

Increased of hypoglycemic effect and pancreatic regeneration of *Pandanus amaryfollius* leaves ethyl acetate extract in streptozotocin-induced diabetic rats

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Article history

Received: 25 September 2017

Received in revised form:

10 November 2017

Accepted: 24 November 2017

Abstract

The number of diabetes mellitus patients is continuously growing. Diabetes is a chronic disease and/or a major endocrine disorder caused by a deficiency of insulin production by pancreas or low insulin productivity. Hypoglycemic potentials have been widely examined from plant ingredients to substitute diabetes medicines and treatments. The objective of this research was to investigate a hypoglycemic activity and pancreatic cell regeneration of ethyl acetate extract from *Pandanus amaryfollius* leaves in streptozotocin-induced diabetic rats. The ethyl acetate extract of pandan leaves was prepared by crushing, macerating, filtrating and evaporating the leaves. Forty rats were grouped into five groups: (1) normal controlled rats, (2) diabetic controlled rats, (3) diabetic rats with feed standard and 0.985 mg/20g diet ethyl acetate extract (K1), (4) diabetic rats with feed standard and 1.97 mg/20g diet ethyl acetate extract (K2), and (5) diabetic rats with feed standard and 2.955 mg/20g diet ethyl acetate extract (K3). The treatments were being conducted for 28 days; blood sampling was taken every 7 days to be analyzed its blood glucose levels, and the final research stage was to analyze its blood insulin levels and MDA levels in the rats' liver and pancreatic histology. The result showed that the intake of ethyl acetate extract in the diabetic rats could lower their blood glucose levels. The higher the dose of ethyl acetate extract resulted in the lower blood glucose levels, the higher blood insulin levels, and the lower liver damage due to the oxidative stress. In addition, the higher the dose of ethyl acetate extract resulted in the lower insulin resistance in the islet of Langerhans, indicated the smaller the volume of pancreatic β -cells. However, the insulin resistance decreased and the insulin secretion increased; hypoglycemic ability of ethyl acetate extract of fragrant pandan leaves remained high.

Keywords

Pandanus amaryfollius
ethyl acetate extract
pancreatic β -cells
hypoglycemic ability

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Introduction

Diabetes mellitus is one of chronic diseases caused by a degenerative disease and/or a deficiency of insulin production by pancreas, or ineffective insulin produced. The number of diabetes patients will double from 177 million in 2000 to 366 million in 2030 (Sarah *et al.*, 2004). WHO reported that 422 million adults aged over 18 years were living with diabetes in 2014, four times higher than the number of diabetics in 1980 that is 108 million people, and the prevalence of diabetes in the Southeast Asia region is 8.6% (WHO, 2016).

Various antidiabetic medicines have been used to cure the diabetic patients. Oral antidiabetic medicines were reported to induce serious side-effects such as liver disorder, lactic acidosis and diarrhea (Rajalaksmi *et al.*, 2009). Therefore, natural ingredients are investigated for a potency of safer diabetes treatments. Sasidharan *et al.* (2011) stated that pandan leaves in Malaysia are widely used

as a traditional medicine for diabetes. However, hypoglycemic potential research on pandan leaves (*Pandanus amaryfollius*) is few. In the others species of pandanus, Kumari *et al.* (2013) stated that methanol extract from pandanus roots (*Pandanus fascicularis* Lamk) have antidiabetic activity.

Hypoglycemic mechanism and antidiabetes of several plant ingredients have been investigated. Extracts of cinnamon, clover, curcuma and laurel leaves have similar activity with insulin that can increase glucose metabolism and autophosphorilation of beta insulin receptors, and lower the activity of phosphate tyrosine; while cinnamon extract is also proven to regulate insulin (Broadhurst *et al.*, 2000). Marsono *et al.* (2002) reported that hypoglycemic of red bean is used due to its ability to inhibit glucose absorption in small intestines. As supported by Qin *et al.* (2004), cinnamon extracts prevent the insulin resistance induction in the rats with high fructose diet, while Cao *et al.* (2010) stated that cinnamon could increase insulin. Kavishankar *et al.* (2011)

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reported that water extract from *Pandanus odurus* could improve insulin levels in blood and increase liver glycogen. Kumara *et al.* (2013) stated that methanol extract from *Pandanus amaryllifolius* could improve a pancreatic beta cell function as an insulin producer.

This research, therefore, was conducted to investigate the effect of several doses of ethyl acetate extract on its diabetes activity and the regeneration of pancreatic β -cells.

Materials and Methods

Preparation of pandan leaves extract

The main material was fresh fragrant pandan leaves obtained from a local market in Beringharjo, Yogyakarta, Indonesia. The pandan leaves were sorted and washed in the flowing tap water, then drained and chopped into 3-4 cm wide. Fifty grams of the chopped leaves was crushed using food processor (Philips). Leaf pulp was put into 500 ml erlenmeyer, added with 250 ml ethyl acetate solvent (p.a), and then shaken for one hour in a shaker until it was mixed, and macerated afterwards for 36 hours. The obtained filtrate was filtered with Whatman 41, then vacuum-evaporated using a rotary evaporator (Buchii R215) at 40°C to obtain a thick pasta-like of pandan leaves' ethyl acetate extract, and stored at -10°C (Suryani and Setyowati, 2008).

Experimental design

The experimental animals used in this research were 40 male Sprague Dawley rats aged two months. Each rat was kept in a stainless steel cage, and all rats were being fed with feed standard and excessive water for three days. The feed standard was based on AIN 1993 (Reeves *et al.*, 1993). The rats were kept under room temperature and humidity with natural lighting. After the adaptation period, 32 rats were induced with diabetes and the remaining 8 were served as normal controls. Diabetes induction was conducted using streptocotocin/STZ (Sigma-Aldrich, Germany) with a dose of 65mg/kg body weight dissolved in 3ml citrate buffer with an intravenous administration method (Szkudelski, 2012). Blood sampling was conducted five days after the diabetes induction namely as a day-0 sample. The rats were then grouped into five groups namely Group 1: normal controlled rats; Group 2: diabetes controlled rats; Group 3: diabetic rats with feed standard and treated with 0.985 mg/20g diet ethyl acetate extract (K1); Group 4: diabetic rats with feed standard and treated with 1.97 mg/20g diet ethyl acetate extract (K2); and Group 5: diabetic rats with feed standard

and treated with 2.955 mg/20g diet ethyl acetate extract (K3). The treatment was being given for 28 days. Ethyl acetate extract solution was given upon force feeding in 1ml of 0.5% ethanol in Group 3, 4 and 5, while the other groups (1 and 2) were given 1 ml of 0.5% ethanol.

Rats blood sampling was conducted at the end of the adaptation period and on day 0, 14 and 28 after diabetic condition was reached. The blood samples were analyzed their blood glucose levels using a kit from Diasys GmbH (Holzhem, Germany). On day 28, blood insulin levels were analyzed from the samples using Elisa kit for insulin (U-Cloud-Clone Corp). At the end of the experiment, the rats were anesthetized with diethyl ether, then cut their abdomens to remove the livers and pancreases. The livers were analyzed for malonaldehyde (MDA) levels using thiobarbituric acid reactive substance (TBARS) assay kit (Cayman, USA).

The animal experimentation was performed after the biological test of the research design established an ethical clearance from Ethical Clearance Committee for preclinical research in Laboratory of Integrated Research and Examination, Gadjah Mada University.

Histology preparation and immunohistochemical analysis (IHC)

Upon removal and isolation, the pancreases were cleaned and fixated in 10% formalin, followed by dehydration, infiltration and implantation in paraffin. Afterwards, the pancreases were slit to 7 μ m using a microtome; the slices were put on glass preparates and colored with Hematoxylin – Eosin. IHC analysis with Histofine Mouse Stain Kit ensued upon finding the islets of Langerhans. Dark brown color showed the presence of insulin hormone secreted by pancreatic β -cells. Insulin hormone intensity was analyzed semi-quantitatively.

Data analysis

All data were presented in the form of average \pm standard deviation. The obtained data were analyzed with a method of One Way Analysis of Variance with $p \leq 0.05$ (SPSS version 17).

Results and Discussion

Average body weight and feed intake of rats during experiment

The observation result of rats' body weight during the experiment is presented in Figure 1. The result demonstrated that initial average body weight of the rats before the treatment was 189.99 \pm 4.36 g, which increased during the adaptation period to 193.70 \pm 4.59

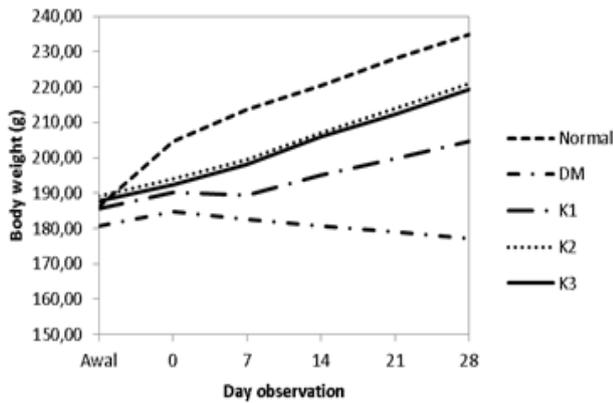


Figure 1. Average body weight of rats during the experiment.

Normal group: normal controlled rats; DM group: diabetes controlled rats; Group K1: diabetic rats with feed standard and treated with 0.985 mg/20g diet ethyl acetate extract; Group K2: diabetic rats with feed standard and treated with 1.97 mg/20g diet ethyl acetate extract; and Group K3: diabetic rats with feed standard and treated with 2.955 mg/20g diet ethyl acetate extract.

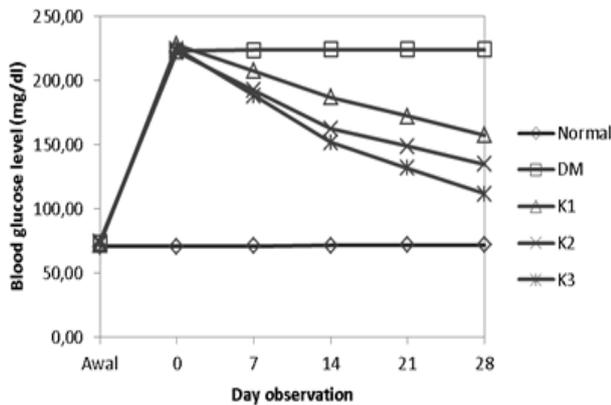


Figure 2. Blood glucose responses of rats during the experiment

Normal group: normal controlled rats; DM group: diabetes controlled rats; Group K1: diabetic rats with feed standard and treated with 0.985 mg/20g diet ethyl acetate extract; Group K2: diabetic rats with feed standard and treated with 1.97 mg/20g diet ethyl acetate extract; and Group K3: diabetic rats with feed standard and treated with 2.955 mg/20g diet ethyl acetate extract

g, however, body weight of controlled diabetic rats after treatment decreased from day 0. Controlled diabetic rats also experienced the highest body weight loss, and obviously the lowest body weight at the end of the experiment, compared to that at the beginning. Meanwhile, other rats' treatments (K1, K2 and K3) with ethyl acetate extract experienced body weight gain. Ethyl acetate extract supplement was, therefore, able to restore metabolism in the rats' body.

Upon a 28-day observation, average feed intake across normal group, K1, K2 and K3 was not significantly different ($P > 0.05$) namely 11.45 ± 0.76 g, 11.34 ± 0.59 g, 11.67 ± 0.50 g and 11.70 ± 0.59 g respectively, but significantly different ($P < 0.05$)

Table 1. Blood glucose, insulin, and MDA levels in livers of experimental rats

Treatments ^{***}	Blood glucose levels (mg/dl) [*]	Blood insulin levels (IU/dl) ^{**}	Liver MDA levels (nmol/g) ^{**}
Normal	72.07 ± 0.83 a	17.25 ± 0.09 e	0.98 ± 0.09 a
Diabetes	224.44 ± 8.35 e	5.90 ± 0.51 a	6.76 ± 1.55 e
K1	157.62 ± 3.56 d	9.00 ± 0.36 b	4.72 ± 0.30 d
K2	134.27 ± 2.58 c	13.03 ± 0.32 c	2.19 ± 0.33 c
K3	111.51 ± 2.95 b	15.44 ± 0.10 d	1.38 ± 0.10 b

^{*}Mean±standard deviation obtained was the mean of 8 replicates

^{**}Mean±standard deviation obtained was the mean of 3 replicates

Mean values followed by different letter within column show a significant difference ($P < 0, 05$)

^{***} Normal group: normal controlled rats; DM group: diabetes controlled rats; Group K1: diabetic rats with feed standard and treated with 0.985 mg/20g diet ethyl acetate extract; Group K2: diabetic rats with feed standard and treated with 1.97 mg/20g diet ethyl acetate extract; and Group K3: diabetic rats with feed standard and treated with 2.955 mg/20g diet ethyl acetate extract

from the controlled diabetic rats namely 13.9 ± 0.27 g. The diabetic rats had the highest feed intake, but their body weight did not increase. It indicated that feed standard without ethyl acetate extract supplement could not improve diabetic condition, leading to abnormal body metabolism and more feed intake.

Blood glucose levels

The blood glucose levels of the experimental rats are presented in Table 1 and Figure 2. Based on the data in Figure 2, it indicates that after three days adaptation, the initial blood glucose levels of rats were 70.97-74.66 mg/dl. It showed that blood glucose levels of rats prior to diabetes induction were normal. Nichols (2003) stated that normal blood glucose levels of rats were 62-175 mg/dl. Three days after diabetes induction in Group 2 (DM), 3 (K1), 4 (K2) and 5 (K3), it showed an extremely significant increase in their blood glucose levels (day 0) up to 221.94-225.73 mg/dl. Meanwhile, in Group 1 (normal controlled rats), there was no significant blood glucose increase. STZ treatment had led to diabetes in the experimental rats and caused damage in pancreatic β -cells. STZ was transported into pancreatic β -cells through glucose transporter GLUT2 that caused necrosis in cells secreting insulin (Szkudelski, 2012).

The effect of ethyl acetate extract supplement during the 28-day experiment on the blood glucose levels of diabetic rats are presented in Figure 2. During the 28-day experiment, the blood glucose levels of the rats experienced a decrease except for normal controlled rats (Group 1). The ethyl acetate extract supplement would lower the blood glucose levels,

indicating that the higher the dose of ethyl acetate extract, the faster the decrease of the blood glucose levels and the higher the dose of ethyl acetate extract supplement, the lower the final blood glucose levels (Table 1). It showed that the supplement of ethyl acetate extract of pandanus leaves would improve the diabetic condition and the higher the dose of ethyl acetate extract resulted in the higher capability of anti-diabetes; this was similar to the findings by Kaneto *et al.* (1999). The results of phytochemical analysis showed that the ethyl acetate extract from *Pandanus amaryfollius* leaves qualitatively containing phenol, flavonoid, alkaloid, steroid and saponin. Based on previous studies shows that the phenolic and flavonoids have hypoglycemic activity. Wangenstein *et al.* (2004) reported that phenolic and flavonoids have antioxidant activity and can to protect oxidative damage, thereby increasing insulin release from pancreatic β -cells. Mukherjee *et al.* (2006) stated that flavonoid and saponin components had anti-diabetic activity. According to Sandhar *et al.* (2011), flavonoid served in increasing regeneration of islet Langerhans and insulin secretion in the diabetic condition due to STZ induction; it also acted as an inhibitor of aldose reductase.

Blood insulin levels

The blood insulin levels are presented in Table 1. The blood insulin level at the end of the experiment showed that controlled diabetes group had the lowest insulin level (5.90 ± 0.51 IU/dl), while in the groups supplemented with ethyl acetate extract, the blood insulin levels were higher. The higher the dose of ethyl acetate extract, the higher the insulin levels in the experimental rats would be. In contrast with the blood glucose levels, the higher the blood insulin levels, the lower the blood glucose levels would be. It might support the assumption that ethyl acetate extract supplement of pandan leaves could improve the diabetic condition so that the insulin production increased. This research indicated a positive effect on the insulin production in the diabetic rats.

Liver malondialdehyde (MDA) levels

Diabetes was related to significant oxidative stress. In this research, oxidative stress was due to STZ induction causing diabetic condition. The oxidative stress was assumed to cause an insulin resistance, beta cell dysfunction, impaired glucose tolerance, and eventually type 2 diabetes (Ceriallo and Motz, 2004). MDA is one of the final products of lipid peroxide formed after the free radicals attack lipid membrane that is affluent with polyunsaturated fatty acids (PUFA). A free radicals analysis was

conducted by measuring the level of malondialdehyde in the liver. MDA levels of the rats are presented in Table 1.

The data in Table 1 shows that the diabetic controlled rats had higher MDA levels than the normal group. The diabetic condition would increase enzyme activity of acyl-coenzyme A oxidase that initiated fatty acid oxidation leading to lipid oxidation (Horie *et al.*, 1981). STZ supplement given to the experimental animals could trigger free radicals and blood glucose level increase. On the other hand, the increasing blood glucose level could increase the oxidative stress. Some mechanism pathways of oxidative stress due to the increased blood glucose levels were polyol pathway of peripheral nerve, increased the production of advanced glycation end products (AGEs) pathway, protein kinase activation pathway, hexosamine pathway flux due to overmodified protein by N-Acetylglucosamine, glucose autooxidation in diabetes mellitus, and oxidative phosphorylation pathway. All the possible pathways could trigger the increase of free radicals in the body (Broge, 2002).

Figure 2 and Table 1 indicate that besides lowering blood glucose levels of the rats, ethyl acetate extract of pandan leaves could lower the liver MDA levels and increase the blood insulin levels (Table 1). MDA levels in Group K1, K2 and K3 were lower than those of controlled diabetic rats, showing that normal antioxidant defense mechanism (without external antioxidant supplement) was insufficient to prevent the damage by free radicals. Animals have internal antioxidative defense system that can be boosted by additional antioxidant which is significantly needed by pancreas because of the weak defense against oxidative stress (Tiedge, 1997). Supplementing ethyl acetate extract of pandan leaves having high antioxidative activity could serve as an oxidative stress repellent.

The higher the dose of ethyl acetate extract, the lower the liver MDA levels in diabetic rats would be. Ethyl acetate extract contains phenol as an antioxidant. Therefore, the higher the dose of ethyl acetate extract, the higher the antioxidative activity, and the lower the MDA levels would be. Phenol antioxidant activity is related to the balance of oxidation-reduction reaction or electron donating substituent bound in an aromatic ring that will accelerate oxidative inhibition reaction by an antioxidant. Natural antioxidant in plants is commonly phenolic compound which is multifunctional and serves as an antioxidant because of its features to reduce, capture free radicals, bind metal and turn singlet oxygen into triplet (Croft, 1999; Estiasih and Andiyas, 2006).

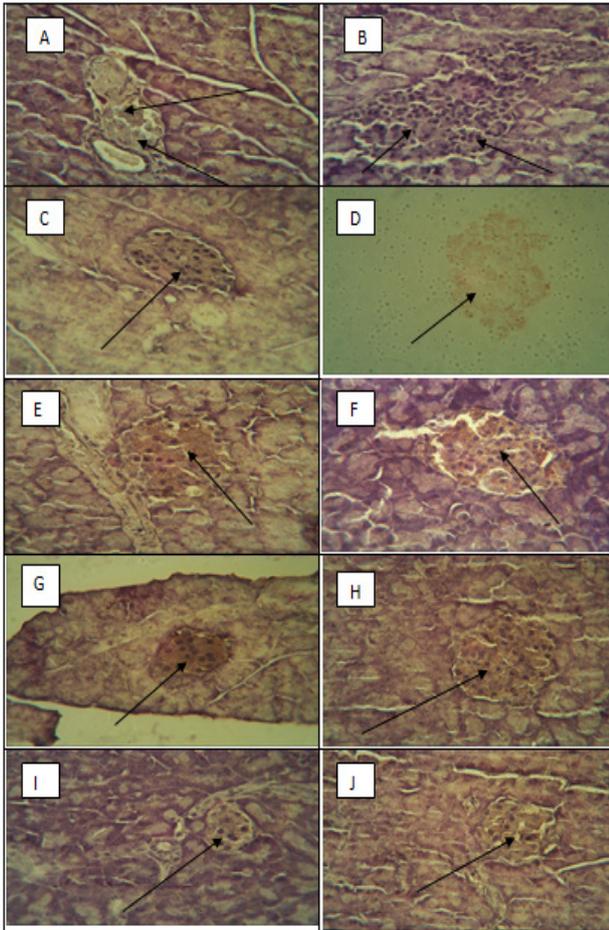


Figure 3. Photomicrograf of insulin-immunoreactive beta cells in the islets of Langerhans in the diabetic, normal, and treatment rats visualized by ABC immunohistochemistry methods (520X). A and B showed no insulin-immunoreactive cells in the islets of diabetic rats, C and D showed the presence of insulin-immunoreactive cells in the islets of normal rats with medium intensity, E and F indicated the high intensity of insulin-immunoreactive cells in the islets of K1 rats. The intensity of insulin-immunoreactive cells decreased in K2 rats (G and H), and disappeared in K3 rats (I and J).

Normal group: normal controlled rats; DM group: diabetes controlled rats; Group K1: diabetic rats with feed standard and treated with 0.985 mg/20g diet ethyl acetate extract; Group K2: diabetic rats with feed standard and treated with 1.97 mg/20g diet ethyl acetate extract; and Group K3: diabetic rats with feed standard and treated with 2.955 mg/20g diet ethyl acetate extract

Histopathology of pancreas

Photomicrograf of insulin-immunoreactive beta cells in the islets of Langerhans in diabetic, normal, and treatment rats visualized by ABC immunohistochemistry methods (520X) is presented in Figure 3. Figure 3.A and B show no insulin-immunoreactive cells in the islets of diabetic rats. HE coloring in the sample did not show any brown color, but indicated more purple and presented swollen cells. Brown color showed pancreatic β -cells, while the purple was not pancreatic β -cells. It was

significantly different from coloring result in the normal rats. Figure 3.C and D show the presence of insulin-immunoreactive cells in the islets of normal rats with medium intensity. Brown color indicated the presence of insulin produced by pancreatic β -cells. It was observable that pancreatic β -cells were not found in the islet Langerhans of controlled diabetic rats. It supported the assumption that STZ induction would cause a damage in the pancreatic β -cells (Szkudelski, 2012; Rajeswari *et al.*, 2012), accordingly insulin production decreased, blood glucose levels increased, and hyperglycemia occurred. Intense brown color indicated the presence of the insulin and pancreatic β -cells. The clearer the brown color intensity indicated the higher the presence of insulin contained.

Figure 3.E and F indicates the high intensity of insulin-immunoreactive cells in the islets of K1 rats. The intensity of insulin-immunoreactive cells decreased in K2 rats (Figure 3.G and H), and disappeared in K3 rats (I and J). The observation on K1, K2 and K3 (Figure 3.E, F, G, H, I, and J) rats showed that the higher the dose of ethyl acetate extract, the lower the intensity of insulin produced by pancreatic β -cells; however, K3 with the highest dose of ethyl acetate extract showed brown color similar to the normal rats group. These phenomena indicated that there was inability of pancreatic β -cells to secrete insulin or volume reduction of pancreatic β -cells. In comparison with the result of the blood glucose and blood insulin levels analysis, it was concluded that the higher the dose of ethyl acetate extract, the higher the blood insulin levels, and the lower the blood glucose levels. In spite of the decreasing insulin intensity in ethyl acetate extract supplement to K2 and K3, the insulin still functioned well.

Accordingly, high dose of ethyl acetate extract (K2 and K3) did not increase pancreatic β -cell regeneration, but it reduced insulin resistance. It was assumed that higher dose of ethyl acetate extract would not increase pancreatic β -cell volume that produced insulin in the islet of Langerhans. In the hyperglycemic condition, pancreatic β -cell degranulation occurred, so that the number of pancreatic β -cells decreased (DeFronso *et al.*, 1992).

Nutrition factor including the antioxidant was significant in the medication of diabetes mellitus and its complications (Alberti *et al.*, 1997; Packer *et al.*, 2000). Defense mechanism against oxidative stress and imbalance antioxidant in diabetes might cause cell and tissue damage, and trigger diabetes complications. A sufficient antioxidant intake could prevent or decelerate the increasing diabetes complications (Packer *et al.*, 2000). The ethyl

Table 2. The intensity of insulin-positive cells in islets of Langerhans of experimental rats

Parameter	Normal group	Diabetes group	Group K1	Group K2	Group K3
Intensity of insulin positive cells	+	-	+++	++	+

Note : Negative : -, low intensity : +, medium intensity : ++, and high intensity : +++

Normal group: normal controlled rats; DM group: diabetes controlled rats; Group K1: diabetic rats with feed standard and treated with 0.985 mg/20g diet ethyl acetate extract; Group K2: diabetic rats with feed standard and treated with 1.97 mg/20g diet ethyl acetate extract; and Group K3: diabetic rats with feed standard and treated with 2.955 mg/20g diet ethyl acetate extract

acetate extract supplement as an antioxidant could repair pancreatic β -cell damage in the diabetic rats. The antioxidant could protect pancreatic β -cells by neutralizing toxin effect from oxidative stress triggered by hyperglycemia, while Donath *et al.* (1999) proved that hyperglycemia induced pancreatic β -cell death in the islet *Psamomys obesus*. It was in accordance with the result of semi quantitative observation on intensity of insulin positive cells as presented in Table 2. Based on the semiquantitative data observation in Table 2, the intensity of insulin-positive cells in diabetes group was negative, K1 had the highest intensity and started to decrease in K2 (medium intensity) and K3 (low intensity). The intensity of the insulin-positive cells in the diabetic rats group with ethyl acetate extract in K3 was low, but it was similar with the normal rats group. It supported the assumption that the higher the dose of ethyl acetate extract, the regeneration of pancreatic β -cells was lesser, but it could lower the insulin resistance and increase the ability to secrete insulin, as indicated from the higher blood insulin levels. It can be concluded that the mechanism of decreasing blood glucose level and regeneration of pancreatic β -cells due to the intake of ethyl acetate extracts from *Pandanus amaryfollius* leaves has the ability of antioxidant so as to decrease liver MDA levels in diabetic rats and can to protect pancreatic β -cells by neutralizing toxins from oxidative stress due to hyperglycemia.

Conclusion

Supplementing ethyl acetate extract to the diabetic rats could lower the blood glucose and MDA levels in the rats' liver and increase the blood insulin levels. The higher the ethyl acetate extract supplement resulted in the lower blood glucose levels and liver damage due to the oxidative stress, and the higher blood insulin levels, but it did not increase the pancreatic β -cells. In the high-dose K2 and K3, in spite of lower pancreatic beta cell volume, the

insulin secretion was high and the insulin resistance decreased, so that the anti-diabetic ability remained high. The results of the research can be recommended that ethyl acetate extract from *Pandanus amaryfollius* leaves can be used for the therapy of diabetics, but the dose of its should be investigated further.

Acknowledgement

Sincerest gratitude goes to Directorate of Higher Education, Ministry of Research, Technology and Higher Education Indonesia for the research grant.

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