

Original Research Paper

Assessment of the *Annona muricata* Leaf Ethanol Extract Effect on The Diameter of Pancreatic Islets in Alloxan-Induced Mice

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Abstract: Diabetes mellitus is one of a non-communicable disease whose prevalence continues to increase from year to year in both developed and developing countries. In Indonesia, it is estimated that 12 million people aged ≥ 15 years old had diabetes mellitus in 2013. Treatment of diabetes mellitus by using plant extract has been reported to have good results. One of the plants that have antidiabetic effects is *Annona muricata*. *Annona muricata* leaf extract was found to increase pancreatic beta-cell regeneration. This study aimed to determine the effect of Soursop Leaf Ethanol Extract (SLEE) on the diameter of pancreatic islets in alloxan-induced mice. This study used a true experimental design with posttest-only control group design. The samples used were 30 male Swiss Webster mice with 12-14 weeks of age and 20-30 grams of body weight. Samples were then randomly divided into 5 groups, induced with alloxan and treated for 14 days. The group consisted of: negative control, positive control that was treated with 0,65 mg/kg BW of glibenclamide, P1 that was treated with 150 mg/kg BW, P2 with 300 mg/kg BW and P3 with 600 mg/kg BW of SLEE. The diameter of pancreatic islets was measured by histopathological examination stained with Hematoxylin-Eosin (HE). Data were analyzed by one-way ANOVA test and followed by Bonferroni post hoc test. Bivariate analysis showed a significant difference between negative control group with P1 ($p = 0.007$) and P2 ($p = 0.004$) group. Therefore, soursop leaf ethanol extract given in a dose of 300 mg/kg BW was the most effective in increasing the diameter of pancreatic islets.

Keywords: *Annona Muricata*, Diabetes Mellitus, *in vitro* Analysis, Soursop Leaf Extract

Introduction

Diabetes mellitus is an endocrine, metabolic disorder characterized by hyperglycemia or an increase in blood sugar levels due to impaired insulin secretion, insulin resistance, or both (Gohari *et al.*, 2018). Diabetes mellitus is one of the major health problems in both developed and developing countries due to its prevalence that continues to increase from year to year. A study states that in 2017, 451 million people were suffering

from diabetes mellitus worldwide (Cho *et al.*, 2018). That number is expected to increase to 693 million by 2045 (Cho *et al.*, 2018). Based on data from the Basic Health Research in 2013, there were 12 million people aged ≥ 15 years old who had diabetes mellitus in Indonesia (Soewondo, 2014).

Untreated diabetes mellitus is associated with several complications that attack various organ systems (AmericanDiabetesAssociation, 2016). The progressive development of the disease makes diabetes mellitus

require proper treatment. Insulin therapy, oral hypoglycemic agents and lifestyle modification are now the recommended treatment regimens. The main target of medical therapy for diabetes mellitus is a decrease in blood sugar levels. This can be achieved through several mechanisms, such as stimulation of insulin secretion, increased use of glucose by the tissues, delayed the absorption of carbohydrates by the small intestine and decreased glucose production by the liver (Bathaie *et al.*, 2012). However, conventional therapy also has some side effects, such as hypoglycemia or weight gain by the exposures of sulfonylurea, hepatotoxicity by the biguanide and gastrointestinal disorders due to flatulence or abdominal colic by the α -glucosidase inhibitor (Kokil *et al.*, 2015).

Traditional treatments using plant extracts in the treatment of diabetes mellitus are reported to have good results (Büyükbalci and El, 2008, Prabhakar and Doble, 2011). Ethnobotany information shows that there are currently more than 800 plants that can be used as alternative therapies because they contain various phytoconstituents that are beneficial to health, although some of them have not yet received scientific supervision (El-Abhar and Schaalan, 2014; Moezi *et al.*, 2018). In contrast, some plant species are known to have been tested pharmacologically and proven has antidiabetic effects (Moezi *et al.*, 2018). Some components in plants such as polyphenols are known to suppress the activity of certain enzymes involved in glucose absorption, regulate insulin signaling and increase the regeneration of pancreatic beta cells (Miranda-Osorio *et al.*, 2016).

One of the plants that are widely used in herbal medicine is *Annona muricata* or commonly known as soursop. *Annona muricata* is a lowland tropical plant that belongs to the family Annonaceae (Moghadamtousi *et al.*, 2015). Various parts of the *Annona muricata* tree, such as the roots, leaves and seeds can be used as herbal medicines because they are known to have various benefits, such as antihypertensive, anticonvulsant and antimicrobial (Florence *et al.*, 2014). Several *in vivo* studies regarding the potential of *Annona muricata* leaf in reducing blood glucose levels has been done. *Annona muricata* leaf extract was found to have antioxidant activity, which plays an essential role in the improvement of pancreatic beta cells (Muthu and Durairaj, 2015).

Materials and Methods

Material

This study used a true experimental with posttest-only control group design and has passed the ethical review from The Ethics Committee of FMUI (No.

3730/UN2.F1/ETIK/PPM.00.02/2019). Soursop leaf ethanol extract was obtained from the Biopharmaca Research Center, IPB University. Tissue slides stained with hematoxylin-eosin were made at the Department of Pathological Anatomy, FMUI.

Animal Preparation

Male Swiss Webster mice with 12-14 weeks of age and 20-30 grams of body weight were obtained from Animal Laboratory of the Center for Health Research and Development, Ministry of Health Republic of Indonesia. Animals were acclimatized for 7 days. The environment was maintained at 25°C with a 12 h lighting cycle. Food and water were given ad libitum. Mice were randomly divided into 5 groups, where each group consisted of 6 mice. All mice were induced with 40 mg/kg BW of alloxan by intraperitoneal injection. Three days after the alloxan administration, fasting blood glucose was measured to ensure the levels had reached ≥ 200 mg/dL. Blood glucose measurements were assessed with a glucometer, where blood was drawn from the tail vein.

Mice were treated for 14 days. The negative control group was only given a standard diet, while the positive control group, besides being given a standard diet, it was also treated with 0.65 mg/kg BW of glibenclamide orally. The glibenclamide dose was calculated by converting its dose for human to 30 grams of mice. Glibenclamide function is reported to improve glycemic control in streptozotocin-induced diabetic rats (Erejuwa *et al.*, 2011). Besides being given a standard diet, the P1 group was also treated with 150 mg/kg BW of SLEE, the P2 group with 300 mg/kg BW of SLEE and the P3 group with 600 mg/kg BW of SLEE. Soursop leaf ethanol extract was given orally.

All animals were euthanized on the 15th day after injecting ketamine intramuscularly. Surgery was performed to remove several organs, one of which was the pancreas. The pancreas was then stored in a pot containing formaldehyde. There were only 25 mice that could be analyzed because 5 mice died in the middle of the study. Therefore, the sample size was reduced to 5 mice/group.

Hematoxylin-Eosin Staining

Pancreatic tissues that have been soaked in formalin solution were cut to a thickness of 0.3-0.5 mm. The tissues were put into a machine to be dehydrated and vacuumed. Liquid paraffin was poured on top of the tissues. The paraffin blocks that have been frozen were cut to a thickness of 3-5 μ m and placed on an object glass that has been smeared with adhesive. Slides were soaked in xylol (I, II and III) to remove the attached paraffin, followed by ethanol (90%, 75% and 70% consecutively) to remove the remaining xylol and

rinsed with water. Slides were stained in hematoxylin solution, rinsed with water, soaked in lithium carbonate solution, rinsed with water and stained in eosin solution. Slides were dehydrated by soaking them in ethanol (70%, 75% and 90% consecutively) and xylol (I, II and III). One drop of adhesive liquid was added and slides were covered with cover glass.

Diameter of Pancreatic Islets Measurement

Slides that have been stained with hematoxylin-eosin were observed on a computer connected to an Olympus microscope and Axiocam camera using the Axiovision software. The diameter of the pancreatic islets was observed in all fields of view. At 400 times magnification, pancreatic islets measurement was done by drawing a line from one point to another through the center of an area that looks paler and has round-shaped cells.

Statistical Analysis

The Shapiro-Wilk test was used to determine the data normality given the sample size of ≤ 50 samples. All groups were normally distributed ($p > 0.05$), thereby the comparative hypothesis test used was one-way ANOVA followed by Bonferroni post hoc analysis.

Results

P2 group that was treated with 300 mg/kg BW of SLEE had the largest mean diameter, followed by P1 group, which was treated with 150 mg/kg BW of SLEE and positive control group (Fig. 1).

The detail results of the calculation of the mean of pancreatic islet diameters are listed in Table 1. Bonferroni post hoc test showed significant differences between the negative control group with P1 ($p = 0.007$) and P2 ($p = 0.004$) group. The three groups that were treated with SLEE showed no significant differences when compared to the positive control group.

Histopathological appearances of the diameter of pancreatic islets are depicted in Fig. 2. glucose-stimulated insulin secretion in pancreatic islets showed that P2 treatment has the highest density of insulin secretion in the pancreatic islets. Samples were stained with hematoxylin-eosin, observed in 400 times magnification of microscopic observation. The treatments were divided into 5 group mice, including: A = negative control. B = positive control. C = P1 group treated with 150 mg/kg BW of SLEE. D = P2 with 300 mg/kg BW of SLEE. E = P3 with 600 mg/kg BW of SLEE.

It can be seen that P2 treatment is the most potential in enhancing insulin secretion in pancreatic islets.

Table 1: Mean of diameter of pancreatic islets

Groups	Treatment	Mean of pancreatic islet diameters (μm)*	<i>p</i> -score
K (-)	Streptozotocin (STZ)	16.83 \pm 3.55	0.001**
K (+)	STZ + glibenclamide	27.29 \pm 9.78	
P1	STZ + SLEE 150 mg/kg	31.31 \pm 3.85	
P2	STZ + SLEE 300 mg/kg	32.29 \pm 4.14	
P3	STZ + SLEE 600 mg/kg	21.24 \pm 4.95	

*Data are expressed as mean \pm standard deviation; ***p*-score was significantly different

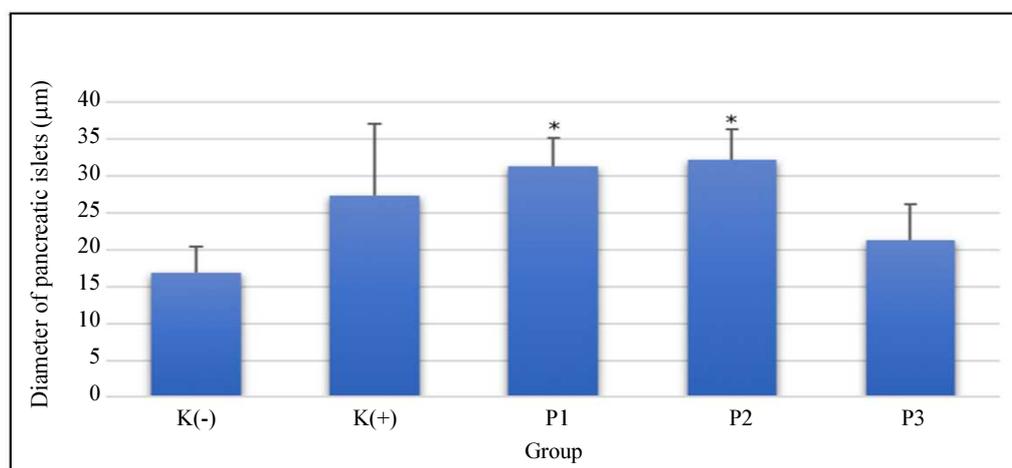


Fig. 1: Mean diameter of pancreatic islets in all groups. K (-) = negative control. K (+) = positive control. P1 = treated with 150 mg/kg BW of SLEE. P2 = 300 mg/kg BW of SLEE. P3 = 600 mg/kg BW of SLEE. * = significant difference with negative control group

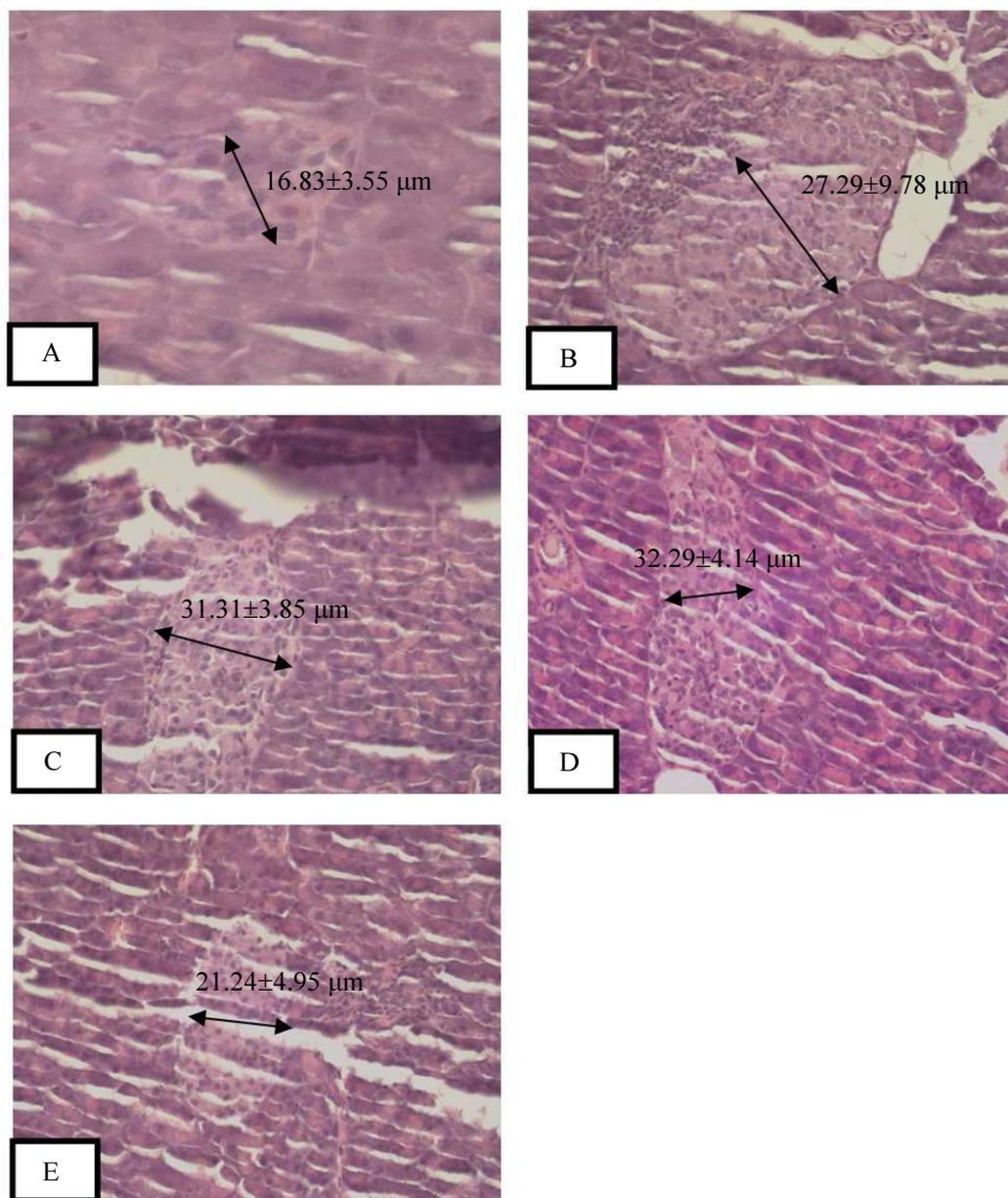


Fig. 2: Histopathological appearances of pancreatic islets. Samples were stained with hematoxylin-eosin, observed in 400 times magnification. A = negative control. B = positive control. C = P1 group treated with 150 mg/kg BW of SLEE. D = P2 with 300 mg/kg BW of SLEE. E = P3 with 600 mg/kg BW of SLEE. Black arrows represent the diameter size limit of pancreatic islets

Discussion

Pancreas ability to release insulin is mainly determined by the size and neogenesis of pancreatic islets and the rate of beta-cell replication and apoptosis. Pancreatic islets have a variety of sizes, ranging from several to thousands of cells. In rodents, beta cells are located in the nucleus, whereas alpha and delta cells are located peripherally. The size of pancreatic islets in mammals generally range from 50-200 μm, regardless

of species. The size of pancreatic islets is relatively in the same range, although its total number increases along with the size of the species (Hosseini *et al.*, 2015; Jo *et al.*, 2012; Kim *et al.*, 2009; Deng *et al.*, 2004). Direct measures of insulin resistance such as steady-state plasma glucose concentration or hyper-insulinemic-euglycemic clamp could be necessary to reduce measurement errors (Li *et al.*, 2010, Justino *et al.*, 2018).

The architecture and composition of pancreatic islets can be influenced by physiological and pathological

processes. Increased metabolic demand, such as in obesity and pregnancy is generally followed by an increase in beta-cell mass. During pregnancy, beta-cell mass is found to increase two to five times through neogenesis. In adult rodents, beta-cell replication is very slow and neogenesis no longer occurs. A study demonstrated in nonobese mice with type 1 diabetes mellitus (DM), revealed a loss of smaller pancreatic islets and regeneration of larger pancreatic islets to compensate for the beta-cell damage. In contrast, subjects with type 2 DM experienced impairment of larger pancreatic islets and changes in composition marked by a decrease in beta-cell mass and an increase in the proportion of alpha cells (Deng *et al.*, 2004; Jo *et al.*, 2012; Kim *et al.*, 2009).

Bonferroni post hoc test carried out in this study showed that there were significant differences between the negative control group with P1 (treated with 150 mg/kg BW of SLEE) and P2 (treated with 300 mg/kg BW of SLEE) group. Administration of soursop leaf ethanol extract in low and moderate doses can increase beta cell regeneration, which then increases the diameter of pancreatic islets. In contrast, the negative control group that did not receive therapy had the smallest diameter. These results are in line with research conducted by Adeyemi *et al.* (2010). The group treated with *Annona muricata* leaf extract had a larger diameter than the group that did not receive therapy thus indicating that the bioactive compounds contained in *Annona muricata* leaf could increase beta-cell proliferation and stimulate insulin release (Adeyemi *et al.*, 2010).

The enzymatic and non-enzymatic antioxidants contained in the *Annona muricata* leaf extract can inhibit the formation of reactive oxygen species. Enzymatic antioxidants such as glutathione reductase, catalase and superoxide dismutase as well as non-enzymatic antioxidants such as carotenoids, vitamin C, flavonoids, tannins and lycopene can prevent apoptosis and trigger beta cell regeneration. Increased antioxidant capacity of beta cells will inhibit lipid peroxidation and accumulation of reactive oxygen species, thus protects it from apoptosis, necroptosis, or autophagy. Aside from being an antioxidant, flavonoids can affect insulin signaling and inhibit alpha-glucosidase enzyme thereby reducing glucose absorption. *Annona muricata* leaf is also found to contain chromium, zinc and magnesium, which play a role in insulin production (Mohamed *et al.*, 2017; Ghorbani *et al.*, 2019; Hosseini *et al.*, 2015; Muthu and Durairaj, 2015).

Several phytochemicals, such as acetogenin derivatives in *A. muricata* leaves have shown neurotoxicity (coreximine with EC₅₀ at 13 µM, reticuline EC₅₀ at 304 µM and annonacin EC₅₀ at 0.018 µM) in vitro and in vivo studies due to its viability reduction of mesencephalic dopaminergic neurons (Lannuzel *et al.*,

2003, Wahab *et al.*, 2018). However, it has been investigated that the synergistic interactions among flavonoids and acetogenins in *A. muricata* leaves confer protection against prostate cancer (Yang *et al.*, 2015).

Conclusion

Soursop leaf ethanol extract can increase the diameter of pancreatic islets in alloxan-induced mice. Administration of low (150 mg/kg BW) and moderate (300 mg/kg BW) doses of SLEE were statistically significant in increasing the diameter, but administration of moderate dose was the most effective. This phenomenon might happen to medicinal plant extracts due to the higher dose might be interfering the efficacy of the bioactive compounds in the plant extracts. Thus, these bioactive compounds which responsible as antidiabetic agents might have a dose-related response.

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Author's Contributions

Syafira N. Dewi: Performing the experiment, visualizing, analyzing and validating the data.

Supri I. Handayani: Conceptualizing, designing the experiments, writing-review and editing the manuscript.

Marini Stephanie: Performing the experiment and writing the manuscript.

Siti Nurbaya: Performing the experiment, analyzing and validating the data.

Vivitri D. Prasasty: Analyzing the data, writing-review and editing the manuscript.

Ethics

The authors agree with their equal contributions; thus, declare that there is no conflict of interest in this matter.

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