2021 IEEE International Conference on Health, Instrumentation & Measurement, and Natural Sciences (InHeNce) | 978-1-6654-4181-0/21/$31.00 ©2021 IEEE | DOI: 10.1109/InHeNce52833.2021.9537215

Potential of *Gnetum gnemon* L. Seed Extract Against Insulin Levels and PDX-1 Expression in Diabetes Mellitus Rats Model

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***Abstract*— Diabetes mellitus (DM) is a condition in which blood glucose levels rise as a result of reduced insulin secretion. DM caused complications such as retinopathy, angiopathy, nephropathy, and neuropathy. Melinjo (*Gnetum gnemon* L.) seeds are high in polyphenols and flavonoids, and also have anti-inflammatory, antioxidant, and antibacterial properties. The aim of this study was to examine Melinjo (*Gnetum gnemon* L.) Seed Extract (MSE) activity in DM rat model. The MSE (200, 400, 600 mg/kg BW) administered orally for 21 days on DM rats. Levels of serum glucose was measured using colorimetry method and serum insulin was measured using Enzyme-Linked Immunosorbent Assay (ELISA). Density of Langerhans islet was measured using hematoxylin-eosin (HE) staining method and PDX-1 expression was measured using immunohistochemistry method. After treatment, it showed that giving MSE 600 mg/kg BW could reduce serum glucose levels from 372.22 to 273.70 mg/dL and increase insulin levels from 68.81 to 253.11 µIU/mL. The density of Langerhans islet cells increased from 54.36 to 64.15 cells/10^4µm^2 and PDX-1 expression in Langerhans islet increased from 73.49 to 114.80. Melinjo (*Gnetum gnemon* L.) seed extract has antidiabetic activities through improving glucose and insulin levels, increase Langerhans cells density, and Langerhans PDX-1 expression.**

Keywords—melinjo seed extract, diabetes mellitus, Langerhans islet, PDX-1

I. Introduction

Diabetes mellitus (DM) is a metabolic condition in which blood sugar levels are abnormally high and usually accompanied by disorders of lipid, carbohydrate, and protein metabolism. These disorders are caused by insulin resistance and insulin deficiency [1].

Insulin resistance is a condition where insulin can't work optimally on its target cells (muscle cells, fat cells, and liver cells) and there is a malfunctioning in β-pancreatic cells. β-pancreatic cells are cells found in the islets of Langerhans, which function is to produce insulin [2]. Hence, malfunctioning in β-pancreatic cells can increase glucose in the blood due to the absence of insulin and caused DM [3].

Environmental and genetic factors have a role in the pathogenesis of diabetes. Another factor that has an impact on diabetes development is mutations of key transcription. PDX-1 (pancreatic and duodenal homeobox 1) is one of the transcription factors and diabetes genes. PDX-1 plays an important role in pancreatic endocrine and insulin-secreting β-cell growth and function in mice and humans [4].

The activation of the PDX-1 transcription factor is regulated by glucose levels in pancreatic β-cells. Chronic hyperglycemia and dyslipidemia, two primary complications of type 2 diabetes, reduce PDX-1 expression, resulting in pancreatic cell dysfunction [5].

Pharmacological therapies used to treat DM are sulfonylureas, biguanides, and α-glucosidase inhibitors. However, this therapy has side effects such as weight gain and hypoglycemia [1]. Therefore, it is necessary to look for other alternatives that are safer and have fewer side effects to treat DM.

Melinjo (*Gnetum gnemon* L.) fruit has high antioxidant and antibacterial qualities and is known to contain a large amount of polyphenol compounds [6]. In a study conducted by Ira and Ikhda [7], infusion of melinjo seeds could reduce blood glucose levels in mice hyperglycemic. Another study by Oniki et al. [6] stated that melinjo seed extract reduce weight gain as well as reduce fasting blood glucose levels in rats fed a high-fat diet. According to Yoneshiro et al. [8], administration of melinjo seeds extract can suppress body fat accumulation and adipose inflammation. Thus, it could prevent high-fat diet-induced fatty liver and insulin resistance in mice.

Study on the expression of PDX-1 in diabetic rats has been carried out previously using immunohistochemistry (IHC) methods. In a study conducted by Tsai et al. [9] showed that powdered *Eurycoma longifolia* root increased PDX-1 expression and beta-cell number.

Study on antidiabetic activity on melinjo seeds extract (MSE) has been widely conducted. However, study on the effect of MSE on PDX-1 expression, serum glucose and serum insulin levels, also Langerhans cell density needs further investigation. Therefore, the focus of this study is to examine the effect of MSE on PDX-1 expression, as well as the effect of MSE on serum glucose levels, serum insulin levels, and Langerhans cell density in male Sprague Dawley (SD) rats induced by Streptozotocin (STZ) as a DM rat model.

II. Methods

A. Extract Preparation

Melinjo plant used in this study was obtained from Rende Village, Bandung, West Java, Indonesia. Identification of melinjo plant was performed in *Lembaga*

Ilmu Pengetahuan Indonesia (LIPI). The melinjo plant was identified as *Gnetum gnemon* L. For extraction, melinjo seeds were grounded into powder and were extracted using a maceration technique with 70% ethanol as a solvent. The filtrate was collected every 24 hours until it became colourless. The filtrate obtained was being evaporated using a rotary evaporator at 50°C until the (MSE was obtained in the form of paste and stored at -20°C [10] [11] [12] [13].

B. Phytochemicals Assay

To identify the presence of phenols, steroids/triterpenoids, saponins, tannins, terpenoids, flavonoids, and alkaloids, phytochemical assays were done on MSE using a modified Farnsworth method. [13] [14] [15] [16] [17].

C. Phenolic Content

A 10 mg of MSE dissolved in 5 mL ddH2O. 500 µL FeCl3 1% added into mixture. If green/red/purple/blue/black color formed, then the sample contained phenol [13] [17] [18].

D. Steroid/Triterpenoid Content

Glacial acetic acid added into 10 mg extract MSE in the dropping plate, let the mixture for 10-15 minutes. In the mixture added 1 drop of concentrated H_2SO_4. If there was greenish-blue color formed then the sample contained steroid, but if there was purple/red/orange color formed then the sample contained triterpenoid [13] [17] [18].

E. Saponin Content

A 10 mg of MSE extract dissolved in ddH2O in the reaction tube. The sample was heated for 5 minutes until it was boiling. Before adding 1 N of HCl, the mixture was filtered and vigorously shaken. If the bubble still formed and still exist after HCL 1 N was added, then the sample contained saponin [13] [17] [18].

F. Tannin Content

A 10 mg of MSE with 2 mL of HCl 2N was added to the reaction tube and heated for 30 minutes in the water bath. Then, 500 µL of amyl-alcohol was added after the mixture cooled down. If there was an orange/red color in the amyl-alcohol layer, then the sample contained tannin [13] [17] 18].

G. Terpenoid Content

Vanillin was added into 10 mg MSE sufficiently into the dropping plate. 1 drop of concentrated H_2SO_4 was added then homogenized. If there was a purple color formed, then the sample contained terpenoid [13] [17] [18].

H. Flavonoid Content

A 10 mg extract of MSE was mixed with HCl 2 N in the reaction tube. Then, Mg/Zn was added sufficiently. Before adding 1 mL amyl-alcohol, the mixture was heated for 5-10 minutes, cooled, and filtered. If a red/orange color formed then the sample contained flavonoid [13] [17] [18].

I. Alkaloid Content

A 10 mg of MSE extract dissolved in 5 mL ddH_2O and evaporated in the water bath. The residue from the evaporation dissolved in 5 mL HCl 2N. Then the mixture is divided into two reaction tubes. Three drops of HCl 2N were added to the first tube as a blank. After that, one drop of the mixture in the second tube was put in the dropping plate with three drops of Dragendorff mixture. If the sample formed an orange precipitate, it contained alkaloids [13] [17] [18].

J. Preparation of Diabetic Rats

Sprague Dawley (SD) male rats weighing 105-120 grams were acclimatized for one week in a cage under constant environmental conditions and given daily food and drink. Rats were then injected via intraperitoneally with STZ at a dose of 60 mg/kg BW. Glucose levels were checked three days after injection using an Autocheck Glucometer to ensure the rats had diabetes [19]. Rats were randomly divided into six groups. Each group consisted of six rats with the following treatment: normal rats (negative control group), diabetic rats induced by STZ 60 mg/kg BW (positive control (group), positive control with metformin treatment at a dose of 85.02 mg/kg BW (metformin group), and positive control with MSE treatment at a dose of 200 mg/kg BW, 400 mg/kg BW, 600 mg/kg BW respectively (MSE group). This research already got ethical approval from *Universitas Prima Indonesia* (013/KEPK/UNPRI/XI/2020).

K. Biochemical Assays

The rat serum was collected on day-10 and day-21 after the rats were given treatment (200, 400, 600 mg/kg BW, metformin 85.02 mg/kg BW). The blood sample was taken through a vein at the back of the eye (retroorbital). Then, the blood was inserted into a microtube and was left to stand for 15 minutes. After that, the blood was centrifuged at 3000 rpm for 15 minutes. The light clear layer at the top was the serum used for biochemical assays [19]. Serum glucose levels were determined using Glucose GOD FS* Kit (Dia Sys, 125509910 023). Serum insulin levels were determined using Rat INS (Insulin) ELISA Kit (ElabSci E-EL-R3034).

L. Rats Termination

The rats were given ketamine (80-150 mg/kg BW) and xylazine (10-20 mg/kg BW) via intraperitoneal. Then, pancreatic organs were taken and put in 10% formalin for histopathological test preparation [20].

M. Hematoxylin-Eosin (HE) Staining

Histopathology of pancreas was carried out using hematoxylin-eosin (HE) staining method. First, the organ was fixed with 10% formalin for 2-3 days, then dehydrated with alcohol 70%, 80%, 90%, and absolute alcohol for 1-2 hours in the incubator, respectively. After the dehydration process, the remaining alcohol was removed with xylol 1 and xylol 2. Then, the organ was put in liquid paraffin at 60ºC inside an incubator. Block paraffin was made at room temperature. Paraffin blocks were sliced into 5 micrometers and stained with Hematoxylin-Eosin (H.E.) and covered with a cover glass. The observation was done using a light microscope [20].

Quantitative data analysis was carried out with ImageJ software's help to calculate the area of Langerhans Island and the number of cells in Langerhans Island. The cell density was calculated as follows:

$$\text{Density (cell/10\^4um2)} = \frac{\text{The number of Langerhans Island cells}}{\text{Langerhans Island area (um2)}} \times 10\text{\^4} \quad (1)$$

N. Immunohistochemistry Assay

The immunohistochemical staining carried out in this study refers to the method developed by [21] using the

MACH 1 Universal HRP-Polymer Detection kit (Biotin-Free Detection Polymer Detection Kit 901-M1U539-080717 (Biocare Medical®, USA). The staining process began with deparaffinization and rehydration processes. After rehydration, the tissue was soaked in distilled water then phosphate buffered saline (PBS). The tissue was immersed in 10% H_2O_2 with methanol as a solvent to remove endogenous peroxides, then rinsed with PBS. The protein blocking process was carried out by immersing the tissue using a Background Sniper solution (Biocare Medical®, USA) and 10% normal serum in PBS.

The tissue was then immersed in trypsin solution with 0.2% $CaCl_2$ solvent then rinsed with PBS. Anti PDX1 (PDX1 Polyclonal Antibody (Elabscience®, E-AB-15824, USA) was applied at a concentration of 1:50 with PBS solvent, then incubated for two nights (2x24 hours) at 4ºC then rinsed with PBS. MACH 1 Mouse Probe application (UP537L10, Biocare Medical®, USA) was carried out by incubating the tissue at 37ºC for 15 minutes, then rinsed with PBS.

After that, MACH 1 Universal HRP-Polymer (MRH538L10, Biocare Medical®, USA) was applied and incubated at 37ºC for 30 minutes then rinsed again with PBS. Chromogen 3,3'-Diaminobenzidine (Betazoid DAB chromogen, BDB900G, Biocare Medical®, USA) was added in the amount of 4 µL in 1000 µl of Betazoid DAB substrate buffer solution (DS900L10, Biocare Medical®, USA) to see the visualization of antigens in tissues. After that, the tissue was rinsed with distilled water and immersed in a solution of hematoxylin as a counterstain. The tissue was then covered with a cover glass and Entellan® (mounting) [20].

O. Statistical Analysis

Statistical analysis used in this study was One Way ANOVA followed by Tukey's HSD Post Hoc Test, with data requirements that were normally distributed and homogeneous variant. If the data didn't meet these requirements, a non-parametric statistical test was used, namely the Kruskal Wallis test, and continued with the Mann-Whitney U test with $\alpha = 0.05$ using the SPSS Statistics 20.0 computer program. The significance value was determined with a p-value ≤ 0.05.

III. RESULTS

A. Phytochemicals Assay

Based on the results shown in Table I, MSE contained a group of phenolic compounds like steroids, terpenoids, tannins, flavonoids, and alkaloids. The highest phytochemical compounds in MSE were alkaloids, while the MSE didn't contain triterpenoids and saponins compounds.

IV. PHYTOCHEMICAL ASSAY OF MSE

Phytochemical Content	MSE Extract
Phenols	++
Steroids / Triterpenoids	+/-
Terpenoids	+
Saponins	-
Tannins	+
Flavonoids	+
Alkaloids	+++

Notes: ++++ = very high content; +++ = high content; ++ = medium content; + = low content; - = negative content

A. Effect of MSE on Serum Glucose and Insulin Levels in Diabetic Rats

The test results of serum glucose levels in Fig. 1 and showed that giving MSE could reduce serum glucose levels in DM rats. MSE 600 mg/kg BW could lower serum glucose levels on the 21st day, and almost as good as metformin, a commercial drug for diabetes.

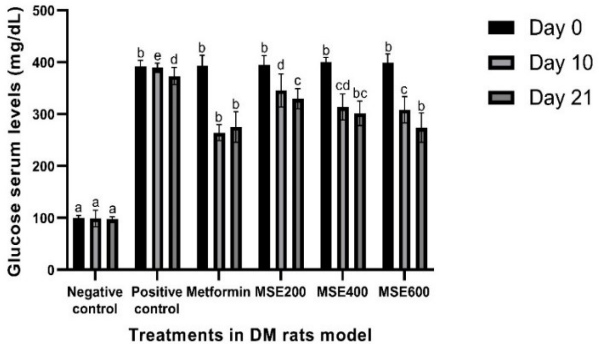

Fig. 1. Effect of MSE on glucose serum levels in DM rats model.
Notes: *Lowercase differences (a, b) on day-0 showed significant differences among treatments ($p < 0.05$, Anova-Tukey's HSD post hoc test)
*Lowercase differences (a, b, c, cd, d, e) on day-10 showed significant difference among treatments ($p < 0.00$, Mann Whitney)
*Lowercase differences (a, bc, c, d) on day-21 showed significant difference among treatments ($p < 0.00$, Mann Whitney)

The study results on serum insulin levels could be seen in Fig. 2. The results showed that MSE could increase insulin levels in DM rats. MSE administration at a dose of 600 mg/kg BW significantly increased insulin levels compared to positive controls and was better than metformin.

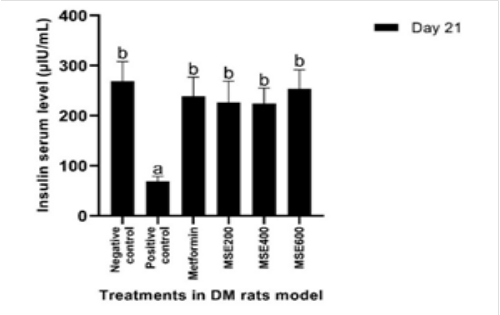

Fig. 2. Effect of MSE on insulin serum levels in DM rats model.
Data presented as mean ± standard deviation. Lowercase differences (a,b) showed significant difference among treatments ($p < 0.00$, Mann Whitney).

B. Effect of Melinjo Extract on Density of Langerhans Island Levels in Diabetic Rats

Based on the study results, the STZ-induced sample had a lower cell density when compared to the negative control. The results of histopathological observations on Langerhans Island showed that MSE at a dose of 600 mg/kg BW could reduce β-pancreatic cell damage when compared with the positive group. The result was supported by the density value of Langerhans Island in Table II, which showed that MSE could increase the density to 64.15±8.72 cells/10^4µm^2 compared to the positive control, namely 54.36±10.04 cells/10^4 µm^2.

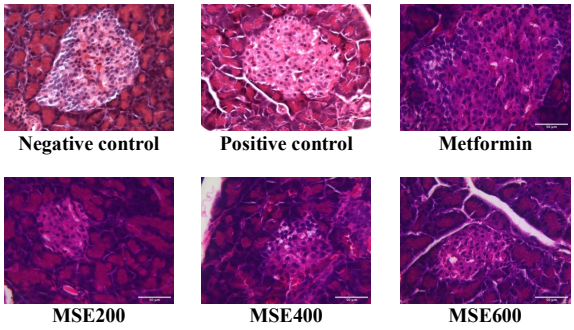

Fig. 3. Effect of MSE on the density of Langerhans island in DM rats model.

V. Effect of MSE on the Density of Langerhans Island in DM Rats Model

Density of LCs	Treatment Group					
	(-) control	*(+) control*	*Metformin*	*MSE 200*	*MSE 400*	*MSE 600*
1	81.87	58.75	63.90	49.34	59.82	74.03
2	90.39	68.92	61.50	52.23	57.91	70.22
3	77.68	53.68	67.73	66.73	45.26	63.42
4	67.46	43.81	75.59	40.33	79.91	61.71
5	99.34	46.62	68.77	53.36	45.40	51.36
Mean ± STD	83.35± 12.16[b]	54.36± 10.04[a]	67.50 ± 5.39[ab]	52.40 ± 9.50[a]	57.66 ± 14.18[a]	64.15 ± 8.72[ab]

Notes: LCs:Langerhans Cell (cells/10^4um^2); (-)control:negative control; (+) control:positive control. Data were presented as mean ± standard deviation. Different small letters (a,ab,b) in the same column showed significant difference between treatment at $P < 0.05$ (Tukey HSD post hoc test).

A. Effect of Melinjo Extract on PDX1 Expression in Diabetic Rats

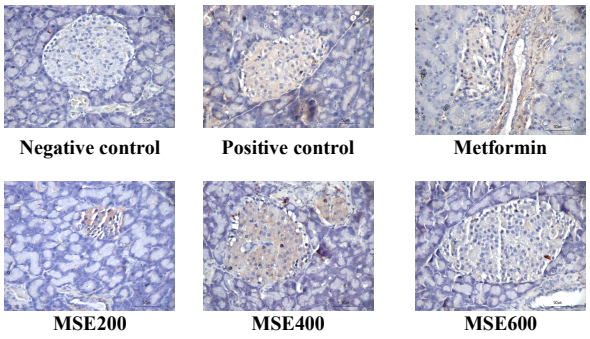

Fig. 4. Effect of MSE on the expression of PDX-1 in Langerhans Island cells.

The IHC test results showed that there was regeneration in Langerhans Islet cells treated by MSE. The brown color indicated the presence of PDX-1 protein. There was some oddity that the positive and negative control looked reversed because in the negative control, PDX-1 should be produced but in the positive control, PDX-1 shouldn't be produced. At MSE600 the concentration of PDX-1 was lower than MSE400. But based on the quantitative results, PDX-1 expression increased in Langerhans Islet cells as shown in Table III.

VI. Effect of MSE on PDX-1 Expression in DM Rats Model

PDX-1	Treatment Group					
	(-) control	*(+) control*	*Metformin*	*MSE 200*	*MSE 400*	*MSE 600*
1	160	91	83	59	106	164
2	94	75	91	139	115	70
3	143	74	135	122	101	128
4	114	96	92	49	120	101
5	104	31	101	123	69	111
Mean ± STD	123.00 ± 27.62	73.40 ± 25.60	100.40 ± 20.37	98.4 ± 41.24	102.2 ± 19.99	114.8 ± 34.67

Notes: (-)control:negative control; (+) control:positive control. Data were presented as mean ± standard deviation.

VII. Discussion

Melinjo (*Gnetum gnemon* L.) seeds contain several bioactive compounds such as phenols, alkaloids, saponins, tannins, and flavonoids [7] [22] [23]. The results of this study indicate that MSE contains phenols, steroids, terpenoids, tannins, flavonoids, and alkaloids. The differences in compound content can be caused by climate, temperature, humidity, and soil conditions. In addition, the level of maturity can also affect the synthesis of secondary metabolites in plants [24] [25] [26] [27].

According to the results on serum glucose and insulin levels, MSE 600 mg/kg BW could lower serum glucose levels increase insulin levels in DM rats. This probably due to secondary metabolites in MSE, especially polyphenols, which acted as antidiabetic agents. Polyphenol compounds in MSE also could reduce oxidative stress [28], which occurred due to hyperglycemia conditions in DM [29] [30] [31].

Preventing the reaction of superoxide to hydrogen peroxide could reduce oxidative stress. Hydrogen atoms from the aromatic hydroxyl group (-OH) in polyphenol compounds could bind free radicals and remove them from the body through the excretory system. The role of these polyphenolic compounds is thought to protect pancreatic β-cells from the effects of free radicals produced under hyperglycemic conditions [7]. This decrease in oxidative stress could help in reducing insulin resistance and protect β-pancreatic cells [31]. The effect of MSE in reducing serum glucose and increasing serum insulin levels is because MSE could increase high molecular weight adiponectin, which decreased the risk of diabetes mellitus [6].

DM caused damage characterized by a decrease in pancreatic β-cells and a decrease in the size of the islets of Langerhans [3]. The low cell density indicated that there was damage due to cell vacuolization on Langerhans islands, arrow in Fig. 3. The results of this study showed that MSE at a dose of 600 mg/kg BW could reduce pancreatic β-cell damage when compared to the positive group. This was due to secondary metabolites in MSE that could regenerate pancreatic β-cells [32] [32].

In the qualitative results of IHC assay, the negative control didn't have PDX-1. It might be because the antigen retrieval method is not sufficient enough to expose PDX-1 so it couldn't be detected by antibody. The dark color in positive control happened because the β-cell already regenerated and released the PDX-1. The function of PDX-1 is to regulate the expression of β-cell specific genes and assist in the maintenance of blood glucose levels. PDX-1 also plays important role in pancreas development [34].

Chronic hyperglycemia caused β-pancreatic cell dysfunction through reduced PDX-1 expression [5]. In the positive control group, the PDX-1 expression value was lowest compared to the others group. This indicated that PDX-1 expression in β-pancreatic cells was reduced in DM rat model. MSE600 had ability to increase in PDX-1

expression compared with positive control. This showed that MSE could increase PDX-1 expression.

VIII. Conclusion

In conclusion, Melinjo (*Gnetum gnemon* L.) seed extract has antidiabetic activities through decrease serum glucose and increase insulin levels, increase Langerhans cells density, and PDX-1 expression of Langerhans.

Acknowledgment

This study was facilitated, and supported by Aretha Medika Utama Biomolecular and Biomedical Research Center, Bandung, Indonesia. The author also thankful to Muhammad Aldi Maulana, Cahyaning Riski Wijayanti, Hanna Sari Widya Kusuma, and Ervi Afifah from Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia for their valuable assistance.

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