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LANGERHANS ISLET CELLS REGENERATION IN TYPE-1 DM MICE (*Mus musculus*) TREATED WITH METHANOL EXTRACT OF GAYAM (*Inocarpus edulis* Forst) STEM BARK

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ABSTRACT

Type-1 diabetes mellitus (Type-I DM) is a metabolic disorder caused by a deficiency of the insulin hormone due to the destruction of pancreatic β cells. The active compound in the bark of gayam tree (*Inocarpus edulis* Forst) extracted with methanol is thought to regenerate β-cells on pancreatic Langerhans island in *M. musculus* with Type-1 DM. Mice weighing ± 20 g were divided into 4 treatment groups. Each group consisted of 3 mice. Furthermore, each group was induced with streptozotocin in a dose of 500 mg/50 ml in 0.02 M buffer citrate at 1 ml/g BW interperitoneally for 28 days. Blood sugar levels were measured every week using glucotest. Type-1 DM mice were treated with methanol extract of bark in doses of 0.4; 0.8 and 1.6 ml/g BW daily for 2 weeks. The results showed that methanol extract from the bark of *Inocarpus edulis* Forst tree was able to regenerate pancreatic β-cells of the diabetic mice so that the glucose content decreased. Saponin and flavonoid compounds in the bark of *Inocarpus edulis* Forst have hypoglycemic effect that can lower blood sugar levels and regenerate pancreatic β-cells.

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INTRODUCTION

Today diabetes mellitus (DM) is a disease that many people suffer. The population of Indonesia over the age of 20 is 15 million and when it is assumed

that the prevalence of diabetes mellitus is 4.6%, it is estimated that by 2020 there will be 178 million people aged over 20 years and 8.2 million diabetics will be present (Soegondo, et al. 2005).

Diabetes mellitus type-1 is a metabolic disorder caused by insulin deficiency or pancreatic β-cell damage. Other types of diabetes are due to hormone insulin dysfunction and diabetes due to pregnancy. Type-1 DM is a type of disease caused by a decrease in the hormone insulin produced by the pancreas gland. Insulin can be regarded as a regulator of blood sugar levels, because the hormone can process sugar levels consumed by the body into energy. This decrease in the hormone results in glucose consumed by the body cannot be processed perfectly, so that glucose levels in the body will increase (Utami, et al. 2003)

Increased blood sugar levels can actually be prevented if people with diabetes mellitus control blood sugar levels to always be within normal limits. Implementation of healthy lifestyle is done by running a good diet, exercise regularly and adequately, and not hesitate to check blood glucose regularly. Diabetes mellitus patients have the threat of complications such as kidney disorders, visual disturbances, sexual disorders, nerve damage, circulatory and cardiac disorders, and susceptibility to infection (Rina, 2008).

People with diabetes mellitus should undergo frequently used medications such as insulin therapy and oral anti diabetic drugs (OAD). However, such oral medications will have negative effects such as severe hypoglycemic, nausea, anorexia and long-term complications that may harm the brain and require very expensive costs (Zubaidah and Widayana, 2015). Therefore, in general people with diabetes mellitus use herbal medicine as an additional. The use of herbal medicine has been used for generations by the community because, in addition to its relatively small side effects, the price is more economical. One of the plants commonly used by the people in Maluku who suffer from diabetes mellitus is bark of *Inocarpus. edulis* Forst tree (*I. edulis* Forst). The bark of the *I. edulis* Forst tree contains saponin and flavonoid compounds (Widjanarko, et al. 2011).

Saponin is a phytochemical compound that can inhibit the increase of blood glucose by inhibiting the absorption of glucose in small intestine and inhibits gastric emptying, so that food absorption will be longer and blood glucose levels decrease. Tannin contained in the bark of the *I. edulis* Forst bark plays a role in: (1) the action of increasing tyrosine phosphorylation of the insulin receptor β subunit and inhibiting tyrosinephosphatase, (2) stimulating glucose transport activity (Bruneton, 1999). The active compounds contained in the bark of the gayam tree may lower blood glucose levels because they are able to regenerate the Langerhans islet cells in DM Type-1.

METHODS

Extract production

The bark of the *I. edulis* Forst tree was cut into small pieces and then air-dried and smoothed. As much as 100 gram of granulated bark leaf shoots were included in 500 ml methanol and left for 24 hours. Then, the solution was filtered to obtain liquid extract, then evaporated to dry using a rotary evaporator until a concentrated extract of methanol was obtained.

Induction of diabetes with Streptozotocin (STZ)

The induction of diabetes in *M. musculus* was performed using STZ doses of 500 mg/50 ml of 0.02 M buffer citrate at 1 ml/g BW interperitoneally twice weekly for 3 weeks. Then, blood sugar levels were measured every week using glucotest.

Dose determination of methanol extract from the bark of *I. edulis* Forst tree

As an initial dose, if used for humans weighing 70 kg, the required dose was 70/50 × 100 ml = 140 ml. The conversion factor for humans (70 kg) to mice (20 g) = 0.0026 g (Elya, et al. 2010), so the dose for mice was 0.0026 g × 140 ml = 0.4 ml. Based on the conversion result, we used dose I as much as 0.4 ml/20 g BB, dose II as much as 0.8 ml/20 g BB and dose III 1.6 ml/20 g BB.

Research implementation

Twelve mice were divided randomly into 4 groups, each consisting of 3 mice. The mice were weighed and then acclimatized for a week. During acclimatization the mice were given with feed and drink. Then, the methanol extract preparation of the bark of *I. edulis* Forst tree was made and the tested animals were treated as follows: Treatment group I: received methanol extract of the bark of *I. edulis* Forst tree as much as 0 ml/20 g BW (control animals); Treatment group II: received methanol extract of 0.4 ml/20 g BW *I. edulis* Forst bark; Treatment group III: received methanol extract of 0.2 ml/20 g BW of *I. edulis* Forst bark; Treatment group IV: received methanol extract of the bark of *I. edulis* Forst tree as much as 1.6 ml/20 g BW. Administration of methanol extract of the bark of *I. edulis* Forst tree was done every day for 14 days.

Measurement of blood sugar levels

Measurement of blood glucose stage I was done at the beginning or before treatment by cutting the tip of the mice's tail. Blood coming out from the tail end was dropped on glucotest strip to be tested and the measurements were recorded. Measurement stage II was done after the extract test to identify the difference of blood sugar value (Siscawati, 2012).

Preparing pancreatic surgical preparations

Preparation of pancreas incisions was done by the procedure according to Nadzifa (2010) in Latumahina (2012): The first stage was coating, then immersion in 70% alcohol at least overnight. Then, the

glass object was immersed in a 0.5% gelatin solution for 30-40 s/s. In the second stage, the pancreas organ that had been stored in 4% formalin solution was washed with alcohol and continued by leaching with 90%, 95%, ethanol absolute (3 times), xylol (3 times), respectively for 20 minutes. The third stage was the infiltration process. The fourth stage, embedding. The cutting stage with microtom. The selected slices were taken with glass object that had been coated then dried over hot plate. The stage of paraffization, preparations were included in xylol as much as 2 times for 5 minutes. The rehydration stage, the preparation was inserted into ethanol solution in stages from absolute ethanol (2 times), 95% ethanol, 90%, 80%, and 70% respectively for 5 minutes. Then, the preparations were immersed in distilled water for 10 minutes. During staining stage, the preparations were dripped with hematoxylin for 3 minutes or until the best color results are obtained. Thereafter, the preparations were incorporated in the alcoholic eosin dye for 30 minutes and rinsed with distilled water for 5 minutes. During the dehydration stage, the preparations were immersed in 80%, 90%, 95% ethanol and absolute ethanol (2 times) each for 5 minutes. During clearing stage, the immersion was in xylol solution 2 times for 5 minutes, then dried. Mounting stage was done using ethylene. The end result was observed with a microscope for each group of mice, photographed, then analyzed for pancreatic damage.

Data analysis

The data of blood glucose measurement were analyzed using Analysis of Variance (ANOVA) at 95% level of confidence, while pancreatic photomicrograph was analyzed descriptively.

RESULTS

Blood sugar levels

The administration of *I. edulis* Forst bark methanol extract in mice suffering from type-1 DM can lower blood sugar levels. The mean results of blood glucose measurements before and after administration of methanol extract of *I. edulis* Forst bark can be seen in Table 1.

Table 1. Mean blood sugar levels of mice (*Mus musculus*) before, after STZ induction and after treatment.

Treatment groups (ml/g)	Mean of mice (*Mus muscullus)* blood glucose in mg/dL			Difference Blood glucose (mg/dL)
	Early	SZT Administration	Treatment (X±SD)	
Control	140.33 ± 7.76	442.00 ± 14.18	581.33 ± 28.54[a]	139.33 ± 21.07 [a]
0.4	149.33 ± 9.07	407.00 ± 42.57	319.00 ± 54.62[b]	-68 ± 6.08 [b]
0.8	140.67 ± 6.03	421.00 ± 40.58	238.33 ± 53.14[c]	-182.66 ± 35.81[c]
1.6	152.67 ± 3.05	448.00 ± 30.81	158.33 ± 3.06[d]	-289.66 ± 33.71 [d]

Note: Superscripts with the same letter were not significantly different (α=0.05)

The results of measurement of blood glucose level in mice (Table 1) showed that in control group mean blood glucose level of 442.00 mg/dL rose by 139.33 mg/dL to 581.33 mg/dL, while blood sugar in group of DM type-1 who received methanol bark extract dose 0,4 ml/g BW decreased by 68 mg/dL so that the blood sugar level decreased from 407.00 mg/dL to 319.00 mg/dL. At a dose of 0.8 ml/gr BW there was a decrease of 182.66 mg/dL so that blood sugar levels decreased from 421.00 mg/dL to 238.33 mg/dL, and at a dose of 1.6 ml/gr BW decreased by 289.66 mg/dL so that blood sugar levels decreased from 448.00 mg/dL to 158.33 mg/dL. More details can be seen in Fig 1.

Based on the results of Analysis of Variance (ANOVA) by using SPSS 16 program, the administration of *I. edulis* Forst bark methanol extract had effect on decreasing blood sugar level of diabetes mellitus mice. Further test results showed that each treatment group with dose 0.4 ml/gr BW, 0.8 ml/gr BW, 1.6 ml/gr BW showed significant difference between control and each treatment group.

Increased and decreased blood sugar levels of mice (*Mus musculus*) in each treatment group can be seen in Fig1.

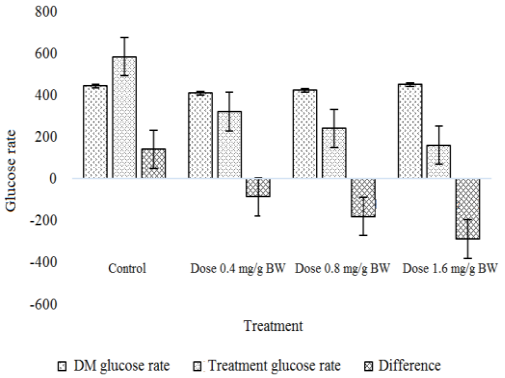

Figure 1. Mean of decreased blood sugar level of DM Type-1 treated by methanol extract of the bark of *I. edulis* Forst.

Histologic profile of pancreatic organs of mice with Hematoxylin-Eosin staining (HE) showed that the negative control group mice suffered β-cell damage to pancreatic langerhans island (Fig. 2B). In the group of mice given with methanol extract of *I. edulis* Forst bark at a dose of 0.4 ml/g BW (Fig 2C) showed β-cell regeneration and β-cells in Langerhans islet although damage are still visible in the form of necrosis, edema, atrophy and cariopicnotis nuclei. Whereas, the group of

mice receiving methanol extract bark in doses 0.8 and 1.6 ml/g BW showed cell regeneration (Fig 2 D and E).

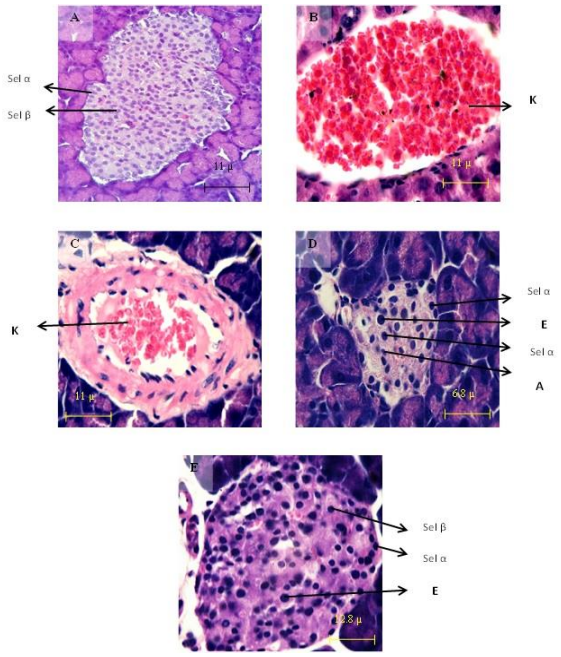

Figure 2. Histology of mice pancreas. (A) Control mice group (+), (B) Control mice group (-), (C) Group of mice receiving methanol extract of *I. edulis* Forst bark 0.4 ml/g BW, (D) Group of mice receiving methanol extract of *I. edulis* Forst bark in a dose of 0.8 ml/g BW, (E) Group of mice receiving methanol extract of *I. edulis* Forst bark in a dose of 1.6 ml/g BB. E = Edema; A = Atrophy; K = Cariopicnotis.
Note: Figure A. 200x magnification; B, C, D and E. 400x magnification.

DISCUSSION

Streptozotocin (STZ) is an alkylation agent that breaks the DNA sequence in Langerhans islet cells in the pancreas of mice. STZ was used for the induction of type-1 DM in this study because STZ is a toxic substance capable of destroying β-cells in pancreatic Langerhans islet directly so as to inhibit the production of diabetes mellitus. Damage to β cells will lead to conditions of hyperglycemia in mice (Joeliana, 2007). Hyperglycemia occurs by increasing blood sugar levels of mice ≥ 400 mg/dL (Table 1).

In this study, injection of STZ resulted in damage of constituent cells of Langerhans islet in the pancreas. The damage was definitely cytopicnotic because the cells are indistinguishable (Fig. 2B). In the pancreas, STZ is presented by macrophages so it is recognized by T-helper cells. Furthermore, macrophages secrete IL-1 to induce proliferation and activation of T-helper cells whivh then activate IFN-γ to increase macrophage activation. During activation, macrophages produce superoxide and NO, which, together with IL-1 and TNF-α, damage pancreatic β cells from free radicals (Siscawati, 2012).

Furthermore, Hanan (2004) stated that giving in vivo streptozotocin causes increased levels of malondialdehyde (MDA). Increased plasma MDA levels indicate an increase in the oxidation of unsaturated fats mainly found in pancreatic β cells, causing disruption to insulin secretion, which then causes hyperglycemic to the following structures: (1) STZ causes DNA chain breaks in pancreatic langerhans islet cells, stimulating polynuclei (ADP-ribose synthetase) resulting in lower levels of intracellular NAD+ and NADP+ that inhibit the synthesis of proinsulin and induce hyperglycemia, and (2) Activating reactive oxygen species (ROS) such as superoxide (O2), hydroxyl radical (OH), and hydrogen peroxide (H2O2). Damage to the pancreas due to streptozotocin depletes the intracellular antioxidants in the cell through a mechanism that undergoes oxidative stress (Latumahina, 2012). Furthermore, oxidative stress causes lipid peroxidation that will cause cell membrane damage. The loss of integrity of the cell membrane causes the buildup of fluid in the cell (edema) which is the phase leading to cell death (necrosis). In this study, edema and necrosis of β-cells and β-cells resulted in the formation of empty spaces within the pancreatic langerhans island.

Nurdiana (1998) states that the empty spaces on Langerhans islets are due to β-cell necrosis. Changes in cells caused by substances that have a cytotoxic effect are the reduction of the langerhans islets in the pancreas, the reduction of β-cell count and degranulation, and the vacuolization of these cells. The results of this study indicated that the administration of STZ for 3 weeks caused an increase in blood sugar levels, then decreased after treatment with *I. edulis* Forst bark tree extract for two weeks.

According to Widyatmoko (2009), antioxidant compounds will deliver one or more electrons to free radicals, so as to stop the damage caused by free radicals. Free radicals will soon react with antioxidants to form a stable and harmless molecule. This is what causes the concentration of blood sugar levels in type-1 DM tends to decrease due to regeneration of pancreatic β cells so that the pancreas re-produce insulin that serves to absorb glucose.

Administration of *I. edulis* Forst bark methanol extract at a dosage of 0.4; 0.8 and 1.6 ml/g BW had an effect on reducing mice blood sugar levels induced by Streptozotocin. This can be seen in the blood sugar level of the group of mice treated with methanol extract of the bark of *I. edulis* Forst in doses of 0,4 ml/g BW of 319,00 mg/dL, in 0,8 ml/g BB equal to 238,33 mg/dL and in dose of 1.6 ml/g BB the blood sugar level was 158.33 mg/dL. Decrease in blood sugar levels of type-1 DM after treatment of *I. edulis* Forst bark methanol extract in this study occurred because the bark of *I. edulis* Forst tree contains secondary metabolite compounds in the form of saponins, tannins and flavonoids. Saponin has a function as an aglicon glycoside that can lower blood glucose levels by inhibiting the release of α-glucosidase enzymes in the small intestine thus inhibiting the polysaccharide reaction to simple sugars. Inhibition of this system can effectively reduce the digestion and absorption of carbohydrates so as to reduce the increase

in postprandial glucose levels. Thus, the release of glucose becomes slower and the glucose absorption in the blood becomes lower, less rapid and less evenly, so that high blood sugar levels can be avoided (Istiani, 2008). Tannin also has a very large role in lowering blood sugar levels because it increases the phosphorylation of tyrosine from the insulin receptor β subunit, and inhibits tyrosine phosphatase and then stimulates glucose transport activity, thereby increasing the activity of insulin receptors. Flavonoids stimulate 16% increase in insulin secretion from pancreatic β cells. The action is obtained through the regulation of peroxisome proliferators activated receptors (PPAR α and PPAR γ). The action of beneficial flavonoids in diabetes mellitus is through its ability to avoid glucose absorption or improve glucose tolerance. Furthermore, flavonoids stimulate the removal of glucose in peripheral tissues, regulating the activity and expression of enzymes involved in the carbohydrate metabolism pathway and acting like insulin, by affecting the mechanisms of insulin signaling (Novrial 2012).

Cell neogenesis can occur due to normalization of insulin-mediated blood sugar levels. Two types of precursor cells will appear on the langerhans islets that regenerates. One type expresses glucose transporter-2 (Glut2) and another type expresses insulin from stomatostatin. Both cells then become monospecific cells containing insulin and fill the empty or broken islets of langerhans (Arjadi and Susatyo, 2010).

The decrease in blood sugar levels due to methanol bark extract can be explained through two mechanisms. First, the intra pancreatic mechanism. This mechanism works by regenerating damaged pancreatic β cells and protecting β cells from damage and stimulating insulin release. Flavonoids are thought to play a role in this mechanism. Second, the extra mechanism of the pancreas. This mechanism can take place through various mechanisms, such as those done by saponins and tannins.

Regeneration of β cells and β cells in pancreatic langerhans islets also occurred in the treatment group of 0.4 ml/g BW (Fig. 2C). Treatment with an extract of 0.8 ml/g BW (Figure 2D) showed better cell regeneration than in the previous treatment. While the treatment of 1.6 ml/g BB (Fig. 2E) showed better regeneration compared to the previous treatment because the micrograph photographs showed large langerhans islets. Increased regeneration of these langerhans islet cells was along with increasing dose of the extract. This opinion is consistent with Siregar (2013); Prameswari and Widjanarko (2014) which states that increased doses results in an increase in the amount of bioactive compounds contained in the extract.

Regeneration occurring in the treatment group was gradual, probably due to the presence of flavonoid bioactive compounds. This opinion is supported by Prameswari (2014) which states that flavonoids have antidiabetic activity capable of regenerating cells in the langerhans islets. Research conducted by Ifridah (2014) also suggests that flavonoids can contribute to regulate blood sugar decrease and improve the distribution of β-producing cells of insulin langerhans islets through HE staining. Further Arjadi and Susatyo (2010) added that flavonoids can lead to regeneration of langerhans islet cells, stimulate insulin secretion and or as insulin-like compounds.

CONCLUSION

Methanol extract of the bark of *I. edulis* Forst tree is able to regenerate β cells of the pancreatic langerhans islets, thus lowering blood sugar levels along with the increased dosage of the extract. The higher the dose of the extract, the lower the blood sugar level in DM type 1.

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