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The Effect of *Annona muricata* Leaf Ethanolic Extract on Insulin Expression and Glucagon-Like Peptide-1 Level in Alloxan-Induced Mice

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ABSTRACT

Annona muricata (Annonaceace) or commonly known as soursop is known to have an antidiabetic effect. Soursop leaf extract was found to increase pancreatic beta-cell regeneration. This study aimed to determine the effect of soursop leaf ethanolic extract (SLEE) on fasting blood glucose (FBG), insulin expression and plasma glucagon-like peptide-1 (GLP-1) level in alloxan-induced diabetic mice. This study used a post-test-only control cohort design. The designed animal cohorts of 30 male Swiss Webster mice were used in this study by dividing into six cohorts consisted of healthy control, negative control, and positive control that was treated with 0.65 mg/kg BW of glibenclamide. The other three cohorts were P1, P2, and P3 that were treated with SLEE in 150 mg/kg, 300 mg/kg and 600 mg/kg BW, respectively. Histopathological examination with immunohistochemical (IHC) staining of pancreatic beta-cells was observed to obtain the morphological insulin expression. The results showed that the SLEE administration performed significantly decreased FBG from day 7 to day 14 for all cohorts ($p < 0.05$). It also indicated significantly different in CN cohort and P2 cohort ($p < 0.05$) for the insulin expression. Moreover, statistical differences were also found in CN cohort and other 5 cohorts for plasma GLP-1 level ($p < 5$). Histopathological changes also confirmed insulin expression significantly increased in pancreatic islets after treatment with medium dose of SLEE in P2 cohort (300 mg/kg), continued with P3, CP, CN, and P1 cohorts, respectively. It can be concluded *A. muricata* leaf ethanolic extracts were potential in treating diabetic disease.

Keywords: Diabetes mellitus, *Annona muricata*, glucagon-like peptide-1, insulin expression

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INTRODUCTION

The prevalence of diabetes mellitus throughout the world has continued to increase over the past two decades. The International Diabetes Federation estimates there are 451 million people who suffer from diabetes mellitus worldwide in the year 2017 [1]. The prevalence is estimated to increase to 693 million people in the year 2045 [1]. Indonesia is known to be the seventh-largest number of diabetic patients with age > 20 years (around 7.6 million) in the world in 2012, in which more than 70% of cases were estimated to have not been diagnosed [2].

Diabetes mellitus disease is associated with several complications in various organ systems, such as kidney, eyes, nervous system, and heart [3]. The progressive disease development of diabetes mellitus requires appropriate treatments, including insulin therapy, oral hypoglycemic remedy, and healthy lifestyle are now the recommended treatment regimens [4]. The main target of medical treatment for diabetes mellitus is to reduce blood sugar level. It could be achieved by several mechanisms: stimulating insulin secretion, penetrating blood glucose by body tissues, delaying the absorption of carbohydrates by the small intestine, and lowering glucose production by the liver [5]. However, conservative therapy also has several severe impacts, for example, hypoglycemia or weight gain body, which can be caused by sulfonylureas, hepatotoxicity by the biguanide cohort, and gastrointestinal disorders in the form of flatulence or abdominal colic by the α-glucosidase inhibitors [6].

Traditional treatments using plant extracts in the treatment of diabetes mellitus are reported to have good results. Current ethnobotany presents that more than 800 plants are used as alternative therapies due to they contain various phytoconstituents such as flavonoids that are beneficial to health [7]; however, some of them have not yet received scientific report of antidiabetic effect [8]. 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 [9].

One of the medicinal plants that are widely used in traditional medicine is *Annona muricata* or soursop. *A. muricata* is a lowland tropical plant that belongs to the family of Annonaceae [10]. Parts of the *A. muricata* tree from the roots, leaves, until the seeds can be used as traditional medicines due to it has various benefits, such as antihypertensive, anticonvulsant, and antimicrobial properties [10]. *A. muricata* is also known to have potential as an anticancer [11]. Many in vivo studies on the effect of *A. muricata* leaves on decreasing blood glucose levels in subjects

with diabetes mellitus have been conducted [12]. *A. muricata* leaf extract was found to have antioxidant activity that plays an essential role in the repair of pancreatic beta-cells [12, 13]. This study aimed to investigate the effect of *A. muricata* leaf ethanolic extract on insulin expression and GLP-1 level in the plasma. Insulin is mainly produced by pancreatic beta-cells [14]. The enhance in insulin expression might be affected by the increasing glucagon-like peptide-1 (GLP-1) level, which is considered as one of the incretin hormones that play a role in stimulating insulin release [15].

MATERIALS AND METHODS

Extraction of A. muricata Leaves

A. muricata leaves were washed and dried at room temperature. The simplicia of *A. muricata* leaves (dried leaves) were ground and soaked in ethanol solution. After soaking for several hours, the supernatant was separated from the precipitate and evaporated to obtain a concentrated ethanolic extract [16] of 500 g. The extract of 50 mg was dissolved in 5 ml of distilled water before fed to mice models. The SLEE was screened its phytochemical substituents which may contribute as antioxidant agents to promote antidiabetes using qualitative analysis [17]. Total flavonoid content was determined by using colorimetric analysis [17].

Treatment of SLEE in Mice Cohorts

Thirty male Swiss Webster mice (12-14 weeks old, with body weight within 20-30 grams) were obtained from Animal Laboratory of the Center for Health Research and Development, Ministry of Health Republic of Indonesia. The mice were acclimatized for seven days in the maintained environment at 25°C with 12-hour light and dark cycle before being treated. Food and water were given *ad libitum.* Mice were randomly divided into six cohorts (Each cohort contained five mice): 1. Healthy/normal cohort (N), 2. Negative control (CN), 3. Positive control (CP), 4. Treatment 1 of 150 mg/kg (P1), 5. Treatment 2 of 300 mg/kg (P2), and 6. Treatment 3 of 600 mg/kg (P3). All mice were induced with alloxan 40 mg/kg body weight (BW) by intraperitoneal injection. Three days after administration of alloxan, fasting blood glucose (FBG) measurements were carried out to assure the FBG level had reached ≥ 200 mg/dL. The FBG measurements were done using a glucometer at the veins of mice tails [12].

The calculation to estimate the minimum sample size in this study was used Federer's formula [18, 19], as follow:

$$(t-1)\,(n-1) \geq 15$$
$$(6-1)(n-1) \geq 15$$
$$n \geq 4$$

Where:
t = Number of groups
n = Minimum sample size
15 = general degree of freedom

Mice with FBG levels ≥ 200 mg/dL were confirmed to have diabetes and subjected to the treatments for 14 days. The normal control (N) is healthy mice cohort without any treatment. The negative control cohort was only given a standard diet, while the positive control cohort was treated with glibenclamide 0.65 mg/kg per day. The glibenclamide was administered at 0.65 mg/kg BW. The treatment cohort 1, P1 was treated with soursop leaf ethanolic extract (SLEE) of 150 mg/kg BW, while treatment cohort 2 & 3 (P2 & P3) were daily treated with SLEE at 300 mg/kg BW, and 600 mg/kg BW, respectively. Each plant extract dose was given once a day to the mice. The FBG levels were determined at day-0, -7 and -14. All mice underwent dehydration conditions on the day-15. Euthanasia was done using ketamine drug injection intramuscularly. The pancreas was taken out and stored using formaldehyde. Arterial blood extraction from the aorta was also done and kept in a tube containing heparin. This research has approved by the University Health Research Ethical Committee of Universitas Indonesia with ethical letter number: KET-3730/UN2.F1/ETIK/PPM.00.02/2019.

Preparation of Immunostaining with Immunohistochemistry

The paraffin block was cut to a thickness of 3-5 mm and then placed on a glass object that has been smeared with poly-L-lysine. Deparaffinization was carried out in xylol solution. The preparations were immersed in ethanol solution with decreasing concentration to remove xylol residues. The preparation was rinsed with water flow and incubated in a Tris-EDTA buffer solution. The preparations were washed with phosphate-buffered saline (PBS) and incubated with the primary antibody kit (anti-insulin rabbit monoclonal antibody, ab181547, Abcam) for 1 hour.

The preparations were second washed with PBS and incubated with the secondary antibody kit (anti-rabbit peroxidase polymer, ab205718, Abcam) for 30 minutes. Next, the preparations were washed with PBS and incubated for 1 minute with diaminobenzidine tetrahydrochloride (DAB), then rinsed, immersed in hematoxylin, rinsed back, immersed in lithium carbonate solution, and rinsed back with water flow to remove lithium carbonate solution. Dehydration with ethanol solution with increasing concentration was done, followed by xylol immersion. In a wet state, the preparation was dripped with adhesive liquid and covered with a clean glass slide [20]. Placental tissue and the paraffin block in each cassette were selected as positive and negative controls, respectively.

Determination of Insulin Expression

The tissue preparations that have been daubed with immunohistochemical staining [21] were observed on a computer connected to an Olympus binocular microscope and Axiocam camera through the Axiovision program. The insulin expression from pancreatic beta-cells, which was marked with brownish color [22], was searched throughout the field of view and captured at 40x magnification to

determine the morphological focus assay. Calculation of insulin expression was done by the histoscore (H-score) formula with a semi-quantitative assessment of both values: the percentage of the etched cells with a range of values from 0-100% and the intensity of staining with the etched cell appearance within the range 0 to 300 (0 = unstained; 100 = weakly stained; 200 = medianly stained; 3 = strong stained). The range of possible scores was from 0 to 300 [23].

Determination of Plasma GLP-1 Level by The Enzyme-linked Immunosorbent Assay (ELISA)

Blood samples of mice were centrifuged to separate blood plasma. The anti-GLP-1 (ab184857, Abcam) coated plate was washed by adding a washing buffer in the microplate wells. The 50 µL sample and standard solutions were added and incubated in the microplate well, then rinsed with a washing buffer. The 50 µL anti-GLP-1 labeled biotin was added and incubated to all microplate well, then rinsed with washing buffer. The 50 µL of conjugated horseradish peroxidase (HRP) was added and incubated in the microplate well, then rinsed with washing buffer. The 50 µL chromogenic substrate was added and incubated before mixing the 50 µL reagent solution to stop the reaction. The absorbance was read at a wavelength of 450 nm using an ELISA reader device [21].

Statistical Analysis

The statistical data analyses were processed using the IBM SPSS (version 24) program. The one-way ANOVA was performed with a significant difference at $p < 0.05$. All data were expressed as mean ± standard error of mean (SEM).

RESULTS AND DISCUSSION

The fasting blood glucose (FBG) levels of mice were measured at day 0, day 7, and day 14, as depicted in Figure 1.

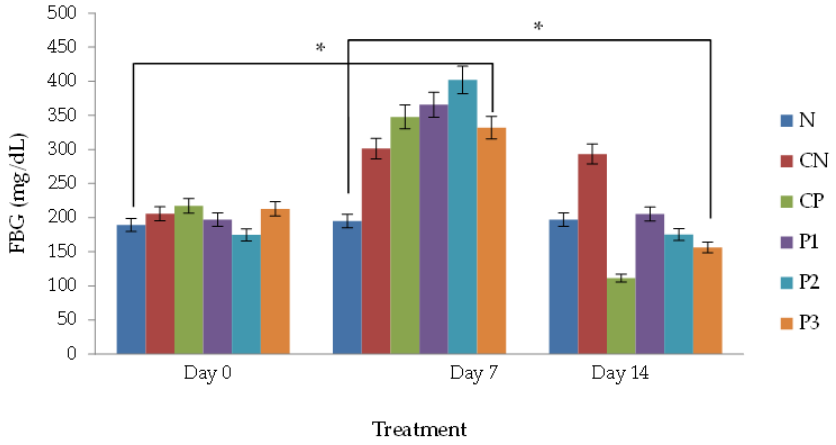

Figure 1. FBG levels of treated mice with SLEE. Data is displayed as a mean ± standard error of mean (SEM). Significant difference was marked as asterisk (*) with $p < 0.05$. All legend symbols are: N = healthy control; CN = negative control; CP = positive control, treated with 0.65 mg/kg BW of glibenclamide; P1 = SLEE 150 mg/kg BW; P2 = SLEE 300 mg/kg BW; P3 = SLEE 600 mg/kg BW.

FBG levels of all cohorts showed normal levels at day 0 (< 200 mg/dL). However, all cohorts were found to have significantly increased FBG levels over than 200 mg/dL which indicating diabetes from day 0 to day-7 ($p < 0.05$). After the SLEE treatments were given to P1, P2 and P3 cohorts, they were found to have significantly decreased FBG levels from day 7 to day-14 ($p < 0.05$). After mice cohorts being treated for 14 days, the index of insulin expression was measured by the H-score formula, as shown in Figure 2.

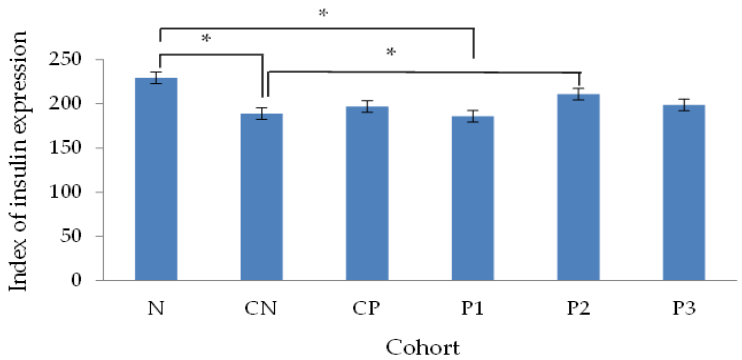

Figure 2. Insulin Expression of treated mice with SLEE. Data is displayed as a mean ± standard error of mean (SEM). Significant difference was marked as asterisk (*) with $p < 0.05$. All cohort symbols are: N = healthy control; CN = negative control; CP = positive control, treated with 0.65 mg/kg BW of glibenclamide; P1 = SLEE 150 mg/kg BW; P2 = SLEE 300 mg/kg BW; P3 = SLEE 600 mg/kg BW.

The P2 cohort was treated with a medium dose of SLEE 300 mg/kg had the largest average insulin expression index, while the P3 cohort treated with high dose SLEE had the second-highest average index of insulin expression followed by positive and negative controls. The low dose of P1 cohort had the smallest average insulin expression index. Figure 2 shows significantly different between CN and P2 cohorts ($p < 0.05$). Moreover, statistically different also found in N cohort with CN and P1 cohorts ($p < 0.05$).

We performed histological change of insulin expression with immunohistochemical (IHC) staining of mice pancreatic beta-cells in brown colors. The insulin staining significantly increased in pancreatic islets of mice after treatment with SLEE in P2 cohort, continued with P3, CP, CN, and P1 cohorts, respectively. The area of insulin expressions in pancreatic beta-cells were indicated with white arrows. The morphological insulin expression of pancreatic beta-cells can be observed in focus assay of immunohistochemical staining. as depicted in Figure 3.

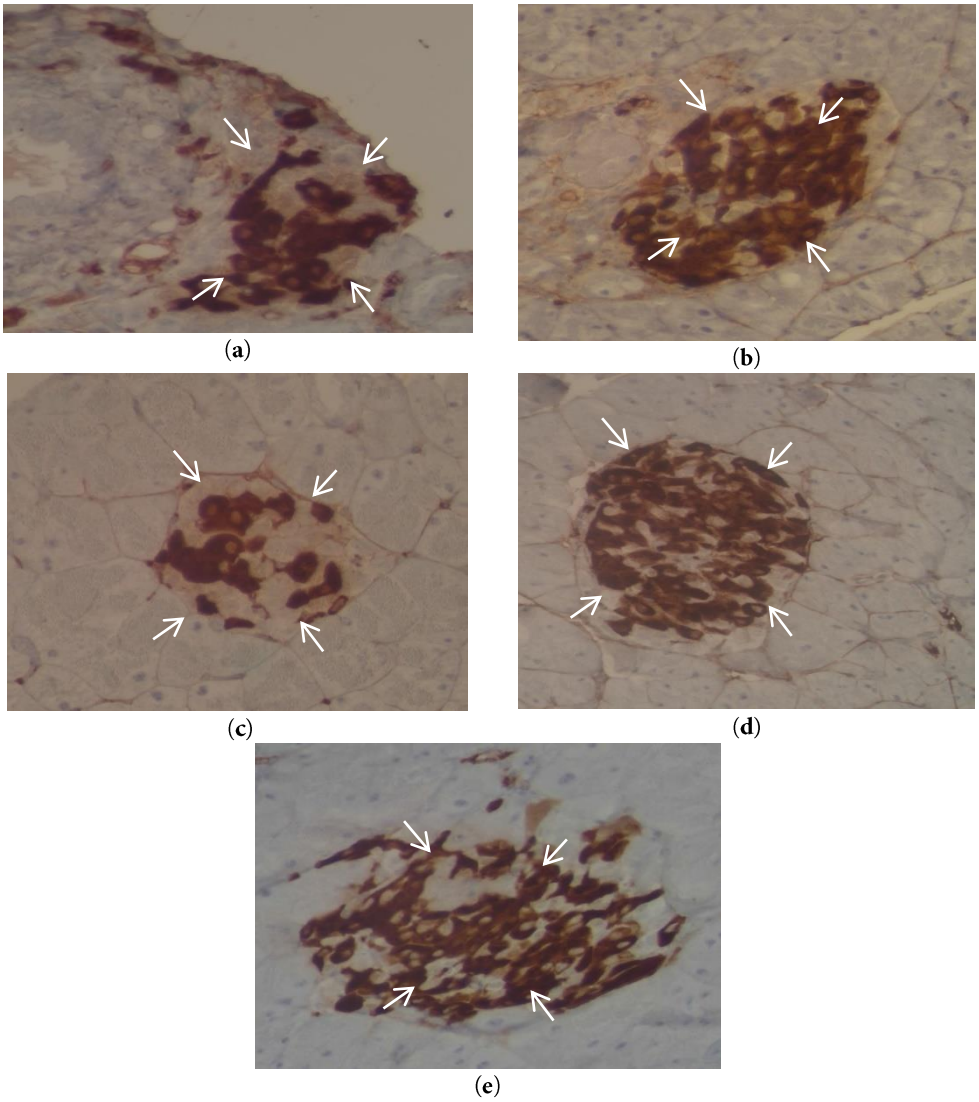

Figure 3. The morphology of insulin expression by pancreatic beta-cells. The cell preparations were daubed with immunohistochemical staining, observed at 40x magnification, in which: (**a**) negative control (CN); (**b**) positive control (CP); (**c**) low dose of 150 mg/kg (P1); (**d**) medium dose of 300 mg/kg (P2); (**e**) high dose of 600 mg/kg (P3).

The results of the one-way ANOVA test showed no significant differences, so that post hoc analysis was not necessary. The negative (CN) and positive (CP) cohorts did not show significant differences when compared to the treatment (P1, P2, P3) cohorts. The effect of SLEE on the plasma GLP-1 levels of the five mice cohorts treated for 14 days was measured by ELISA reader, as depicted in Figure 4.

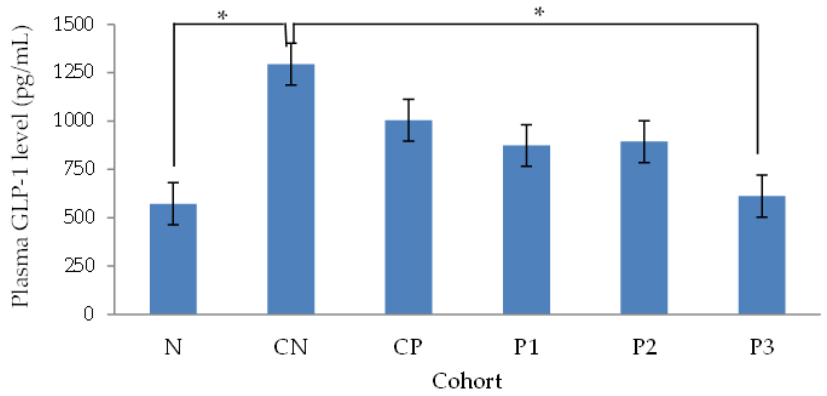

Figure 4. Plasma GLP-1 levels of treated mice with SLEE. Data is displayed as a mean ± standard error of mean (SEM). Significant difference was marked as asterisk (*) with $p < 0.05$. All cohort symbols are: N = healthy control; CN = negative control; CP = positive control, treated with 0.65 mg/kg BW of glibenclamide; P1 = SLEE 150 mg/kg BW; P2 = SLEE 300 mg/kg BW; P3 = SLEE 600 mg/kg BW.

The negative control as the normal mice (non-alloxan-induced mice) cohort, which did not receive SLEE treatment, had the highest average plasma GLP-1 level, followed by positive control, moderate dose (P2), and low dose (P1) cohorts. The high dose cohort (P3) had the lowest average plasma GLP-1 level. Healthy control (N) and the treatment of P3 cohorts showed a significant difference with the negative control cohort (CN) at $p < 0.05$.

Diabetes mellitus induction in this study was done by injecting alloxan into mice models. Alloxan stimulates the formation of reactive oxygen species, one of which is hydroxyl radicals, which cause beta-cell degradation, thereby reducing the quantity and quality of insulin [24]. Moreover, pancreatic beta-cells have low levels of free radical scavenger enzymes and sensitive to damage by oxidative stress [25, 26]. Alloxan can depolarize pancreatic beta-cells, thus opening calcium channels that are dependent on the stress condition, allowing calcium to enter, and accelerating the destruction of pancreatic beta-cells [27]. Another mechanism is to inhibit glucokinase and interfere with the formation of microtubules [24].

The P2 cohort treated with moderate-dose SLEE had the highest average insulin expression index, followed by the high dose cohort (P3), positive control (CP), and low dose (P1). It showed that the administration of SLEE, especially in moderate and high doses, can increase insulin expression in pancreatic beta-cells. The one-way ANOVA analysis showed that there was a significant difference between the insulin expression of the treatment cohort P2 with negative control ($p < 0.05$). These results were similar to some previous studies regarding the antidiabetic potential of the *A. muricata* leaves. Soursop leaf extract was reported to be able to increase insulin release by pancreatic beta-cells and decrease fasting blood glucose levels of diabetic rodents [28, 29]. It is also confirmed in our study that soursop leaf ethanol extract given in a dose of 300 mg/kg BW was the most effective in increasing the diameter of rat pancreatic islets [30].

The enzymatic and non-enzymatic antioxidants contained in the *A. muricata* leaf extract could 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 may prevent apoptosis and trigger beta-cell regeneration [31, 32]. Increased antioxidant capacity of pancreatic beta-cells could inhibit lipid peroxidation and accumulation of reactive oxygen species to protect it from apoptosis, necroptosis, or autophagy [33]. Aside from being an antioxidant, flavonoids can affect the insulin signaling pathway and inhibit the alpha-glucosidase enzyme, thereby may reduce glucose absorption [34, 35]. Some flavonoids have been found to increase insulin secretion by influencing the cAMP/PKA signaling pathway [36]. *A. muricata* leaves are also found to contain chromium, zinc, and magnesium, which play essential roles in insulin production [13, 37]. Pancreatic beta-cell regeneration itself occurs through several mechanisms, including replication of pre-existing pancreatic beta-cells and beta-cell neogenesis from other cells such as stem cells, Ngn3-labeled endocrine progenitors, pancreatic alpha-, delta-, and acinar cells [13, 38].

Glucagon-like peptide-1 (GLP-1) is an insulinotropic hormone secreted by enteroendocrine L-cells, which are mainly located distally in the small intestine in responding the nutrient stimuli [39]. The N terminal of the active GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase-4 so that it has a short half-life [40, 41]. In addition, GLP-1 can also induce proliferation and inhibit the death of pancreatic beta-cells to trigger its release through the increased insulin transcription. Moreover, GLP-1 release is triggered by ingestion. Triacylglycerol, glucose, several types of proteins and fructose are the useful nutrients that trigger GLP-1 secretions [42, 43]. Carbohydrates, especially glucose, can trigger the release of GLP-1 through several mechanisms, such as the activation of sweet taste receptors, K_{ATP}-channel closure, and SGLT1 activity [43, 44]. Carbohydrates that

cannot be digested by the human gastrointestinal tract, that have been found to increase GLP-1 release. Fat is another effective stimulator, although the response takes a longer time to appear than carbohydrates. Plasma GLP-1 level in the fasting state is generally in the range of 5-10 pmol/L in 10-15 minutes after eating and could reach up to 15-50 pmol/L in 40 minutes [43-45]. Patients with type 2 diabetes mellitus are reported to have a lower response to incretin release; thus postprandial insulin release is reduced. Several studies reported that fasting and postprandial GLP-1 level of type 2 diabetes mellitus patients are lower than subjects with normal glucose tolerance. The results of the one-way ANOVA test in this study showed that there was no significant difference of GLP-1 levels between the control cohorts (CN and CP) and the SLEE treatment cohorts. The CN cohort had the highest plasma GLP-1 level, followed by CP, P2, and P1 cohorts, respectively. There are several factors that might influence these results. The reversal effect of blood glucose to normoglycemia in the CN cohort caused plasma GLP-1 level to be higher than the other cohorts [44, 46]. The content of primary metabolites, such as fatty acids, glucose, and proteins and secondary metabolites in soursop leaves have the capability to stimulate GLP-1; however, it might not be too potent [46]. Moreover, measurement of GLP-1 level is also quite difficult to do in mice due to the hormone is metabolized by endopeptidase quickly. Besides, examinations that are currently available also generally require the large sample volumes around 100-700 µL [44, 46, 47].

The use of alloxan as an inducing agent for diabetes mellitus was found to have several limitations. Hyperglycemia due to alloxan induction is not stable enough and can only last in less than a month [24]. The alloxan-induced animal mortality rate was also reported to be quite high [48]. Diabetes mellitus caused by alloxan has characteristics similar to type 2 diabetes, where complications in the form of ketoacidosis are more common [24]. The mechanism of alloxan action also involves free radicals, notably hydroxyl radicals, which could cause more toxic [24, 27]. The high rate of mortality causes the results of bioactive compound evaluations may have less reliable antidiabetic potential [24]. Streptozotocin, another compound that is also widely used to induce type-2 diabetes mellitus, has several advantages over alloxan. Streptozotocin is relatively more stable due to it has a longer half-life. The duration of hyperglycemia due to streptozotocin also lasts longer, which is 3 months [49]. A previous study reported that streptozotocin-induced diabetic rats were administered with cycloart-23-ene-3b, 25-diol with sitagliptin, and L-glutamine with sitagliptin demonstrated antihyperglycaemic effect after 8 weeks treatment period [50]. Streptozotocin toxicity is reported to be lower due to its selective mechanism of action against pancreatic beta-cells [49]. Alloxan induces damage to all cells expressing GLUT2 [49]. Decreasing blood glucose level down to normoglycemia after diabetes mellitus induction is successfully carried out susceptible to intraperitoneal injection with an alloxan dose of 150 mg/kg BW. However, it was observed several limitations of alloxan, such as the decreasing blood glucose level in the CN cohort and the death of several mice in the middle of the experiment [24]. Therefore, future studies regarding the diabetic inducing agent of streptozotocin could be considered to assess the antidiabetic potential of SLEE, and cytotoxicity of SLEE treatment to evaluate the effectiveness of treatment dosage.

CONCLUSION

The ethanolic extract of soursop leaves in low dose, P1 (150 mg/kg BW) and high dose, P3 (600 mg/kg BW) were not significantly different in the insulin expression when compared with negative and positive controls. However, a moderate dose, P2 (300 mg/kg BW) and negative control (CN) was found significantly different ($p < 0.05$) to enhance insulin expression. Moreover, cohort of P3 (600 mg/kg BW) and negative control (CN) was found significantly different ($p < 0.05$) in increasing of GLP-1 level. These findings can serve as a reference for a potential antidiabetic agent.

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