Twenty four male Wistar rats age 2 months were purchased from The National Agency of Drug and Food Control (Yogyakarta, Indonesia). The animals were maintained at 21-25°C and 50-60% of humidity and kept on a 12 h light-12 h dark cycle with free access to food and water. The research protocol was approved by The Committee of Ethical Clearance for Pre-clinical Research of The Integrated Laboratory of Research and Testing, Gadjah Mada University, Yogyakarta, Indonesia Ref: 441a/KEC-LPPT/III/2016.

Table 1. Composition and energy values of ND and HFFD

Ingredient	ND		HFFD	
	g	kcal	g	Kcal
Cornstarch	62	248	47.5	190
Sucrose	10	40	-	-
Fructose	-	-	10	40
Casein	14	56	14	56
Soybean oil	4	36	3	27
Beef tallow	-	-	15	135
Cholesterol	-	-	0.5	-
Alphacel	5	-	5	-
AIN-93-MX	3.5	-	4.13 ^a	-
L-cystine	0.18	-	0.18	-
AIN-93-VX	1	-	1.18 ^a	-
Choline bitartrate	0.25	-	0.25	-
Tert-butylhydroquinone	0.0008	-	0.0008	-
Total	99.93	380	100.74	448
% total energy				
Carbohydrate	75.8		51.3	
Fat	9.5		36.2	
Protein	14.7		12.5	

Source: [26,27]

ND = normal diet; HFFD = high fat and fructose diet

^a mineral mix and vitamin mix were adjusted to provide equal amounts of micronutrients per unit of energy, resulting in a 0,81% increase in the total weight of the HFFD (100.74 g instead of 99.93 g).

2.3. Insulin Resistance Induction by HFFD

After three days of adaptation, the rats were randomly divided into six groups based on their fasting blood glucose. One group (n=4) were fed normal diet (AIN93M), whereas five groups (n=4 per group) were fed HFFD diet for 4 weeks. Table 1 shows the composition of normal diet (ND) and HFFD.

2.4. Treatment Protocols

Wistar rats were randomly divided into six groups, consisted of four rats for each group:

1. NLC (normal control); rats were given ND and distilled water by forced feeding for 10 week-treatments
2. NC (negative control); rats were given HFFD and distilled water by forced feeding for 10 week-treatments
3. PC (positive control); rats were given HFFD for 10 week-treatments and forced feeding of pioglitazone 1.35 mg kg^{-1} bw (body weight) for the last 6 week-treatments
4. LA (low AEPC); rats were given HFFD for 10 week-treatments and forced feeding of 6.25 mg total anthocyanin kg^{-1} bw for the last 6 week-treatments
5. MA (medium AEPC); rats were given HFFD for 10 week-treatments and forced feeding of 12.5 mg total anthocyanin kg^{-1} bw for the last 6 week-treatments
6. HA (high AEPC); rats were given HFFD for 10 week-treatments and forced feeding of 25 mg total anthocyanin kg^{-1} bw for the last 6 week-treatments

Distilled water was prepared fresh daily as well as feed was changed every day.

2.5. Plasma and Serum Collection and Tissue Samplings

At the end of the experiments, the rats were fasted overnight (12 h) and then anesthetized using sodium pentobarbital. At the 0th, 4th, and 10th week of experiment, blood samples were collected from retro-orbital sinus of rats. The blood was collected into heparinised or non-heparinised tubes and centrifuged at 2000 rpm for 20 min, respectively. The plasma and serum were collected and stored at -80°C until analysis to determine the biochemical parameters. The pancreases were removed, washed in ice-cold phosphate buffer saline (PBS) 0.01M pH 7.4, and the tissue were collected and fixed in 10% formalin for histopathological analysis. For GLP1R analysis, after washing, pancreatic tissues were collected and kept at -80°C until analysis.

2.6. Serum Fasting Glucose, Insulin and HOMA Analysis

Serum fasting glucose levels were determined at the 0, 4th, and 10th week of the experiments using a GOD-PAP method. Serum insulin levels were determined at the 10th week of the experiments using an ELISA Kit (Elabscience, Wuhan, Hubei, China) according to the manufacturer's

protocol. Homeostasis model assessments for insulin resistance (HOMA-IR) and HOMA- β values were calculated by using the following formula [28]:

$$\text{HOMA-IR} = [\text{fasting insulin (mU L}^{-1}) \times \text{fasting glucose (mmol/L)}] / 22.5$$

$$\text{HOMA-}\beta = [20 \times \text{fasting insulin (mU L}^{-1})] / [\text{fasting glucose} - 3.5]$$

While HOMA-IS values were calculated using formula of Matsuda (1999) as follows:

$$\text{HOMA-IS} = 405 / (\text{fasting insulin (IU L}^{-1}) \times \text{fasting glucose (mmol L}^{-1}))$$

2.7. Plasma GLP1 and Pancreatic GLP1R Analysis

Plasma GLP1 and pancreatic GLP1R levels were determined by using an ELISA Kit (Elabscience, Wuhan, Hubei, China) according to the manufacturer's protocols.

2.8. Histopathological Analysis of Pancreas

Small pieces of pancreas were fixed with formalin 10% in PBS and embedded in paraffin. Section (8 μm) were cut and stained with hematoxylin and eosin and observed under a microscope (Olympus BX-52 Microscope). Images were captured using a camera (Olympus DP-21 Camera) at a 400x magnification [29].

2.9. Statistical Analysis

The results are expressed as the means \pm standard deviation (SD). The statistical analysis data were based on a one-way ANOVA followed by Duncan's multiple range test (DMRT). Correlation between parameters were

measured using Pearson correlation test. The data were analysed using the SPSS statistical package (IBM SPSS statistics, version 18.0).

3. Results

3.1. Effect of AEPC on Fasting Serum Glucose

The administration of AEPC affected the fasting serum glucose of rats as seen on Figure 1. At the beginning of research, all groups had the same value of fasting serum glucose, meant that all rats were randomly divided (Figure 1A). HFFD treatment made fasting serum glucose higher than AIN93 diet, which increases approximately 152% (Figure 1B). AEPC treatment for six weeks at medium dose lowered fasting serum glucose as much as pioglitazone groups, i.e. decreases approximately 40%. Low and high dose of AEPC could also lower fasting serum glucose though not as much as medium dose (Figure 1C).

3.2. Effect of AEPC on fasting serum insulin

Fasting serum insulin of rats fed HFFD were not influenced by the administration of AEPC (Figure 2). As shown in Figure 2, after ten-week treatment, all groups with HFFD had the same fasting serum insulin, independent on treatment given to the groups. Fasting serum insulin of rats fed HFFD were higher than rats fed normal diet. Fasting serum insulin of pioglitazone and high dose of AEPC groups were the same as normal control groups.

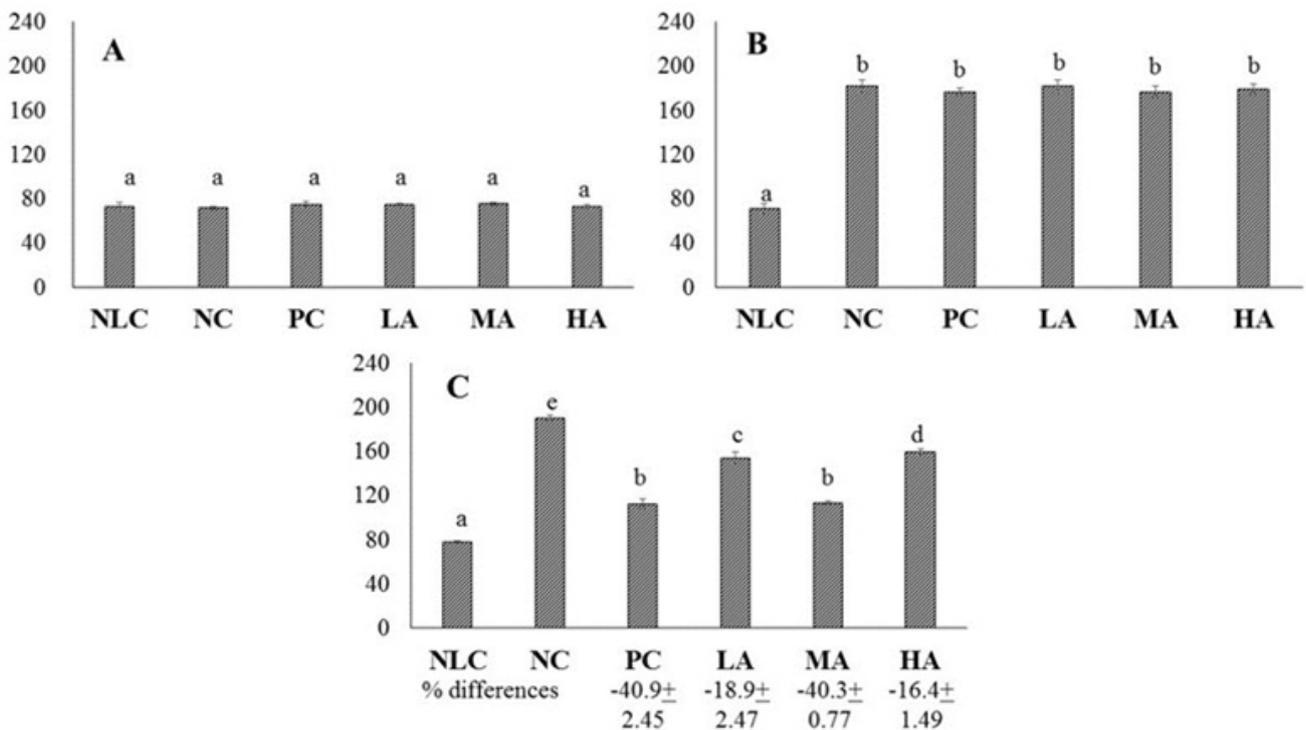


Figure 1. Effect of AEPC treatment on the fasting serum glucose (mg dL⁻¹) of rats at week 0 (A), week 4 (B) and week 10 (C). (NLC=normal control/normal diet; NC=negative control/HFFD; PC=positive control/ HFFD+ pioglitazone 1.35 mg kg⁻¹ bw.; LA=HFFD + AEPC 6.25 mg kg⁻¹ bw.; MA=HFFD+ AEPC 12.5 mg kg⁻¹ bw.; HA=HFFD+ AEPC 25 mg kg⁻¹ bw; n=4; difference letter means difference value at 5% significance, % differences are compared to NC group)

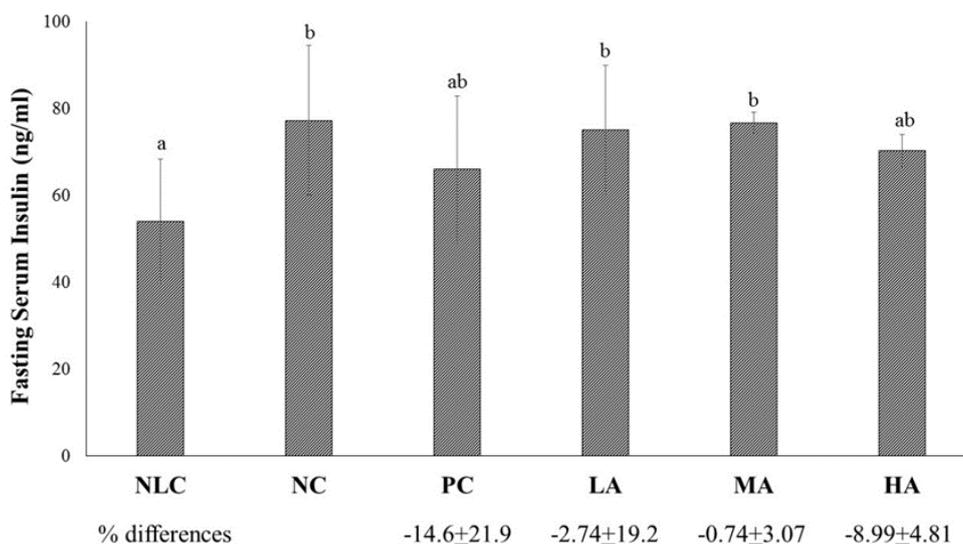


Figure 2. Effect of AEPC on the fasting serum insulin of rats after 10 weeks of treatment. See Figure 1 for further explanation

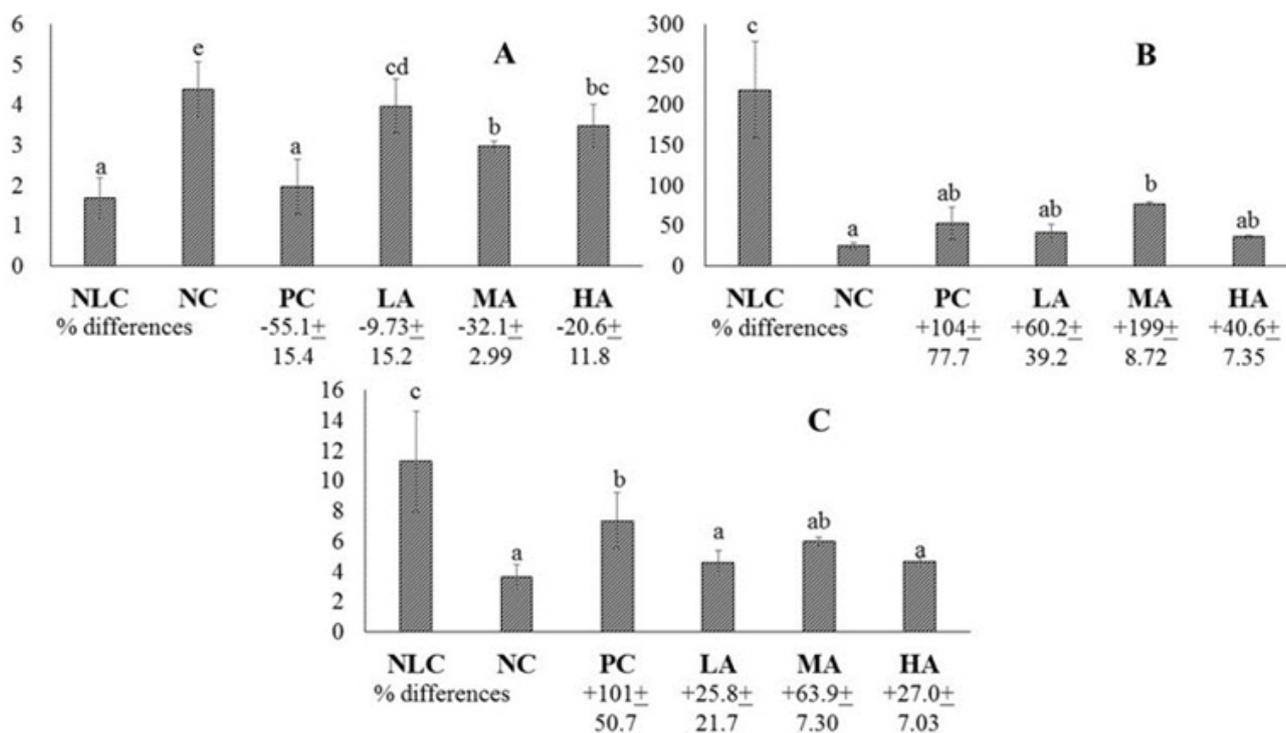


Figure 3. HOMA-IR (A), HOMA-β (B), and HOMA-IS (C) scores after 10 week-treatment of rats with or without AEPC. See Figure 1 for further explanation

3.3. Effect of AEPC on HOMA-IR, HOMA-β, and HOMA-IS Scores

The administration of AEPC decreased HOMA-IR, while it was increased in HOMA-β and HOMA-IS of rats fed HFFD (Figure 3). Figure 3 showed HOMA-IR scores after 10 week-treatment of rats with or without AEPC. After 10 week-treatment, rats of normal and positive control groups had the lowest HOMA-IR score, while negative control group had the highest one. AEPC treatments could improve HOMA-IR, the best condition was by giving AEPC of 25 mg kg⁻¹ bw, which could decrease in 32% of HOMA-IR score (Figure 3A).

High fat and fructose diet decreased level of pancreatic beta-cell function. Pioglitazone and AEPC treatments at low and high doses were not enough to increase in

HOMA-β score, while AEPC treatment at medium dose improved pancreatic beta-cell function with 200% increase of HOMA-β score (Figure 3B). HOMA-IS decreased after feeding of HFFD for 10 weeks, meaning that insulin sensitivity of rats fed HFFD was decreased. AEPC treatment at low and high doses could not improve insulin sensitivity, while pioglitazone and AEPC treatments at medium dose could increase 100% and 64% HOMA-IS scores, respectively (Figure 3C).

3.4. Effect of AEPC on Plasma GLP1 and Pancreatic GLP1R

The administration of AEPC to rats fed HFFD improved plasma GLP1 and pancreatic GLP1R (Figure 4). High fat and fructose diet lowered plasma GLP1 level as

seen in Figure 4A. Treatments with pioglitazone and medium and high doses of AEPC increased plasma GLP1 level by 70, 52, and 42%, respectively. It meant that AEPC could improve plasma GLP1 of rats fed HFFD. HFFD reduced pancreatic GLP1R level (Figure 4B). Treatment with pioglitazone and low and medium doses of AEPC increased pancreatic GLP1R levels by 44, 38, and 31%, respectively. It meant that AEPC could improve sensitivity of pancreatic GLP1R of rats fed HFFD.

3.5. Correlation between HOMA-IR and Other Parameters

Correlation between HOMA-IR score at 10 week-treatment and other parameters were seen in Table 2. HOMA-IR as an indicator of insulin resistance had strong positive correlation with fasting serum glucose, and fasting serum insulin, and strong negative correlation with HOMA-β score, HOMA-IS score, plasma GLP1, and pancreatic GLP1R levels. The higher the fasting serum glucose and fasting serum insulin, the higher insulin resistance incident, while the higher the HOMA-β and HOMA-IS score, GLP1 plasma, and pancreatic GLP1R level, the lower insulin resistance incident.

Table 2. Correlation between HOMA-IR Score and Other Parameters

Parameters	Pearson's Correlation Score with HOMA-IR
Fasting serum glucose	0.825**
Fasting serum insulin	0.661**
HOMA-β	-0.534**
HOMA-IS	-0.830**
Plasma GLP-1	-0.740**
Pancreatic GLP-1R	-0.508*

**means that there is a correlation at 1% significant.

*means that there is a correlation at 5% significant.

3.6. Pancreatic Histopathological Analysis

Figure 5 shows pancreatic morphology of rats after treatment with or without AEPC to rats fed HFFD. Negative control group (NC) showed presence of necrosis with lymphocyte and neutrophil infiltration, while the other four treatment groups (PC, LA, MA, and HA) showed there was no change in pancreatic histopathologic features. This suggested that pioglitazone and all of three doses of AEPC were capable of providing repair of pancreatic cell damage caused by HFFD. The effect of all three doses of AEPC on pancreatic tissue repair damaged by HFFD was proportional to pioglitazone (Figure 5).

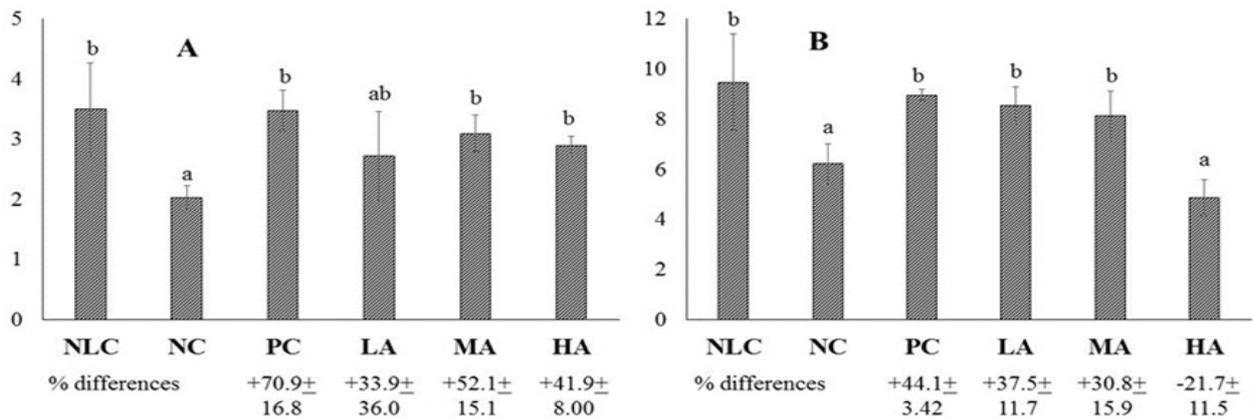


Figure 4. Plasma GLP1, ng mL⁻¹ (A) and pancreatic GLP1R, ng g⁻¹ (B) of rats with or without AEPC after 10 weeks of treatment. See Figure 1 for further explanation

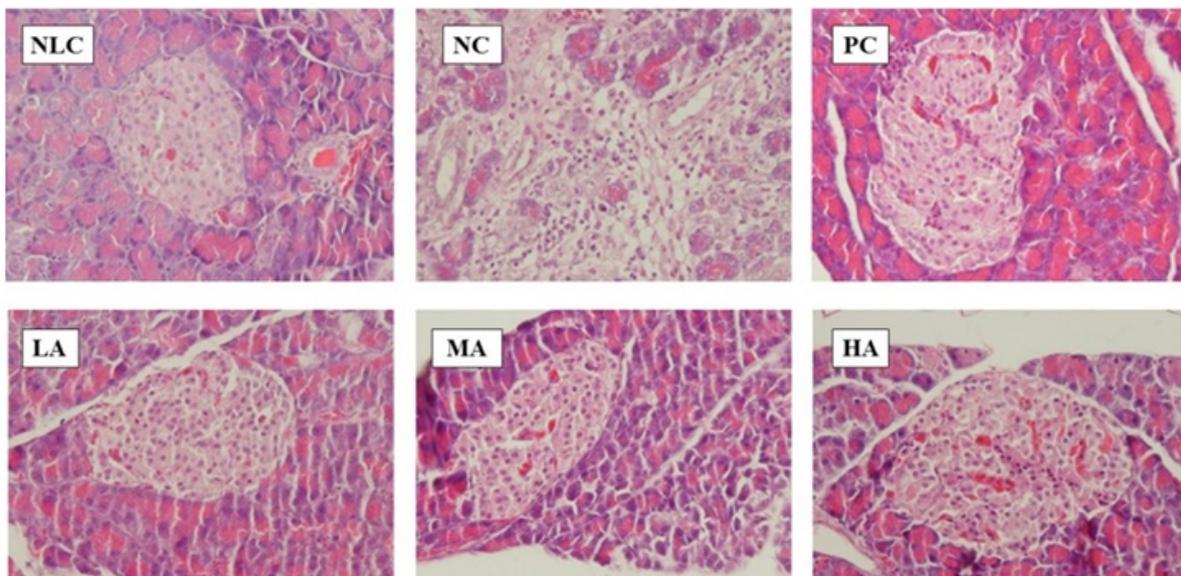


Figure 5. Histopathology of pancreatic islet (NLC=normal control/normal diet; NC=negative control/HFFD; PC=positive control/ HFFD+ pioglitazone 1.35 mg kg⁻¹ bw.; LA=HFFD + AEPC 6.25 mg kg⁻¹ bw; MA=HFFD+ AEPC 12.5 mg kg⁻¹ bw; HA=HFFD+ AEPC 25 mg kg⁻¹ bw)

4. Discussion

The blood glucose levels between groups at the beginning of the study (week 0) were homogeneous. Therefore, it could be interpreted that the rats had been randomly divided into groups based on their blood glucose levels. Administration of HFFD caused an increase in blood glucose levels compared to rats fed standard AIN93M (week 4). The increase in blood glucose levels was very significant which indicated that the provision of HFFD made blood glucose levels higher. This result is in line with other studies that overconsumption in fat and fructose lead to blood glucose increase [30,31,32]. At the end of the 10th week in which the treatment was completed, normal group blood glucose levels had the lowest blood glucose levels. Administration of HFFD without other treatment (negative control group) had the highest blood glucose levels. Standard drug administration of pioglitazone (positive control group) caused blood glucose levels to approach the normal group. Similarly, treatment of AEPC 12.5 mg kg⁻¹ bw produced the same blood glucose levels as standard drug administration. It meant that high blood glucose level caused by HFFD could be normalized by AEPC. While AEPC of 6 and 25 mg kg⁻¹ bw of rats also caused blood glucose levels to decrease compared to the negative control group, but the decrease was not as high as AEPC 12.5 mg of rat. This result was similar to previously research, which showed that purple corn decreased blood glucose level in mice fed high fat diet [33].

At the end of week 10, insulin level of rats fed HFFD were higher than normal control. This result was the same as some researches, who stated that insulin concentrations were higher in the rats fed high fat diet compared to control [34-39], although their results were slightly different. Le et al said that high fructose diet in offspring of type 2 diabetic patients increased fasting plasma concentration of insulin [40]. Tsuda et al. also got the same result that consumption of purple corn color containing cyanidin-3-glucoside, which decreased insulin level of mice fed high fat diet [33]. AEPC feeding prevented the increase in serum insulin caused by HFFD consumption. The lower insulin levels were achieved by the group with a dose of AEPC 25 mg kg⁻¹ bw (Figure 2). A study in human showed that ones who have insulin resistance will release insulin fourfold to fivefold higher than insulin-sensitive individuals [41]. Xu et al. said that at the first phase insulin-resistant state, in a respond to glucose load, there is a loss of initial insulin secretion, resulting in postprandial hyperglycemia. Afterwards, hyperinsulinemia is happened because of an excessive insulin response in the second phase insulin-resistant state. Subsequently, insulin resistance become worse and also pancreatic β -cell become dysfunction [42].

The HOMA-IR is well-defined biomarker for the assessment of insulin resistance [43]. Feeding of HFFD increased insulin resistance, while pioglitazone improved it. AEPC feeding also ameliorated insulin resistance although their HOMA-IR scores were still higher than pioglitazone group. Medium dose of AEPC was better than low and high dose of AEPC in improvement of insulin resistance (Figure 3).

From Figure 3, it can be seen that the best HOMA- β score was shown by the group receiving an AEPC at a

dose of 12.5 mg kg⁻¹ bw, approaching the positive control, although this level was still lower than the normal control. A low-dose AEPC resulted in a lower HOMA- β level. This indicated that this AEPC dose were not been able to repair the damage of pancreatic β cells. The highest dose of AEPC also showed a low HOMA- β score, probably because the dose actually served as a pro-oxidant.

It is reported that GLP-1 action is deficient in subjects with type 2 diabetes mellitus [44]. GLP-1 is a protective cytokine against inflammatory events, which in this study is caused by oxidative stress due to the HFFD diet. The presence of GLP-1 inhibits various pro-inflammatory cytokines that trigger insulin resistance conditions. The higher the level of GLP-1 content, the protective effect is more prominent. HFFD diet (negative control) causes the lowest GLP-1 levels, whereas positive controls show higher GLP-1 levels. Giving AEPC for all doses can increase plasma GLP-1 levels. This shows the protective nature of AEPC in preventing damage, which caused by HFFD administration, starting from a dose of 6.25 mg kg⁻¹ bw.

In line with GLP1, pancreatic GLP1R as a receptor of GLP1 in pancreas, also have a negative correlation with insulin resistance. GLP1 activate the expression of GLP1R on beta cells and therefore trigger glucose-induced insulin secretion. Furthermore, GLP1 inhibit glucagon secretion from pancreatic alpha cells [45].

Based on Table 2, then the next discussion was done by using the HOMA-IR score as a reference to know the relationship between all other parameters. This HOMA-IR score was chosen because it correlates strongly with insulin tolerance test (ITT), so it can be used as a predictor for insulin resistance conditions in Wistar rats. HOMA-IR can detect insulin resistance with 90% sensitivity. Compared with other methods for assessing insulin resistance, HOMA-IR has advantages in terms of ease of testing, stressing fewer test animals, and avoiding the risk of hypoglycemia in test subjects [46].

Administration of HFFD increases serum blood glucose and insulin levels, as well as HOMA-IR. HOMA-IR scores correlated positively with blood glucose and serum insulin levels. This result is in line with previous study, where high-fat dietary administration led to significant increases in serum glucose and insulin levels compared with normal control [47]. High fat and fructose diets cause an increase of free fatty acid and glucose blood level, resulting in insulin resistance that stimulates lipolysis and insulin secretion. The compensatory responses of insulin resistance include adipogenesis which can lead to diabetes [48].

This study has shown that AEPC administration was able to suppress blood glucose and serum insulin level compared to high fat and fructose diet group. The best suppression of blood glucose levels was obtained in the group with an AEPC dose of 12.5 mg kg⁻¹ bw, while the lowest serum insulin at AEPC was 25 mg kg⁻¹ bw.

There was a negative correlation between HOMA-IR and plasma GLP1 levels, which indicates that the lower plasma GLP1 levels, the higher the incidence of insulin resistance. GLP1 is a hormone produced by the gastrointestinal tract and stimulates insulin secretion due to elevated blood glucose level. Aulinger et al. found that GLP1 levels were correlated with the incidence of insulin resistance in healthy subjects [49]. One mechanism proposed why GLP1 improve insulin resistance is that

GLP1 improve β -cell function, reduce gluconeogenesis, and improve insulin sensitivity [50].

In patients with type 2 diabetes mellitus and insulin resistance, there was a decrease in GLP1 levels. It is known that insulin resistance is associated with a chronic inflammatory process in fatty tissue, one of which is caused by tissue oxidative stress due to HFFD administration. The accumulated macrophages in fatty tissue secrete various pro-inflammatory cytokines, resulting in an inflammatory reaction. GLP1 is able to inhibit macrophage infiltration in fat, heart, and liver tissue, thus plays an important role in inhibiting the secretion of these pro-inflammatory cytokines [51]. Thus, in conditions of insulin resistance (high HOMA-IR), low levels of GLP1 will be found. AEPC also improve GLP1R levels, means that there is an improvement in beta cells condition.

5. Conclusions

The results of the study showed that AEPC administration improved insulin resistance, pancreatic- β cells, and hepatic sensitivity to insulin, reduced blood glucose levels, increased plasma GLP1 levels, pancreatic GLP1R level and improved pancreatic histopathology. HOMA-IR scores positively correlated with blood glucose levels and blood insulin levels, but negatively correlated with HOMA- β and HOMA-IS scores. The condition of insulin resistance was negatively correlated with plasma GLP1 and pancreatic GLP1R. This result, indicated that the improvement of insulin resistance due to AEPC administration via improved plasma GLP1 and pancreatic GLP1R mechanism. Giving AEPC of 12.5 mg kg⁻¹ bw yielding the best conditions approaching standard drug administration pioglitazone 1.35 mg kg⁻¹ bw.

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Statement of Competing Interests

The authors have no competing interests.

List of Abbreviations

AEPC anthocyanin extract of purple corn
ANOVA analysis of variance

bw body weight
DMRT Duncan's multiple range test
GLP1 glucagon-like peptide 1
GLP1R glucagon-like peptide 1 receptor
HA high AEPC group
HFFD high fat and fructose diet
HOMA- β homeostasis model assessments for β -cell function
HOMA-IR homeostasis model assessments for insulin resistance
HOMA-IS homeostasis model assessments for insulin sensitivity
LA low AEPC group
MA medium AEPC group
NC negative control group
ND normal diet
NLC normal control group
PBS phosphate buffer saline
PC positive control group
SD standard deviation

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