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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2017.28.35

Research Article
Levels of Blood Glucose and Insulin Expression of Beta-cells in Streptozotocin-induced Diabetic Rats Treated with Ethanolic Extract of *Artocarpus altilis* Leaves and GABA

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Abstract

Background and Objective: Information about the *Artocarpus altilis* leaf as an antidiabetic associated with the active compounds Gamma Amino Butyric Acid (GABA) is still limited. This study was conducted to determine the effects of ethanolic extract of *A. altilis* leaves decoction and GABA on blood glucose levels and insulin expression of beta-cells in streptozotocin-induced diabetic rats. **Material and Methods:** This study was done by using completely randomized design and male Sprague Dewley rats. The rats were devided into normal control group and diabetic rats groups. Levels of bood glucose were measured using strip rapid test. The insulin expression in beta-cells was assessed using immunohistochemistry. Quantitative data were analyzed using ANOVA at 5% confidence level. **Results:** The result indicated that 50 mg kg^{-1} b.wt., GABA, 400 and 800 mg kg^{-1} b.wt., ethanolic extract of *A. altilis* leaves decreased the level of blood glucose and increased the insulin expression in pancreas beta-cells. **Conclusion:** The GABA and ethanolic extract of *A. altilis* leaves with a minimum dose of 400 mg kg^{-1} b.wt., can be used as an antidiabetic. Pancreas is the target organ was affected by GABA and *A. altilis* leaves as antidiabetic agents. Results of this study may support the development of research on the potency of GABA in natural materials as antidiabetic particularly type 1 diabetes.

Key words: Diabetes, streptozotocin, *Artocarpus altilis*, GABA, IHC, insulin, beta-cell, blood glucose, pancreas

Received: August 26, 2016 Accepted: November 05, 2016 Published: December 15, 2016

Citation: Meti Indrowati, Rarastoeti Pratiwi, Rumiyati and Pudji Astuti, 2017. Levels of blood glucose and insulin expression of beta-cells in streptozotocin-induced diabetic rats treated with ethanolic extract of *Artocarpus altilis* leaves and GABA. Pak. J. Biol. Sci., 20: 28-35.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus is a carbohydrate metabolism disorder caused by insulin deficiency or ineffective insulin. This metabolic disease has become health concern with morbidity rate up to 150 million people in 2000, 221 million in 2010 and estimated in 2025 will reach 300 million people[1]. Around 5-10% of diabetes cases are type 1 diabetes[2]. Type 1 diabetes is signed by little to no insulin production, which caused by rupture or destruction of more than 70% of pancreas beta-cells[3,4].

The study on therapy of diabetes is important to be conducted because the therapy itself is complex and also causing side effect. The treatment of this disease by working out, diet management and administration of oral hypoglycemia drugs are not enough. Those treatment must be conducted in addition with injection of insulin continuously or pancreas transplantation surgery[5]. Insulin injection from synthetic product has flaw such as the need to increase the dose because of the insensitivity, complexity of exogenous insulin mechanism regarding time, acute hypoglycemia risk, mortality potential andalso the increase chance of certain cancer risk[6]. Related with those facts, a new treatment are required to be developed from natural alternative sources such as natural compound consist of active compound which had a potential to become antidiabeatics for diabetes.

One of the alternative source from nature which had potential used as antidiabetic is breadfruit *Artocarpus altilis* (Park.) Fosberg leaves[7,8]. Extract of *A. latilis* leaves can decrease blood triglycerid, cholesterol and glucose level of rats were glucose loaded[9,10]. Leaves of *A. altilis* was traditionally used in Western India as a material to make tea and believed to have antidiabetic effect[11]. The *A. altilis* leaves potentially used as antidiabetic because it contains active compound Gamma Amino Butyric Acid (GABA)[11,12].

Active compound GABA can be synthesized by plant[13], lactic acid bacteria[14] and also animal[15]. In mammal, GABA is the main neurotransmitter in central nervous system[16]. This neurotransmitter take part in inhibitory synapses[17] by inducting $GABA_A$ receptor which resulted in hyperpolarization of post synapse membrane[15,18]. Besides in central nervous system, GABA also exists in high concentration in pancreas beta-cells with insulin[19] but inside different vesicle which are Large Dense Core Vesicles (LDCVs) for insulin and Synaptic Like Micro Vesicles (SLMVs) for GABA[20,21]. Earlier research reported that GABA in pancreas had correlation with diabetes in regards with regulating glucose homeostasis condition[22] and also including insulin and glucagon in their regulation process[23].

Testing the use of synthetic GABA in the treatment of diabetes had been done by Jester[24], which was combined with xylitol. In general, synthetic GABA has not been commonly used in the treatment of diabetes because the pancreas GABA function had not been known comprehensively[4,20,21], including possible side effects. Research on the leaves of *A. altilis* as antidiabetic had been conducted but information about the *A. altilis* leaf as an antidiabetic associated with the active compounds GABA is still limited. This study was conducted to determine the effects of ethanol extract of *A. altilis* leaves decoction and GABA on blood glucose levels and insulin expression of beta-cells in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Extraction of *A. altilis* leaves: The leaves of *A. altilis* was collected from Karanganyar Central Java Indonesia. The *A. altilis* leaves were previously washed and cut into small pieces. They were dried in oven at 45°C for 5 days and crushed into fine powder. The powdered part (354.19 g) was macerated with 3600 mL ethanol 50%, stirred for 30 min and incubated for 24 h, then filtered. This process was twice repeated. The filtrate that produced in this process was distilled at 70°C using a rotary vacuum evaporator in water bath. This step produced the viscous extract. Then the viscous extract was poured in a porcelain dish and heated in a water bath at 70°C while constantly stirred. This process produce 47.47 g ethanolic extract of *A. altilis* leaves that ready to used.

Diabetes induction and treatment: The experiments was conducted in accordance with the ethical rules of the Animal Ethics Committee Universitas Gadjah Mada. Twenty five male Albino rats (*Rattus norvegicus* Berkenhout, 1769) Sprague Dewley strain, 2 months old, weighing 100-200 g were purchased and housed in stainless steel cages at the Integrated Research and Testing Laboratory (LPPT) UGM Unit 4. The rats were maintained at room temperature 22-24°C with light and dark periods at 12 h light/12 h dark intervals. The animals had free access to food (standar pellet diet) and water *ad libitum*. They were acclimatized for 7 days before conducting the experiments.

Single dose intraperitoneal injection of STZ streptozotocin (N-methyl nitroso carbamoyl) alpha-D-glucosamine, from Nacalai, at the dose of 50 mg kg^{-1} b.wt. was used for diabetes induction. Freshly made solutions of STZ dissolved in 0.1 M citrate buffer pH 4.5. Citrate buffer made by fixing of trinatrium

citrat dihydrateand citric acid. Five days following the injection, tail blood samples from overnight fasted rats were obtained to measure blood glucose levels. Rats with blood glucose levels above 200 mg dL^{-1} were selected as diabetic rats.

This study was done by using Completely Randomized Design (CRD). Twenty five rats were devided into five groups, each containing five rats. The rats were devided into a group of normal rats as a normal control group and four groups of Streptozotocin (STZ)-induced diabetic rats 50 mg kg^{-1} b.wt., dose. The four groups of diabetic rats were administrated by treatments as follow: Saline as a diabetic control, 50 mg kg^{-1} b.wt., GABA per oral, 400 and 800 mg kg^{-1} b.wt., ethanolic extract of *A. altilis* leaves per oral. Levels of bood glucose were measured before induction as the initial level (pre treatment), after STZ induction on days 0.21 and 42, using strip rapid test from GlucoDR. The measurements of body weight was employed on the pre-treatment day 0, 7, 14, 21, 28 and 42 using scale.

Immunohistochemical staining of insulin: The pancreases were fixed using Bouin's solution. Histological examination of the pancreas was performed on paraffin sections with immunohistochemical staining of insulin, using insulin antibody from Biogenex. The microanatomy of the pancreas was observed under the microscope and analyzed descriptively. Slides were examined and photographed using binophoto microscope fitted with Nikon Eclipse 50 idigital camera.

Data analysis: The statistical analysis were carried out using SPSS 16.0 for windows. Blood glucose levels were expressed as Mean±SD. Values in all group were compared using the analysis of variance (ANOVA) and the level of significance was set at $p<0.05$.

RESULTS

Body weight: The measurements of body weight of diabetic rats with ethanolic extract of *A. altilis* leaves and GABA treatments are shown at Fig. 1. The measurements of body weight was employed on pre-treatment, treatment day 0, 7, 14, 21, 28 and 42 using scale.

Levels of blood glucose: The results of blood glucose level with GABA and ethanolic extract of *A. altilis* leaves treatments on streptozotocin-induced diabetic ratsare well listed in Table 1.

Number of animals was 25 males SD *Rattus norvegicus* were devided into 5 groups (n = 5). The measurements of blood glucose level was employed on pre-treatment, treatment day 0, 21 and 42 using strip strip rapid test.

Blood glucose level were expressed as Mean±SD. Values in all groups were compared using the analysis of variance (ANOVA), statistical significance was fixed at $p\leq0.05$. Changes in the treatment groups were compared with the normal control group using Duncan test (DMRT) to control for

Table 1: Effects of GABA and ethanolic extract of *A. altilis* leaves treatments on the level of blood glucose streptozotocin-induced diabetic rats

	Blood glucose level (mg dL^{-1}) days			
Groups	Pre-treatment	0	21	42
Normal	123.6±13.9	135.6±6.1^{a}	129.0±14.9^{a}	140.0±14.5^{a}
Diabetic	136.2±16.3	306.2±13.9^{c}	367.8±14^{c}	3852.0±14.2^{c}
GABA 50 mg kg^{-1} b.wt.	127.4±11.2	370.6±14.4^{bc}	360.6±21.7^{bc}	226.4±15.3^{bc}
A. altilis 400 mg kg^{-1} b.wt.	130.4±19.2	375.2±13.7^{bc}	283.4±19.3^{bc}	292.8±15.9^{bc}
A. altilis 800 mg kg^{-1} b.wt.	131.8±18.28	346.8±14.7^{b}	216.4±12.1^{b}	146.4±5.8^{b}

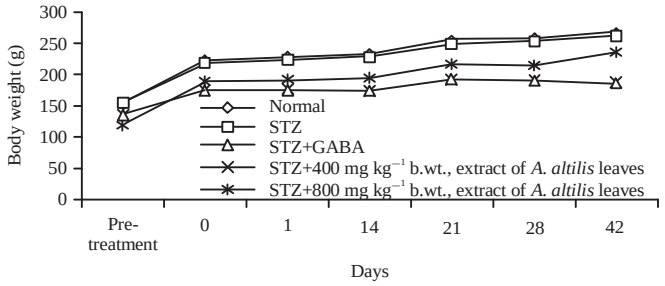

Fig. 1: Body weight on diabetic rats with ethanolic extract of *A. altilis* leaves and GABA treatments

multiple comparison. Statistical calculations were performed using the SPSS 16.00 software. In table, numbers followed by the same letter in the same column indicates no significant difference.

Insulin immunohistochemistry: Immunohistochemistry (IHC) method performed to determine insulin expression in beta-cell. Insulin immunopositive cells shown by dark brown color inside the cytoplasm of beta cell. Reactivity of beta-cell to insulin antibody are divided into three categories i.e., strong (insulin immunopositive cell ≥75-100% of islet), moderate (25-75% of islet) and weak (≤25% of islet)[25].

The result indicated that 50 mg kg^{-1} b.wt., GABA, 400 and 800 mg kg^{-1} b.wt., ethanolic extract of *A. altilis* leaves decreased the level of blood glucose and increased the insulin expression in pancreas beta-cells.

DISCUSSION

Body weight of rats was estimatedin this research to evaluates the health of the rats in general[26]. Based on the results, Fig. 1 i.e., changes in body weight of rats during treatment showed body weight gainduring treatment. The weight gain occurred in all groups, whether in groups of normal, diabetic, treatment of GABA and *A. altilis* leaves ethanol extract treatment. The increase in body weight of rats in control and treatment groups did not differ significantly from day to day during the 42 days of treatment. Ethanol extract of *A. altilis* leaves or GABA orally did not affecton the growth and development of male Sprague Dawley rat body weight for 42 days. On the last day of treatment, none of the group exceeds 300 g b.wt. According to Kohn and Clifford[27] normal mouse weight was no more than 300-400 g. In this study, the body weight of rats was not classified as obese condition. So, the treatment of GABA and ethanolic extract of *A. altilis* leaves in diabetic rats does not cause obesity.

Table 1 showed the changes in glucose levels both in the control and treatment groups. In the pre-treatment, glucose levels of the rats did not reach 200 mg dL^{-1} as diabetes indication. Induction of streptozotocin 50 mg kg^{-1} b.wt., by intraperitoneal injection was able to make the conditions of hyperglycemia in rats. Diabetic status condition was marked by the level of blood glucose levels >200 mg dL^{-1} during fasting period[5]. Streptozotocin induced diabetes by STZ infiltration on beta-cells through the glucose transporter GLUT-2. The infiltration caused alkylating DNA from nitrosourea cluster. The STZ acts as a donor of nitric oxide which plays a role in beta-cell damage through reconstructed the formation of cGMP and increased guanilil cyclase activity. Beta-cells damaged DNA activated poly ADP-ibosylation resulting emphasis cellular of celluler NAD^+, decrease level of ATP and ultimately inhibits the synthesis and secretion of insulin[28]. Arising of hyperglycemic conditions was due to inhibition of the synthesis and secretion of insulin. Based on the research results, STZ dose of 50 mg kg^{-1} b.wt., proved to be capable as an agent to induce diabetes, as indicated by the average blood glucose levels in diabetic and control rats treated on days 0 reaches levels above 200 mg dL^{-1}.

Statistically according to Table 1, control treatment group showed significant difference compared with other groups. The diabetes group showed significant difference with group treated with 800 mg kg^{-1} b.wt., of *A. altilis* leaves. The GABA group and 400 mg kg^{-1} b.wt., of *A. altilis* leaves showed difference with diabetes group but not significantly. This results showed that treatment with ethanolic extract of *A. altilis* leaves 800 mg kg^{-1} b.wt. had the biggest impact in reducing blood glucose level, while the *A. altilis* 400 mg kg^{-1} b.wt., leaves and GABA were able to reduce the glucose blood level but not significant.

The mechanism of GABA that can reduce glucose level in blood occurs because the mechanism is related with beta-cell and insulin. The GABA secreted by pancreas beta-cell can act as autocrine which is acting inside the cell itself and paracrine which is acting inside nearby cell because of the receptor factor location. The GABA as autocrine was known to have regenerative effect, increasing proliferation and suppressing the apoptosis of pancreas beta cell[4,29] and also take part in regulating the insulin secretion especially regulating proinsulin synthesis[21,30]. The GABA coordinates with insulin in mechanism of regulating the function of secretion of pancreas α-cell[31]. The GABA as paracrine[32], along with insulin is one the inhibitor factor of glucagon secretion[33], by inducting hyperpolarization in α-cell membrane and increasing influx[33,34] of Cl^-.

Based on the research results, *A. altilis* leaves ethanol extract confirmed its ability to reduce blood glucose level in diabetic rats. According to Indrowati *et al.*[35], *A. altilis* leaves ethanol extract contained GABA which were detected with thin layer chromatography static phase silica GF_{254} and mobile phase BAW (3:1:1) with average mark of Rf 0.49. Subsequent research conducted by Indrowati *et al.*[9] revealed that in ethanol extract of breadfruit leaves, GABA was detected with quantity 0.0098±0.003%. Ethanolic extract of *A. altilis* leaves contain flavonoids, detected by TLC with static phase silica GF_{254} and mobile phase chloroform:methanol (98:2)[36].

Extract ethanol of *A. altilis* leaves as much as 800 mg kg^{-1} b.wt., confirmed its highest effectivity to reduce glucose blood level. This showed that even though in the extract contained GABA, where GABA was known to inhibit

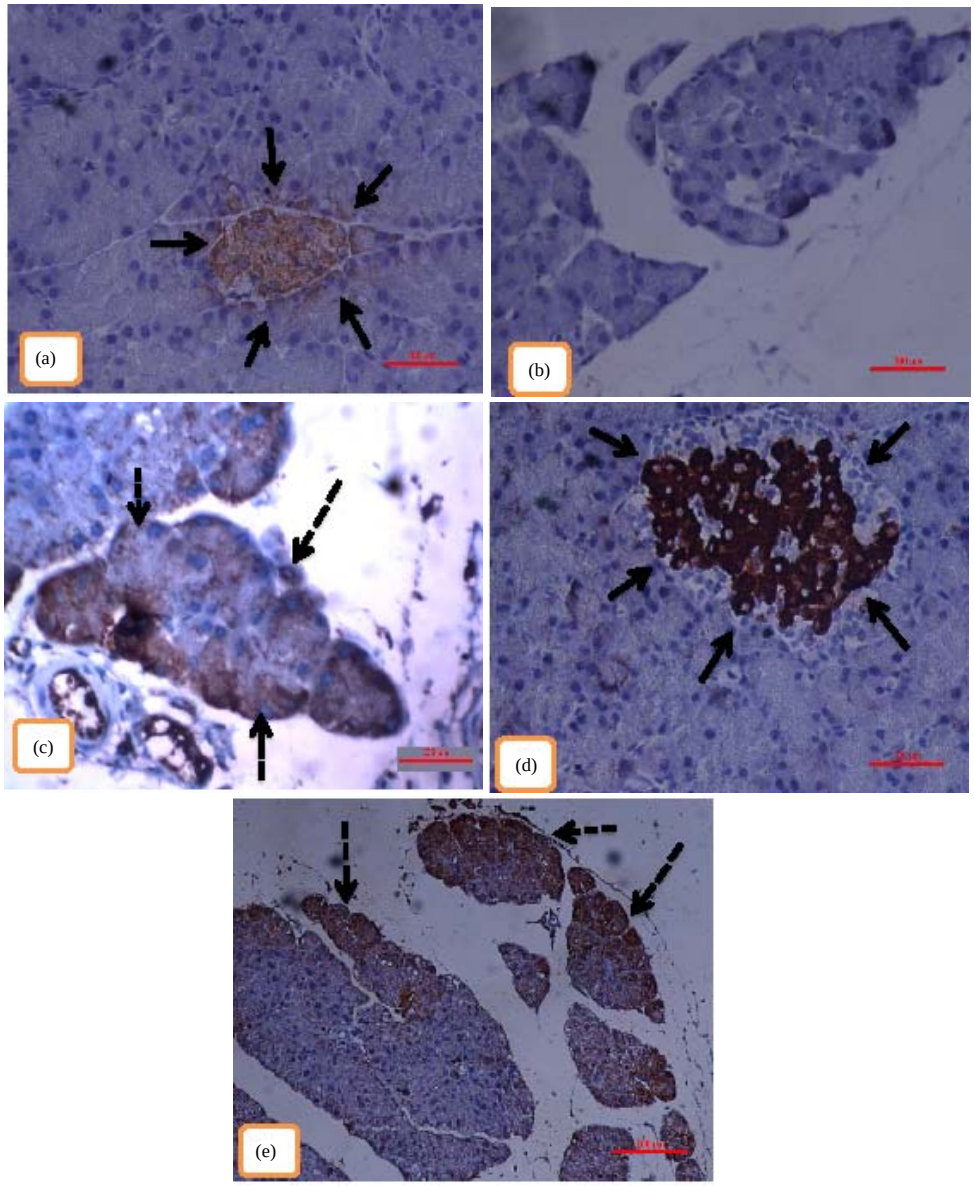

Fig. 2(a-e): Photomicrographs of insulin immunohistochemical staining of pancreatic islets. (a) Normal control group, (b) Diabetic group, (c) GABA treatment, (d) 400 mg kg^{-1} b.wt., ethanolic axtract of *A. altilis* leaves treatment and (e) 800 mg kg^{-1} b.wt., ethanolic axtract of *A. altilis* leaves treatment. Beta-cells, expression which were immunoreactive with insulin was shown by dark brown color inside the cytoplasm of beta-cells, →: Arrows indicate insulin-immunopositive cells with strong reactivity (cell reactivity ≥75-100% of islet),--->: Arrows indicate moderate reactivity (cell reactivity 25-75% of islet), No arrow in (b) Indicate weak reactivity (cell reactivity ≤25% of islet)[25]

insulin secretion mechanism, there were other compound affected the glucose blood level. Lotulung *et al.*[7] stated that inside *A. altilis* leaves was contains flavonoid that also have an effect in glucose level. It can be concluded that pure GABA can decrease blood glucose level but it will have a better effect if GABA is used with other nature compound in *A. altilis* leaves.

In the current study, insulin expression in beta-cell was determined with immunohistochemistry (IHC) method. Beta-cell expression which were immunoreactive with insulin was shown by dark brown color inside the cytoplasm of beta-cell in the histological sections obtained after the treatment of rats.

Figure 2 showed photomicrograph of IHC insulin slides stained in each group. In the normal group it showed the highest beta-cell expression which immunoreactive with insulin (strong reactivity). It reflected the number of beta-cell

cytoplasm stained with dark brown (Fig. 2a). In diabetic groups, the immunoreactive beta-cell expression was lowest (Fig. 2b) with weak reactivity.

The treatment group, GABA and *A. altilis* ethanol extract showed a recovery in beta cell which were immunoreactive with insulin, even though it did not reach the level of the normal condition as in the normal group (Fig. 2c-e). Beta-cell on GABA treatment group has moderate reactivity (Fig. 2c), 400 mg kg^{-1} b.wt., ethanolic axtract of *A. altilis* leaves treatment group has strong reactivity (Fig. 2d) and 800 mg kg^{-1} b.wt., ethanolic axtract of *A. altilis* leaves treatment group has moderate reactivity to insulin (Fig. 2e). Three groups of treatment confirmed their ability to increase insulin expression in beta-cell, eventhoughthe quality could not be observed clearly in the group which showed the highest significant in insulin expression in pancreas beta-cell.

The decrease of beta-cell expression which were immunoreactive to insulin indicates the decrease of insulin synthesized by those cells, so that antibody administration to insulin through immunohistochemistry staining only react with insulin producing cells. The decrease of insulin synthesis mechanism signified a damaged in pancreas beta-cells by induction previously of the STZ. According to Soltani *et al.*[4], STZ is glucosamine-nitrosouren compound like other alkylating agent in nitrosure class. Streptozocin caused toxic effect by destroying cell DNA. Inside cell, streptozocin was like glucose that were transported by glucose protein transporter (GLUT-2) but was not recognized by the other glucose transport mechanism.

Based on the research results, it was known that GABA and *A. altilis* leaves ethanol extract treatment had proven to be able to reduce blood glucose level in diabetes rats induced with STZ andincreased insulin expression in beta-cells by immunohistochemistry observations. This was in accordance with records of Andrali *et al.*[37] which stated that the rises of blood glucose level related with insulin secretion and insulin gene transcription. The GABA compound plays a role in insulin secretion and reducing of glucose blood level. This findind was also stated previously by Dong *et al.*[30]. The latter reference found relationship between GABA and insulin secretion from pancreas beta-cell and the changes of glucose blood level. In addition to that, Bansal *et al.*[31] mentioned that GABA coordinated with insulin in regulating the pancreas cell secretion function. Bailey *et al.*[32] postulated a correlation between pancreas α-cell with $GABA_A$ receptor expression. The GABA was found in high concentration in pancreas beta-cells along with insulin but at different vesicles[19], which was Large Dense Core Vesicles (LDCVs) for insulin and Synaptic Like Micro Vesicles (SLMVs) for GABA[20,21,38]. The GABA takes part as autocrine modulator by triggering depolarization of beta cell so that Ca^{-} influx are increasing then resulted in insulin secretion andalso along with insulin had role as paracrine by increasing influx Cl^{-} in α-cell and suppressing glucagon secretion[23].

According to the current results, it was found that GABA treatment in STZ induced diabetic rats were able to reduce blood glucose level and increased insulin expression in pancreas beta-cell. The treatment of *A. altilis* leaves ethanol extract that contained GABA with dosage 400 and 800 mg kg^{-1} b.wt., proved to be able to reduce blood glucose level and increased insulin expression in pancreas beta-cell. The decrease of blood glucose level showed significant results in ethanol extract *A. altilis* leaves treatment with 800 mg kg^{-1} b.wt., dosage in diabetes rats.

CONCLUSION

It can be concluded that 50 mg kg^{-1} b.wt., GABA and ethanolic extract of *A. altilis* leaves with a minimum dose of 400 mg kg^{-1} b.wt. can be used as an antidiabetic. Pancreas is the target organ which was affected by GABA and *A. altilis* leaves as antidiabetic agents.

SIGNIFICANT STATEMENT

- This study about the potency of *Artocarpus altilis* leaves as antidiabetic and it correlation with GABA
- Research on the leaves of *Artocarpus altilis* leaves as antidiabetic had been conducted but information about the *Artocarpus altilis* leaf as an antidiabetic associated with the active compounds GABA is still limited
- This study was conducted to determine the effects of ethanol extract of *Artocarpus altilis* leaves decoction and GABA on blood glucose levels and insulin expression of beta-cells in streptozotoc in-induced diabetic rats
- The results provided scientific information about the target organ affected GABA and *Artocarpus altilis* leaves as antidiabetic agents

ACKNOWLEDGMENTS

The author thank to Directorate General of Higher Education Ministry of Research Technology and Higher Education, Republic Indonesia for financially this study as part of the Doctoral Programme at Universit as Gadjah Mada. Core facilities and services were supported byIntegrated Research and Testing Laboratory (LPPT) Universitas Gadjah Mada Unit 4.

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