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Original Article

EVALUATING THE EFFECT OF MATOA LEAVES ETHANOL EXTRACTS (*POMETIA PINNATA* J. R. FORST AND G. FORST) ON PANCREATIC BETA-CELLS INSULIN RELEASE

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ABSTRACT

Objective: Matoa Leaves Ethanol Extract (*Pometia pinnata* J. R. Forst and G. Forst) (MLEE) with flavonoid content has been proven to have antioxidant activity that can neutralize free radicals so it can potentially repair damage to pancreatic β-cells that produce the hormone insulin. This study aimed to determine the ability of Matoa Leaves Ethanol Extract (MLEE) to lower fasting blood glucose (FBG) levels and determine the increase in insulin expression of rats' pancreatic β-cells induced by alloxan 150 mg/KgBW.

Methods: Experimental research was conducted using Complete Randomized Design on 6 groups, including normal group, negative control, positive control (glibenclamide 5 mg/KgBW), and MLEE groups with 50, 100, and 200 mg/KgBW for 14 days. The efficacy of MLEE in insulin release can be assessed by its ability to reduce blood glucose levels and modulate insulin production in pancreatic β-cells. Expression is quantified based on the distribution and intensity of staining observed using the ImmunoHistoChemistry (IHC) method. The Fasting Blood Glucose (FBG) data and IHC scores were subjected to analysis using a one-way ANOVA.

Results: The results indicate that administering a dose of 50 mg/KgBW of MLEE for duration 14 days effectively reduced FBG levels to 143.25 mg/dl ($p<0.05$) via enhancing the secretion of insulin in pancreatic β-cells ($p<0.05$).

Conclusion: It was found that MLEE dosages of 50, 100, and 200 mg/KgBW efficiently reduced FBG levels and enhanced insulin expression in pancreatic β-cells in rats.

Keywords: Immunohistochemical, Insulin, Hypoglycemic agents, Pancreas, Pometia pinnata

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by a lack of insulin, resulting in the poor utilization of blood glucose, which leads to hyperglycemia. The main cause of diabetes is lack of insulin production (DM type I) or lack of sensitivity of body tissues to insulin (DM type II). DM disease increases every year, as evidenced by the number of DM events worldwide in 2019 as many as 463 million people predicted to increase by 24.83% in 2030, which is 578 million people and will increase by 51.19% in 2045 with an incidence rate of 700 million people [1]. Uncontrolled blood glucose can increase mortality, morbidity, and complications in diabetes mellitus patients [2]. Diabetes mellitus is managed by administering antidiabetic medications [3].

Glibenclamide is the first choice of sulfonylurea class drugs by increasing insulin secretion of pancreatic β-cells [4, 5]. The receptor surface on the β-cells membrane binds to sulfonylureas, thus inhibiting the ATP-sensitive potassium channel process, which causes potassium to not be able to escape, resulting in cell membrane depolarization. Depolarization of the cell membrane keeps the voltage-dependent calcium channel open, which results in extracellular calcium entering the cell and eventually increasing calcium cytosol, stimulating insulin secretion. Mechanism similar to sulfonylureas to increase insulin secretion in pancreatic β-cells from natural ingredients is flavonoids [6].

Glibenclamide has side effects in the form of weight gain in diabetes patients [4]. Flavonoids are present as an antidiabetic alternative that comes from nature and has the property of controlling body weight in patients, thereby reducing the risk of obesity in diabetes patients. Obesity itself can trigger metabolic syndrome, which triggers insulin resistance and/or hyperglycemia [7]. The success of treatment in diabetes mellitus patients can be influenced by several factors, one of which is the side effects of treatment [8]. Glibenclamide has a large side effect of hypoglycemia, so further research is needed on whether the hypoglycemia side effect of this flavonoid is greater than that of the sulfonylurea. However, many studies have shown that flavonoids can control blood glucose levels in rats induced by streptozotocin. This can be used as a basis for developing flavonoid-based drugs as an alternative to nature-based treatments [9, 10].

One of the natural ingredients that can be used to control blood glucose is matoa leaves (*Pometia pinnata* J. R. and G. Forst). In matoa leaves, there are polyphenol group compounds, namely flavonoids (*quercetin*). Matoa Leaves Ethanol Extract (MLEE) contains phenolic compounds and flavonoids with average levels of 47.80±6.33 µg/ml and 26.8±1.45 µg/ml. This flavonoid compound component shows a very strong antioxidant because activity with an IC50 value of 45.78 ppm<50 ppm 32.05±0.39 µg/ml [11]. Flavonoid compounds function as compounds that can neutralize free radicals so as to prevent damage to the pancreatic of β-cells and increase insulin hormone secretion in pancreatic β-cells. In addition, metabolite flavonoid is effective in lowering Fasting Blood Glucose (FBG), maintaining body weight (BW) due diabetogenic administration as alloxan [6]. This study investigated the efficacy of MLEE reducing FBG levels and determining the enhancement of insulin expression pancreatic β-cells of rats produced by alloxan at dosage of 150 mg/KgBW.

MATERIALS AND METHODS

Research categories and design

Research conducted in the experimental category using the Complete Randomized Design on 6 groups showed a normal group, negative control, positive control Glibenclamide 5 mg/KgBW, and three MLEE groups with 50, 100, and 200 mg/KgBW for 14 d.

Chemical materials

The materials used were matoa leaves (*Pometia pinnata* J. R. and G. Forst) determined in Laboratory of Biology, Faculty of Natural Science,

University Setia Budi, Surakarta, Indonesia, with the letter No: 088/A. E-1/lAB. BIO/I/2019, 50% ethanol, aqua WFI, alloxan (Sigma Aldrich®), NaCl solution 0.9% (WIDA®), Glibenclamide (Indofarma). The materials for Immuno Histo Chemistry (IHC) are rats pancreatic organ preparations, xylene, entellan, absolute ethanol, Phosphate Buffer Saline (PBS), hydrogen peroxide (H_2O_2) 3%, anti-insulin antibodies, DABs (diaminobenzidine), serum blocking.

Plant and simplisia preparation

Matoa leaves were harvested and collected for further wet sorting and washing which aims to remove contaminants such as dust, soil, fertilizer or microbes [12]. The next stage was the chopping process to speed up the drying process of the simplicia. Medicinal plant materials were not chopped too thinly (around 3 mm) to maintain the active compound content [13]. After the matoa leaves were chopped, the next stage was the drying stage using an oven at 45 °C. After drying, dry sorting was carried out to remove damaged simplicia [14]. The simplicia was then extracted using the maceration method with 50% ethanol in a ratio of 1:10 (simplicia: ethanol) for 24 h, stirring occasionally. The resulting macerate was then filtered using a Buchner funnel. The simplicia dregs were re-macerated twice in the same way, and the macerate was collected. The macerate was then evaporated using a rotary evaporator and concentrated over a water bath until a thick extract was formed.

Animal modelling diabetes mellitus test

A total of 25 of the 30 test animals in this study were induced diabetes mellitus. Twenty-five (25) white rats Wistar strain fasted for 18 h, and induced using alloxan dose 150 mg/KgBW with the intraperitoneal route once and received a daily dose of 1 ml of glucose [15]. Rats were confirmed with diabetes when the blood glucose levels exceeded 200 mg/dl. Five (5) non-induced diabetics were given water for injection (WFI) only intraperitoneally. This protocol passed ethics Health Research Ethics Commission Faculty of Medicine, Universitas Muhammadiyah Surakarta, Indonesia, letter number 1741/A.2/KEPK-FKUMS/I/2019.

Treatment test

There were 30 rats divided into 6 groups. Five (5) non-induced diabetic test animals were used as normal controls and were given only aquadest treatment (Group I). Twenty-five (25) other test animals that had been experienced with diabetes were divided into 5 treatment groups, namely negative controls only given treatment aquadest (Group II), positive controls given treatment Glibenclamide 5 mg/KgBW (Group III), and 3 treatment groups (Groups IV-VI) each given MLEE treatment 50, 100, and 200 mg/KgBW. The treatment was given once a day for 14 consecutive days after the rats developed diabetes.

Data collection

Blood samples and FBG measurements were carried out on the day before diabetes induction (used as baseline data), the day after achieving the diabetes condition (used as data on day 0 of treatment), and on the 14th day of treatment. After the treatment was completed, all rats were sacrificed for pancreatic organs, fixed with 10% NBF (Neutral Buffer Formalin), paraffin blocks were made and observations with a microscope of insulin expression β-cell.

Preparation of rats pancreatic organ samples

On the 14th day after the treatment of each group and a significant decrease in FBG levels, the rats were then sacrificed by cervical dislocation. The pancreatic organ is then washed using 0.9% NaCl solution then immersed in a container containing 10% NBF for further preparation of paraffin blocks [16].

Staining of immunohistochemistry (IHC Preparations)

The IHC staining process involves deparaffinization with xylene for 30 min followed by rehydration in a series of alcohol solutions (70%, 95%, absolute) for 5 min each. The preparation rinsed in running water for 5 min and then submerge it in a jar filled with distilled water for another 5 min. The slide is obstructed with a pap-pen and treated with 3% H_2O_2 in methanol for 5 min to remove endogenous peroxidase activity. After 5 min, transfer the prepared mixture to a glass container previously filled with citrate buffer solution and place it in a digital oven for another 5 min. Allowed to cool at room temperature for approximately 30 min, then rinsed with PBS three times for 5 min each.

Apply serum blocking of 50-100 µl for approximately 1 hour at room temperature. Following serum blocking, the primary antibody insulin is applied at a ratio of 1:500 (Insulin Antibody: PBS) and left to incubate for 1 hour. After 1 hour, rinse the slides with PBS three times for 5 min each. Next, the second antibody (poly HRP Goat anti-rabbit IgG) was added and left to incubate for 1 hour. Rinse the tissue with PBS three times. The antigen-antibody reaction results were visualized with diaminobenzidine (DAB) after being washed three times with PBS. This visualization process took place at room temperature for approximately 10 min in the dark. After being washed in running water for 5 min, the sample was counterstained with Haematoxylin-Eosin (HE). The specimen was dehydrated using absolute alcohol (95% and 70%), cleaned with xylene for 5 min, mounted on a slide, and covered with a glass coverslip.

Observation of IHC preparations

Microscopic observation to observe the presence of insulin expression in pancreatic β-cells that have been stained immunohistochemical. Insulin expression is seen with a magnification of 2000×. Photos of preparations that have been taken using a 2000×magnification objective lens. Analysis of insulin expression was quantified based on grading, with the first assessment showing the distribution of insulin expression in pancreatic β-cells: score 1 for focal distribution, score 2 for zonal distribution while on score 3 for spread distribution. The second assessment relates to the intensity of immunohistochemical staining, which is defined as weak (score 1), medium (score 2) and strong (score 3). The sum between the distribution score and the intensity score gave rise to the following final grading system: grade 0 for negative stains, grade 1 for final scores from 1 to 3, and grade 2 from 4 to 5 and grade 3 for final scores>5 [17]. Furthermore, calculations were made on the % area of five fields of view of each organ with Image software.

Statistical analysis

Statistical analysis of body weight parameters and blood glucose levels of rats tested normality with the Saphiro-Wilk test. The data is declared normally distributed if the significance value $p>0.05$. Then proceed with the homogeneity test (Levene test), and if the significance value $p>0.05$ indicates that the data is homogeneous. Furthermore, a statistical test of One Way ANOVA can be carried out with a level of 95%. If there are differences between groups, the Post Hoc Tukey LSD analysis continues.

RESULTS AND DISCUSSION

Result

The hyperglycemic condition developed following the administration of alloxan 150 mg/KgBW. As a result, group II-VI experienced a maximum blood glucose level exceeding 200 mg/dl. Based on the results of FBG, if the data is normally distributed ($p>0.05$), data processing is continued with One-way ANOVA. The results showed that giving 3 various doses of MLEE can reduce FBG levels up to day 14th, when compared to negative controls ($p<0.05$). MLEE of 50, 100, and 200 mg/KgBW were reduced to FBG equivalent positive control ($p>0.05$). There was a significant decrease in blood glucose levels after the rats were given the treatment (fig. 1).

Based on the results of the body weight test, the data is normally distributed ($p>0.05$), so that data processing was continued with One-way ANOVA. Based on the results of body weight measurements, it appears that group II (negative control) showed weight loss until the end of the study because uncontrolled diabetes can cause weight loss, while in groups III-VI (positive control and 3 MLEE dose groups) it was seen that the treatment was able to maintain the body weight of rats experiencing diabetes mellitus (not experiencing weight loss) (table 1).

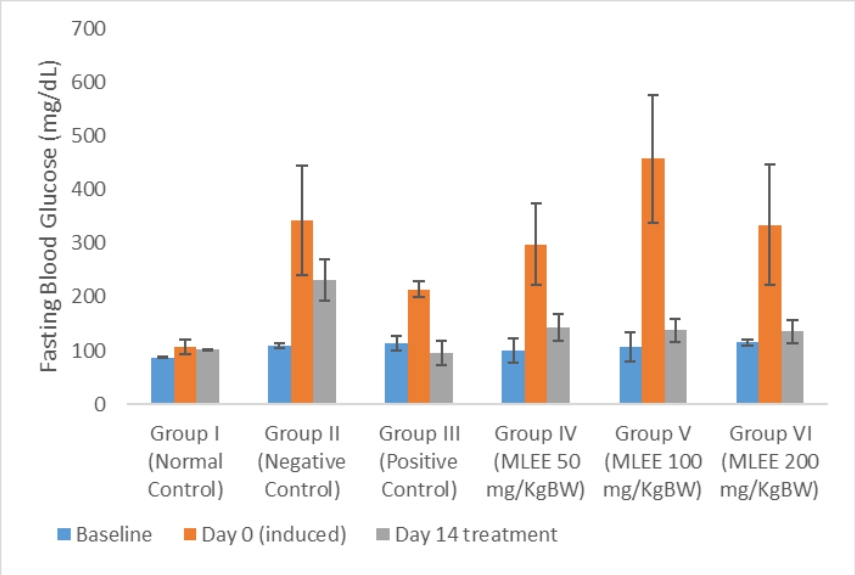

Fig. 1: Relation between time (days) and fasting blood glucose level (mg/dl). Error bars indicate SD values, n=5

Table 1: Average body weight (g) before and after treatment with aquadest, glibenclamide and MLEE in various doses (n=5)

Group	Body weight (g)		
	Baseline	Day 0 (Induced)	Day 14 treatment
Group I (Normal Control)	231.00±8.60	238.33±4.98	233.33±7.58
Group II (Negative Control)	163.33±16.97	154.66±18.62	153.66±19.39
Group III (Positive Control)	166.66±7.54	186.00±10.61	187.66±12.65
Group IV (MLEE 50 mg/KgBW)	188.75± 33.72	191.75±43.22	190.00±50.29
Group V (MLEE 100 mg/KgBW)	223.00±43.76	208.50±37.90	215.00±48.49
Group VI (MLEE 200 mg/KgBW)	167.50±13.48	160.50±24.69	166.25±31.79

Data are expressed as mean±SD, n=5

Due to the destruction with 150 mg/KgBW alloxan induction was causing β-cells pancreas to produce less insulin hormone [18]. The insulin is a hormone produced by the pancreas gland, functioning to regulate the concentration of glucose in the blood. This excess glucose is carried by liver cells further converted into glycogen for storage. Insulin deficiency that causes diabetes is characterized by an increased FBG [19]. The increase in insulin levels is proportional to the number of β-cells in the pancreas. It is hoped that administering MLEE treatment to rats can prevent damage to pancreatic beta β-cells and/or repair damage to pancreatic β-cells due to alloxan induction so that they are expected to produce more insulin than the group that was not given treatment.

Table 2: Score of insulin expression in pancreatic β-cells after treatment in several group

Group	Score of insulin expression in pancreatic β-cells			
	% Area	D	I	G
Group I (Normal Control)	11.66±1.34	3.5±0.2	5.2±0.3	3.8±0.1
Group II (Negative Control)	0.36±0.52	2.3±0.3	1.6±0.7	2.2±0.6
Group III (Positive Control)	6.42±4.53	3.2±0.2	3.9±0.5	4.2±0.4
Group IV (MLEE 50 mg/KgBW)	7.44±3.28	3.1±0.2	$4.7±0.3^{a,b,c}$	$3.5±0.1^{a,b,c}$
Group V (MLEE 100 mg/KgBW)	3.13±2.48	3.8±0.2	$3.4±0.8^{a,b}$	$3.9±0.2^{a,b}$
Group VI (MLEE 200 mg/KgBW)	1.91±1.19	3.9±0.2	$3.1±0.4^{a,b}$	$3.7±0.1^{a,b}$

Data are expressed as mean±SD, n=5, [a](p<0.05 compared with Negative Control), [b](p>0.05 compared with Positive Control), and [c](p>0.05 compared with Normal Control) and Score of D (Distribution 1= focal; 2= zona l; 3= diffuse. Score of I (Intensity) 1=weak; 2= intermediate; 3= strong. Score of G (Grade= addition of distribution and intensity) grade 1 for final scores from 1 to 3, grade 2 from 4 to 5 and grade 3 for final scores>5.

One Way ANOVA is performed to determine the significant difference in area to determine ability to express insulin in pancreatic β-cells. The results of ANOVA test between treatment group compared with control group obtained significant results (p<0.05). While the test between treatment groups with Tukey LSD analysis test not significant data results (p>0.05).

The immunohistochemical methods used to detect various components in cells or tissues having the basic principle of a reaction between antigens and antibodies [20]. The brown color seen on the incision of pancreatic β-cells islets indicates the expression of insulin excreted by pancreatic β-cells. As in the results of immunohistochemical staining (fig. 2), the alloxan administration group as a negative control showed brown color in a small area with weak color distribution and intensity (table 2). The score of grade of insulin expression in the pancreatic cell produced was 2.2±0.6 (table 2) so that only a small fraction of insulin expression was produced.

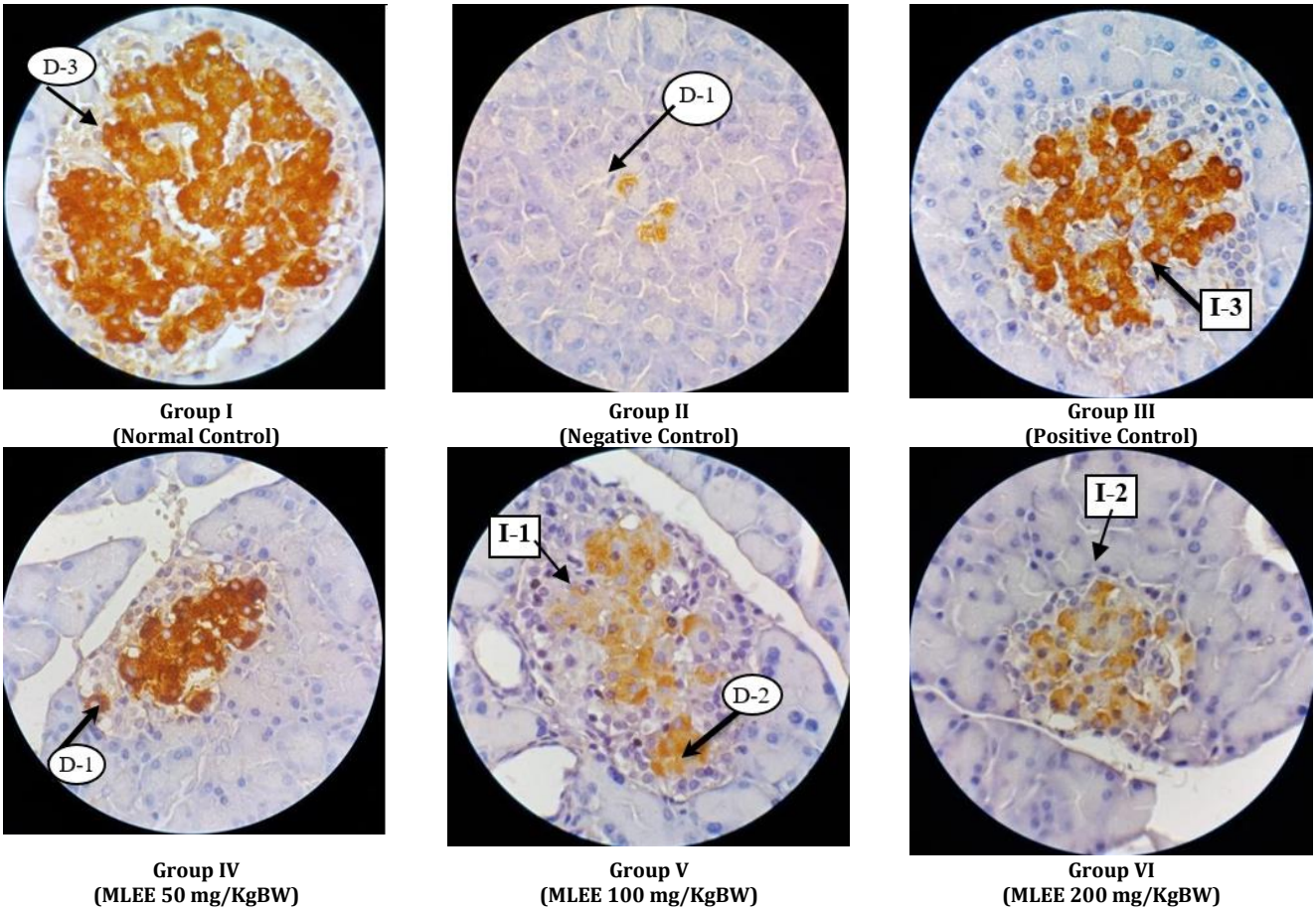

Fig. 2: Immunohistochemical results on pancreatic β-cells with magnification of 2000×. The scale of scoring for distribution is as follows: D-1 = focal, D-2 = zone, and D-3 = spread. The intensity levels are as follows: I-1 represents moderate intensity, I-2 represents medium intensity, and I-3 represents strong intensity.

DISCUSSION

Alloxan as a diabetogenic agent, is used as an inducer of diabetes in test animals with the mechanism of increasing the number of cytosolic-free calcium ions of pancreatic β-cells. Calcium influx alloxan induction results in depolarization of pancreatic β-cells. In this condition, insulin concentrations increase rapidly with high FBG, resulting in significant peripheral insulin sensitivity disturbances in a short time. In addition, alloxan administration provides the effect of reactive oxygen formation as a factor in pancreatic β-cells damage [21]. Damage from pancreatic β-cells is seen by the presence or absence of insulin expression secreted pancreas β-cells. This study MLEE against lowering blood glucose, maintaining weight, and expressing insulin pancreatic β-cells.

Fasting blood glucose levels of rats through blood collection through the lacrimal vein of the eye and obtaining blood serum reading using GOD-PAP reagent can be seen (table 1). From the results obtained on baseline, all groups of test animals showed glucose levels in the normal range, so that all test animals were in good health. Four days after alloxan induction, all rats in group II-VI showed high FBG levels (>200 mg/dl), while the normal control group showed the normal range. Groups II-VI were successfully declared as animal models of diabetes mellitus. After that, all test animals were given treatment according to their respective groups for 14 d. After 14 d, the positive control treatment showed a significant decrease in glucose levels when compared with negative control groups, while MLEE 50, 100, 200 mg/KgBW groups experienced decreased glucose but lower than positive control Glibenclamide.

In a type of diabetes, glucose lacks the ability to be utilized as an energy source. In order to regain the body's energy, lipids and proteins in muscles and fat tissues undergo a process of excessive catabolism [22]. On the 14th day, the MLEE treatment group at 100 and 200 mg/KgBW increased compared to day 0, so that the dose was effective in controlling blood glucose. Glibenclamide experienced a slight increase, while MLEE dose 50 mg/KgBW experienced a slight decrease when compared to negative controls, so MLEE dose 50 mg/KgBW was not effective in controlling blood glucose. MLEE 100 and 200 mg/KgBW were more effective than positive controls and MLEE 50 mg/KgBW was less effective in maintaining body weight of rats. Furthermore, observations of insulin expression using immunohistochemical methods were carried out.

The subject of investigation refers to the pharmacological classification of oral sulfonylureas utilized in the management of diabetes mellitus. Glibenclamide is distinguished out among these sulfonylureas because of its ability to inhibit ATP-sensitive K^+channels in pancreatic β-cells. This inhibition causes depolarization of the cell membrane, and this situation will open the Ca^{++} channel. With the opening of the Ca^{++} channel, Ca^{++}ions will enter the pancreatic of β-cells, stimulate granules containing insulin and insulin secretion occurs [6]. Another mechanism of Glibenclamide to stimulate the proliferation of pancreatic β-cells is caused by damage to pancreatic β-cells due to alloxan administration. So that the body responds to forcing pancreatic β-cells to secrete insulin [23, 24]. Glibenclamide is used to control blood glucose levels as an antidiabetic agent by increasing the release of insulin from the pancreas. This was shown by the results of immunohistochemical staining in Glibenclamide (fig. 2) with brown color results with strong distribution and intensity and score of grade was 4.2±0.4 (table 2) as a positive control compared to negative control.

The chemical components of matoa leaves are *proanthocyanidin*, *epicatechin*, *quercetin*, *kaempferol*, *palmitoyl*, *stigmasterol*. There are

also other compounds of tannins, alkaloids, saponins, and steroids. The MLEE has antioxidant activity as determined by the DPPH technique (*1,1-di-phenyl-2-picrylhydrazyl*), with an IC50 value of 45.78 ppm. This indicates that the extract is classified as a highly potent antioxidant with an IC_{50} value below 50 ppm [11]. Antioxidant compounds to control blood glucose levels [25], so that functions as compounds neutralize free radicals, preventing pancreatic β-cells damage [26], stimulating production of the hormone insulin [27–29].

A previous study stated that MLEE treatment 100 and 200 mg/KgBW can increase plasma insulin levels in the blood by 0.16% and 0.13% [30]. This showed a relationship with the results of % area of insulin expression in the MLEE treatment group with 50, 100 and 200 mg/KgBW of 7.44±3.28; 3.13±2.48; and 1.91±1.19 (table 2). In the MLEE 50 mg/KgBW group treatment, the area yield was % area higher, but the % change in insulin levels in the blood decreased; based on this, it can be suspected that MLEE dose 50 mg/KgBW only increased insulin expression in pancreatic β-cells. While in MLEE of 100 and 200 mg/KgBW the results of the area are lower % area, but the result % change in insulin levels in the blood increases, allegedly in MLEE extract 100 and 200 mg/KgBW secrete insulin out of pancreatic β-cells so that insulin in the blood can also increase. Further research is needed for MLEE to find other mechanisms in increasing pancreatic β-cells insulin expression as well as increased insulin sensitivity of pancreatic or peripheral β-cells.

CONCLUSION

Based on the results of the study, it was concluded that MLEE of 50, 100, and 200 mg/KgBW can reduce FBG and maintain body weight equivalent to positive control Glibenclamide ($p>0.05$), showed significant and increase insulin expression in pancreatic of β-cells.

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Nil

AUTHORS CONTRIBUTIONS

Data curation: Arini Fadhilah, Muhammad Labib Qotrun Niam, Formal analysis: Arifah Sri Wahyuni, Arini Fadhilah, Investigation: Arifah Sri Wahyuni, Sella Aprilia, Methodology: Arini Fadhilah, Tista Ayu Fortuna, Project administration: Arini Fadhilah, Resources: Fazleen Izzani Abu Bakar, Software: Arini Fadhilah, Writing–original draft: All authors, Writing–review and editing: All authors.

CONFLICT OF INTERESTS

Declared none

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