

## Antidiabetes of Combination of Fractionated-extracts of *Andrographis paniculata* and *Centella asiatica* in Neonatal Streptozotocin-induced Diabetic Rats

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### Info Article

Submitted: 17-05-2020

Revised: 04-12-2020

Accepted: 30-12-2020

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### ABSTRACT

Many medicinal plants are widely grown in South- and Southeast Asia countries. Some of them are traditionally used for treatment of diabetes mellitus such as *Andrographis paniculata* and *Centella asiatica*. In the study, we provided fractionated-extracts of *A. paniculata* and *C. asiatica* to increase the concentration of their active compounds and eliminate unexpected substances. The aim of the study was to evaluate the antidiabetes effect of the combination of their fractionated-extracts in male neonatal streptozotocin (STZ)-induced diabetic rats. In the study, diabetes was injected intraperitoneally 90 mg.kg<sup>-1</sup> BWSTZ to two day-old rats. At the age of three months, the rats were administered with the combination of both fractionated-extracts for 14 consecutive days. We evaluated antidiabetes with parameters of blood glucose levels, morphology of pancreatic islet,  $\beta$ -cell density as well as immune histochemical pancreatic insulin. The levels of MDA, SOD and GPx were also determined about oxidative stress change after treatment with the combination. In the study, the combination succeeded to lower the blood glucose level in neonatal STZ-induced diabetic rats. In line with fact, improvement of rat pancreatic islets and  $\beta$  cells density, as well as moderate restoration of pancreatic insulin, were observed after treatment with the combination. The substance could decrease the level of MDA, and increase the levels of SOD and GPx. In conclusion, the combination of fractionated-extracts of *A. paniculata* and *C. asiatica* exhibited potential antidiabetic effect to its antioxidative effect in male neonatal STZ-induced diabetes rats.

**Keyword:** *A. paniculata*, *C. asiatica*, Antioxidant, Antidiabetes, Pancreatic Protection

### INTRODUCTION

Diabetes mellitus (DM) is chronic diseases induced by damage/disorder in carbohydrate metabolism that resulting hyperglycemic. The incidence of diabetes mellitus every year is always increasing, especially in developing countries. Based on WHO data, in 2012 about 1.5 million deaths were caused by diabetes mellitus (WHO, 2016). In the condition of diabetes mellitus, the

decrease of pancreatic  $\beta$  cells can be contributed by apoptosis (Butler *et al.*, 2003), resulting in insulin deficiency and turn increasing blood glucose levels.

South- and Southeast Asia countries have big biodiversity together with medicinal plants. Exploration of medicinal plants is very interesting and widely done to provide alternative medicines. *A. paniculata* and *C. asiatica* are two examples of traditionally medicine that used for treating

hyperglycemic in diabetic patients. Ethanolic extract of *A. paniculata* lowered the blood glucose levels in STZ-induced diabetic rats (Zhang and Tan, 2000). The fractionated extract of *A. paniculata* can succeed to lower blood glucose levels, improve and increase the number of pancreatic cells in neonatal streptozotocin (STZ)-induced diabetic rats (Nugroho *et al.*, 2013a). Similar studies were conducted by Zhang *et al.* (2009), andrographolide which is the main active compound in herbal *A. paniculata* exhibit to have blood glucose reduction activity and protection against alloxan-induced pancreatic beta cells. In the other hand, the ethanol extract of *C. asiatica* was able to lower blood glucose levels and inhibited significant damage to pancreatic  $\beta$  cells in alloxan-induced rats (Chauhan *et al.*, 2010), reduced blood glucose levels, and cholesterol in STZ-induced male rats (Gayathri *et al.*, 2011).

Andrographolide and asiaticoside are the main active compounds of *A. paniculata* and *C. asiatica* respectively. Reportedly, andrographolide could prevent diabetes nephropathy by inhibiting renal oxidative stress, inflammation, and also fibrosis through inhibition of Akt/NF- $\kappa$ B signalling pathway in STZ-induced diabetic rats (Ji *et al.*, 2016). Asiaticoside exhibited anti-diabetic, antioxidant, anti-inflammatory and modulated PI3K/Akt/GSK-3 pathway effects (Yin *et al.*, 2015).

In the research, we evaluated the anti-diabetes effect of the combination of their fractionated-extracts in male diabetic rats that induced neonatal streptozotocin (STZ). The parameters were blood glucose levels, pancreatic islets and beta cells morphology, density of beta cells, pancreatic insulin. Besides, the levels of MDA, SOD and GPx were also determined concerning oxidative stress change.

## MATERIAL AND METHODS

Andrographolide analytical standard and asiaticoside functioning as biological markers, streptozotocin (STZ) as a permanent diabetogenic compound for animal model of type 2 diabetes, glibenclamide (a sulfonylurea antidiabetic drug) were purchased from Sigma Aldrich USA. Measurement of blood glucose level was based on enzymatic colour metric method using glucose GOD-PAP reagent purchased from DiaSys, Diagnostic Systems GmbH, Holzheim, Germany. Materials for haematological study such as Hematoxylin, Eosin and Victoria blue were obtained from Sigma Aldrich USA. Insulin expression is determined using primary anti-

insulin antibody and secondary chicken anti-goat IgG antibody (Abcam USA). All other chemicals used in the experiments were high-grade qualified materials.

### Preparation of fractionated-extract of *A. paniculata*

*A. paniculata* was obtained from the area of Special Region of Yogyakarta Indonesia during March 2016. The plant was identified at Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada Indonesia.

Dried powder of *A. Paniculata* herbs was macerated with 90% ethanol (1:10) for 7 days, and the liquid extract was collected. The residue was remacerated with 90% ethanol (1:2) solvent for 24h. Remaceration process was performed twice. To obtain viscous extract, all liquid extracts were evaporated by rotary vacuum evaporator. The extract was fractionated using the *n*-hexane (1:10) to eliminate hydrophobic ballast compounds such as chlorophyll, waxes, fatty acids, or terpenes. The *n*-hexane insoluble fraction was collected and then fractionated with ethyl acetate (1:10) to provide ethyl acetate-soluble and insoluble fractions. The last fraction was collected and evaporated to provide viscous fractionated-extract. Quantification of andrographolide in the viscous fractionated-extract was determined using TLC-Densitometry method.

### Preparation of fractionated-extract of *C. asiatica*

*C. asiatica* was also collected from the area of Special Region of Yogyakarta Indonesia during March 2016. The plant was identified by a botanist at Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada Indonesia. The voucher specimen is kept in this department.

Dried powder of *C. asiatica* herbs was macerated with 90% ethanol (1:10) for 7 days, and the liquid extract was collected. The residue was separated and then remacerated with 90% ethanol (1:2) solvent for 24h. This process was done twice. All collected liquid extracts were evaporated using a rotary vacuum evaporator under reduced pressure to provide a viscous extract. The viscous extract was fractionated using the *n*-hexane (1:10). The *n*-hexane insoluble fraction was fractionated and then evaporated to provide a viscous fractionated-extract. Quantification of asiaticoside in the viscous fractionated-extract was determined using TLC-Densitometry method.

### Induction of neonatal diabetic rats

The use of experimental animal in this study was conducted under the guidance of the basic standard, which has been prepared and approved by the Ethics Committee of Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Indonesia (No.62/04/LPPT/X/2016). Two-day-old neonates were intraperitoneally injected with STZ at the high dose of 90 mg.kg<sup>-1</sup> BW. Control neonates were given with the vehicle of STZ, citrated buffer pH 4.5. After three months of STZ induction, male neonatal rats were selected by determining the blood glucose level. The rats were considered diabetic if their blood glucose levels are more than 1.5 fold of this control rat.

### Experimental groups for assessment of *in vivo* antidiabetic activity

Animals were divided into 8 groups i.e. group 1 was non-diabetic rats, and the other groups were diabetic rats. The neonatal STZ-induced diabetic rats were divided into 7 groups (group 2-8). All treatment was administered once daily for 14 days. Group 1: normal control (non-diabetic rats); Group 2: negative control (diabetic rats received the drug vehicle orally); Group 3: diabetic rats and treated once daily with glibenclamide dose 4.5 mg.kg<sup>-1</sup> BW; Group 4: diabetic rats and treated with fractionated-extracts of *C. asiatica* (FECA) dose 1.0 g.kg<sup>-1</sup> BW orally; Group 5: diabetic rats and treated with fractionated-extracts of *A. paniculata* (FEAP) dose 1.3 g.kg<sup>-1</sup> BW orally; Group 6: diabetic rats and treated with combination of FECA (0.3 g.kg<sup>-1</sup> BW) and FEAP (0.9 g.kg<sup>-1</sup> BW) orally; Group 7: diabetic rats and treated with combination of FECA (0.5 g.kg<sup>-1</sup> BW) and FEAP (0.65 g.kg<sup>-1</sup> BW) orally; Group 8: diabetic rats and treated with combination of FECA (0.7 g.kg<sup>-1</sup> BW) and FEAP (0.4 g.kg<sup>-1</sup> BW) orally;

The fasting blood samples was collected from the orbital sinus of light anaesthetized-rats at days 0 (initial data base), 7 and 14 after 12h fasting. Two hours after oral administration of glucose 1.75 g.kg<sup>-1</sup> BW, blood samples were collected for postprandial glucose levels. The blood was then allowed to stand at room temperature for 30 min. The samples were then centrifuged at 3600 rpm for 10 min to provide serum. The serum glucose levels were determined based on enzymatic colourimetric method using glucose GOD-PAP.

### Measurement of enzyme activity

Fresh liver samples were washed with saline in an ice bath. The samples were then extracted

with 0.1 M Tris/HCL pH 7.4 and homogenized to provide a homogenate for measurement of superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA) activities. SOD measurement is based on generation of superoxide radicals by reaction of xanthine and xanthine oxidase with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride to form a red formazan. SOD activity was evaluated based on percentage of inhibition of this reaction. GPx measurement is performed according to a coupled reaction with glutathione reductase. Process of hydroperoxide reduction by GPx results in oxidized glutathione (GSSG) and recycled to its reduced form by the role of glutathione reductase and NADPH. Process of oxidation of NADPH to NADP<sup>+</sup> is represented by a decrease of absorbance at 340 nm that proportional to GPx activity in the sample (Ismail *et al.*, 2012). Whereas, the measurement of MDA was according to thiobarbituric acid (TBA) reactivity (Wasowicz *et al.*, 1993).

### Histological observation of pancreas

At the end of the experiment period, the rats were sacrificed for histological observation of Langerhans islet of pancreas. Rat's pancreas was then removed and fixed with a solution of 4% paraformaldehyde in 1x phosphate buffered saline (PBS) for 24 hours. The tissue was paraffin block-embedded and sectioned at 4 µm using microtome. The section was deparaffinized by incubating with xylene and washed with different graded ethanol dilution and stained with hematoxylin and eosin (H & E), or 0.1% Victoria blue (pH 0.3) to observe the Langerhans islet and beta cells of pancreas, respectively. After clearing with xylene, the slide was mounted with a mounting medium. The objects were observed under the light microscope (Olympus BX51, Japan) with 40x or 100x magnification. Each section was observation was randomly photographed by four times and the pancreatic content of beta cells evaluated using semiquantitative analysis. The density of pancreatic beta cells per tissue area in each photograph was counted and exhibited as control percentage.

### Immunohistochemistry (IHC) Antibody-Pancreas

The experiment was conducted based on our previous study (Nugroho *et al.*, 2013a). Preparation of pancreatic tissue section used a paraffin sectioning technique. The endogenous peroxidase activity in slide-mounted tissue was blocked using

a solution of 3% H<sub>2</sub>O<sub>2</sub> (in methanol) for 15 min. The tissue sections were washed twice with aquadest and then incubated with 20% horse serum for at least 10 min. The target protein was recognized after two successive incubations with primary antibody against rat insulin at 1:250 dilution for one hour, and then with peroxidase-conjugated secondary antibody at 1:500 dilution for one hour, respectively. Target protein expression was eventually visualized after incubation in a substrate for 15 min. Sections were finally counterstained with hematoxylin for 30 minutes, dehydrated in graded alcohol, and cleared in xylene. After mounted with coverslips, slides were examined and photographed using a light microscope (BX51 Olympus, Japan).

### Statistical Analysis

Data were reported as the mean  $\pm$  standard error mean (SEM). The data were statistically analyzed with one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test. The differences significant was indicated as P-values <0.05.

## RESULTS AND DISCUSSION

### Phytochemical analysis

Fractionated-extracts of *A. paniculata* and *C. asiatica* were obtained by gradual fractionation of an etanolic extract to remove ballast and increase the level of active compounds in the fraction. In the study, yields of fractionated-extracts of *A. paniculata* and *C. asiatica* were 6.80 % and 10.0 % (percentage to weight of simplicia powder). Qualitative identification of fractionated-extracts of *A. paniculata* was conducted using a thin layer chromatography with a chloroform:methanol (9:1) as its mobile phase on a silica gel 60 GF<sub>254</sub> under UV-254. Quantitative analysis was demonstrated by TLC densitometry method. Fractionated-extracts of *A. paniculata* has a spot with a retention factor (Rf) equal to that of standard andrographolide. This fraction contains andrographolide of 17.41% $\pm$ 0.42. Besides, qualitative identification of fractionated-extracts of *C. asiatica* was conducted using a thin layer chromatography with a mobile phase of *n*-buthanol:acetic acid:aquadest (3:1:1) on a silica gel 60 GF<sub>254</sub> under UV-254. After spraying with Liebermann-Burchard (LB) reagent, fractionated-extracts of *C. asiatica* has a spot with a retention factor (Rf) equal to that of standard aciaticoside. The content of aciaticoside in this fraction is 11.34% $\pm$ 0.69.

### Effect on blood glucose levels

In preliminary study using six rats, three-month old neonatal streptozotocin (STZ)-induced diabetic rats exhibited the blood glucose level of 149.12 $\pm$ 5.95 mg/dL. Besides, the normal rats possessed the blood glucose level of 73.56 $\pm$ 6.33 mg/dl (Figure 1). It means that STZ-induced diabetic rats had blood glucose levels two folds higher than that of normal rats.

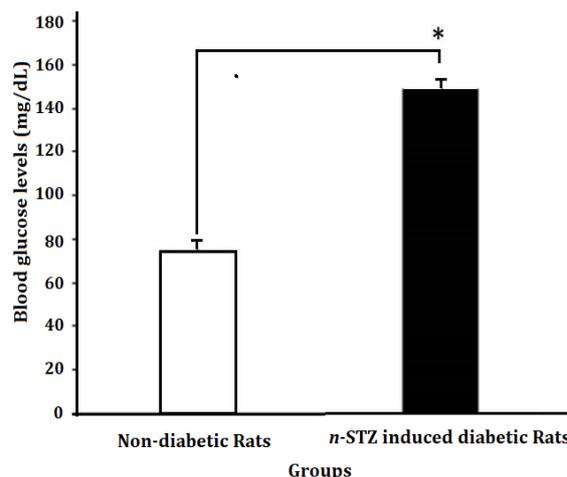


Figure 1. Blood glucose levels (mg/dL) of normal rats and neonatal STZ-induced diabetic rats, a model of type 2 DM rats at three months of age. Data represent mean  $\pm$ SEM.  $P < 0.05$  compared to the value of non-diabetic rats group.

In the study, single treatment of fraction either fractionated-extracts of *A. paniculata* and *C. asiatica* could decrease the blood glucose levels significantly ( $P < 0.05$ ) after 7 and 14 days administration. At the day 14, these fractions lowered the blood glucose levels by 41.23 $\pm$ 12.76% and 33.69 $\pm$ 8.91%, respectively. These values are higher than this of glibenclamide (28.00 $\pm$ 4.23%). Besides, combinations between these fraction (group 6-8) also exhibited lowering effect on the blood glucose levels by 37.77 $\pm$ 10.85%; 41.39 $\pm$ 4.94% and 27.59 $\pm$ 12.48% at the day 14, respectively. Among them, combination of FECA 0.5 g.kg<sup>-1</sup> BW and FEAP 0.65 g.kg<sup>-1</sup> BW (group 6) has highest lowering effect on the blood glucose level (Figure 2). However, the effect of this group was only slightly higher than that of single treatment of FEAP and even equal to that of single treatment. These facts indicate that combination of fractionated-extracts of *A. paniculata* and *C. asiatica* is ineffective to potentiate the blood glucose lowering effect of each fraction.

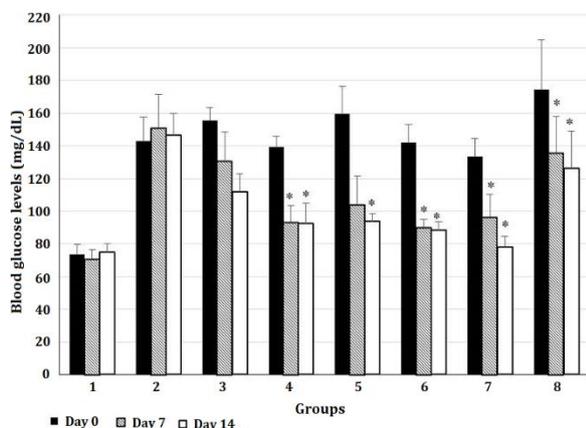


Figure 2: Effect of substance once daily for 14 days on blood glucose levels (mg/dL) in neonatal STZ-induced diabetic rats. Data represent mean±SEM (for each group, n = 5). P<0.05 compared to the value of drug's vehicle-treated diabetic rats group (negative control, Group 2). Group 1 : non-diabetic rats; Group 2 : the diabetic rats received the drug vehicle orally (negative control); Group 3 : the diabetic rats received glibenclamid dose 4.5mg.kg<sup>-1</sup> BW orally, once daily; Group 4 : the diabetic rats received fractionated-extracts of *C. asiatica* (FECA) dose 1.0 g.kg<sup>-1</sup> BW orally, once daily; Group 5 : the diabetic rats received fractionated-extracts of *A. paniculata* (FEAP) dose 1.3 g.kg<sup>-1</sup> BW orally, once daily; Group 6-8 : the diabetic rats received combination of FECA 0.3, 0.5, 0.7 g.kg<sup>-1</sup> BW and FEAP 0.9, 0.65, 0.4 g.kg<sup>-1</sup> BW orally, once daily

**Effect on enzyme activity**

In control group, the diabetic rats received the drug vehicle orally, the MDA value increased and the SOD and GPx values decreased in comparison to these of normal rats group. On the other side, single treatment of fraction either fractionated-extracts of *A. paniculata* and *C. asiatica* could decrease the MDA value and increase the SOD and GPx values significantly (P<0.05) in comparison to these value of control group. in the combination treatment of the two fractionated-extracts (with variations in the percentage combination), there was also an in the MDA value and a decrease in the SOD and GPx values in comparison to these value of control group (P<0.05). In the study, combination of FECA and FEAP exhibited a markedly decreasing effect on the MDA value by 53% (Group 7). The combination also succeeded to restore the GPx value (50.32 nmol.g<sup>-1</sup>±0.98, Group 8) reaching this of normal

value (54.76 nmol.g<sup>-1</sup>±0.51), and also the SOD value of 61% (Group 6) (Table I). Treatment of glibenclamide could also decrease the MDA value and increase the SOD and GPx values significantly (P<0.05). These effect were higher than these of single treatment of fraction either fractionated-extracts of *A. paniculata* and *C. asiatica*, and their combination.

Table I. Effect of substance once daily for 14 days on MDA, SOD and GPx values in neonatal STZ-induced diabetic rats.

Groups	Parameters (Enzyme)		
	MDA (nmol/gr)	GPx (U/mg)	SOD (%)
1	1.38±0.13	54.76±0.51	85.72±2.73
2	9.47±0.20	17.49±0.24	17.26±3.32
3	2.17*±0.19	52.04*±0.49	76.19*±1.58
4	4.08*±0.28	50.73*±0.52	58.33*±4.16
5	3.10*±0.20	50.86*±0.94	68.45*±5.08
6	3.67*±0.22	49.54*±0.70	61.31*±3.62
7	4.47*±0.12	49.11*±0.31	52.97*±2.60
8	4.23*±0.24	50.32*±0.98	54.76*±2.60

Data represent mean ±SEM (for each group, n = 5). P<0.05 compared to the value of drugs vehicle-treated diabetic rats group (negative control, Group 2). Group 1 : non-diabetic rats; Group 2 : the diabetic rats received the drug vehicle orally (negative control); Group 3 : the diabetic rats received glibenclamid dose 4.5 mg.kg<sup>-1</sup> BW orally, once daily; Group 4 : the diabetic rats received fractionated-extracts of *C. asiatica* (FECA) dose 1.0 g.kg<sup>-1</sup> BW orally, once daily; Group 5 : the diabetic rats received fractionated-extracts of *A. paniculata* (FEAP) dose 1.3 g.kg<sup>-1</sup> BW orally, once daily; Group 6-8 : the diabetic rats received combination of FECA 0.3, 0.5, 0.7 g.kg<sup>-1</sup> BW and FEAP 0.9, 0.65, 0.4 g.kg<sup>-1</sup> BW orally, once daily.

**The effect on rat pancreatic islets**

The morphological observations of the Langerhans embodiment with HE staining of the treatment group (Figure 3). Histological observation on STZ-induced Langerhans rats, there were changes in several structures of pancreatic tissues, including changes in the cytoplasmic margins, vacuolization in the cell cytoplasm, reduced size of islets of Langerhans and decreased number of Langerhans cells (Figure 3.B). The normal control group (Figure 3.A) shows regular cells, clearly defined cytoplasmic boundaries and the regular size of islets of Langerhans (Figure 3.C). This result was different in comparison to this of the other groups (Figure 3.D-H). In the *n*-STZ diabetic rats with glibenclamide treatment showed no cellular improvement, there was free space in the tissue section especially Langerhans islets.

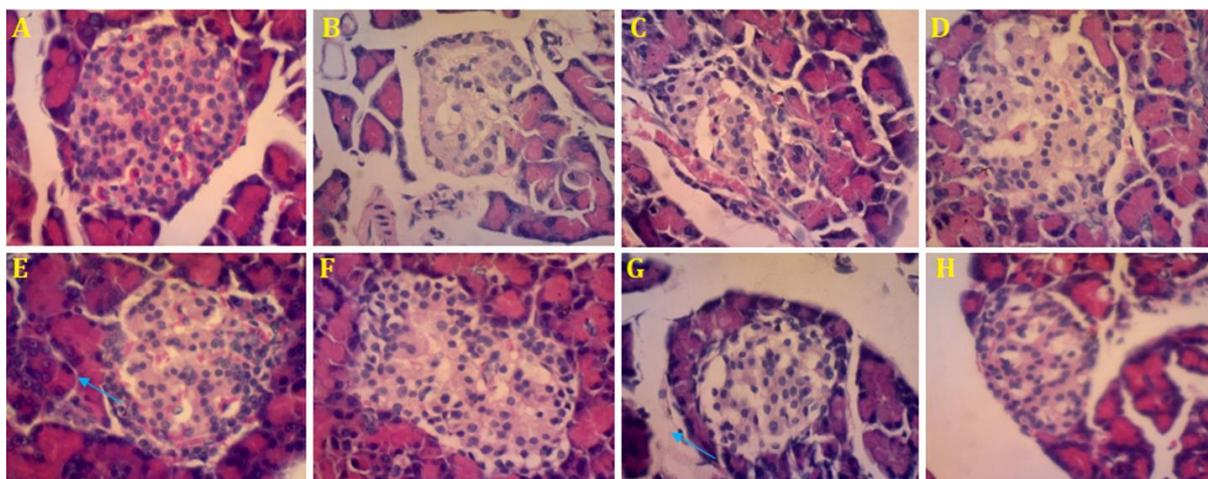


Figure 3. Observation of Langerhans islet for sections of rat pancreas by Hematoxylin and eosin-stained. Magnification  $\times 400$ .

Group A : non-diabetic rats; Group B : the diabetic rats received the drug vehicle orally (negative control); Group C : the diabetic rats received glibenclamide dose  $4.5 \text{ mg.kg}^{-1} \text{ BW}$  orally, once daily; Group D : the diabetic rats received fractionated-extracts of *C. asiatica* (FECA) dose  $1.0 \text{ g.kg}^{-1} \text{ BW}$  orally, once daily; Group E : the diabetic rats received fractionated-extracts of *A. paniculata* (FEAP) dose  $1.3 \text{ g.kg}^{-1} \text{ BW}$  orally, once daily; Group F-H : the diabetic rats received combination of FECA 0.3, 0.5, 0.7  $\text{g.kg}^{-1} \text{ BW}$  and FEAP 0.9, 0.65, 0.4  $\text{g.kg}^{-1} \text{ BW}$  orally, once daily.

This condition did not appear to be different from the negative control group (CMC-Na 0.5%). The *n*-STZ diabetic rat treated with FECA dose  $1.0 \text{ g/kg BW}$  (figure 3.D), combination FECA: FEAP with a ratio of 30:70, 50:50 and 70:30 (Figure 3.F-H), showed a similar result with the negative control group, there are still degeneration and signs of necrosis cells indicated by vacuolization or some spaces between cells. In the *n*-STZ diabetic rat treated with single-dose EAAP of  $1.3 \text{ g/kg BW}$  (Figure 3.E) showed a cellular improvement, characterized by the location of nucleus cell is orderly, almost the same as this of control group, but there is still a slight change in cytoplasmic space or structure.

#### Effect on rats pancreatic $\beta$ cells

Observation and calculation of pancreatic  $\beta$  cell number of rats was done semi-quantitatively on Victoria blue (VB)-stained sections, and using an Image Raster Program. In the VB staining, the pancreatic  $\beta$  cell nucleus is red-colored, and the cytoplasm is blue, so with the colour contrast, the number of pancreatic  $\beta$  cells can clearly be observed and calculated (Kikui *et al.*, 1977). The VB colouring result (Figure 4) the normal control group, the Langerhans are almost blue coloured. It indicates that no pancreatic  $\beta$  cell degeneration occurs; the number of pancreatic

$\beta$  cells is higher than that of the *n*-STZ diabetic rats. Treatment groups with glibenclamide, FECA, and FEAP, both single and combined doses, performed the regeneration of pancreatic  $\beta$  cells. It was demonstrated by the blue contrast formed on the histological preparations of pancreatic Langerhans islets in each treatment group. FECA succeed to restore the beta-cell mass and its function in the diabetic rat (Figure 4.D) because of Asiatic acid content (Liu *et al.*, 2010). FEAP also showed improvement of  $\beta$ -cell pancreas regeneration in the diabetic rat. It is due to andrographolide compound in this *A. paniculata* herb (Zhang *et al.*, 2009)

Based on the semi-quantitative calculation of pancreatic  $\beta$  cell number. The *n*-STZ diabetic rat group without treatment exhibited a decrease in the number of pancreatic beta cells compared to the normal group (non-diabetic rats), the number was only less than half of this of normal group (Figure 5). Treatment with glibenclamide, FECA, and FEAP, both single and combined doses in the *n*-STZ diabetic rats showed an increase of the number of pancreatic beta cells, even though these increases have not been able to reach normal values ( $P < 0.05$ , Figure 5). Another fact, effect of the combination of FECA and FEAP in the *n*-STZ diabetic rats was not significantly different in comparison to this of the single treatment FECA or FEAP ( $P < 0.05$ , Figure 5).

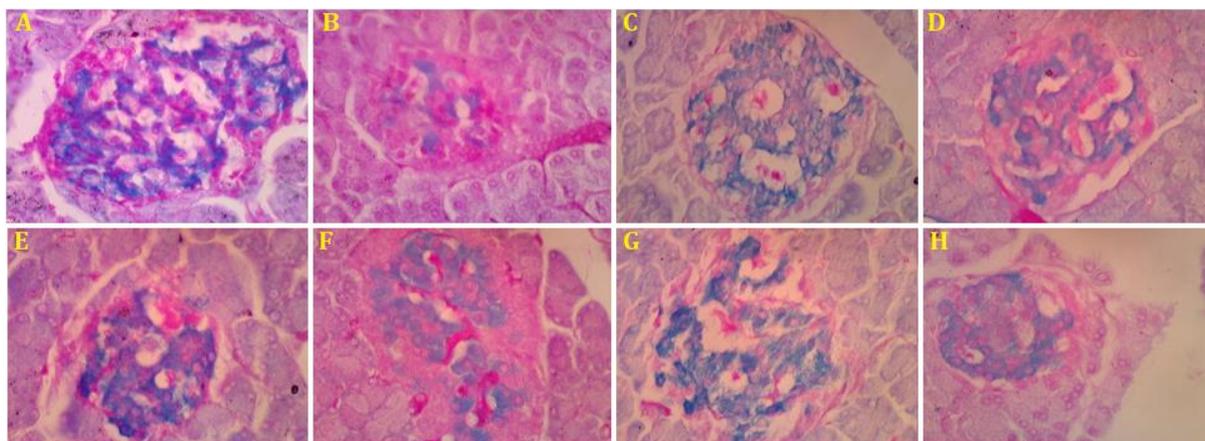


Figure 4. Observation of pancreatic beta cells with victoria blue staining. Magnification  $\times 400$ . Beta cell is showed as parts with blue colour in the section, and the cell with a blue-stained cytoplasm. Group A : non-diabetic rats; Group B : the diabetic rats received the drug vehicle orally (negative control); Group C : the diabetic rats received glibenclamide dose  $4.5 \text{ mg.kg}^{-1} \text{ BW}$  orally, once daily; Group D : the diabetic rats received fractionated-extracts of *C. asiatica* (FECA) dose  $1.0 \text{ g.kg}^{-1} \text{ BW}$  orally, once daily; Group E : the diabetic rats received fractionated-extracts of *A. paniculata* (FEAP) dose  $1.3 \text{ g.kg}^{-1} \text{ BW}$  orally, once daily; Group F-H : the diabetic rats received combination of FECA  $0.3, 0.5, 0.7 \text{ g.kg}^{-1} \text{ BW}$  and FEAP  $0.9, 0.65, 0.4 \text{ g.kg}^{-1} \text{ BW}$  orally, once daily

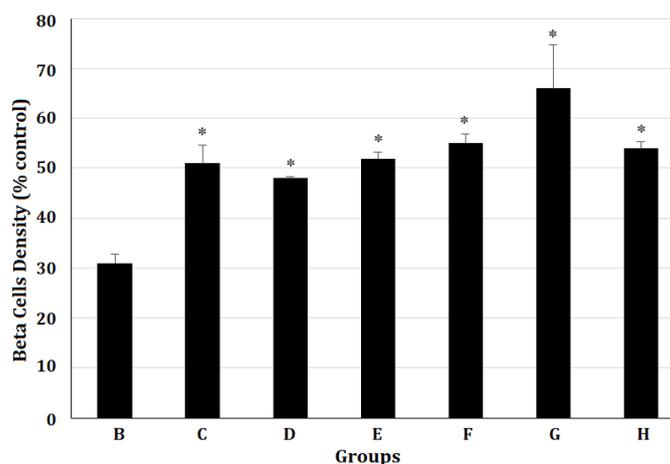


Figure 5. The percentage of beta-cell density (percentage compared with the non-diabetic rats group). Data represent mean $\pm$ SEM (for each group, n = 3).  $P < 0.05$  compared to the value of the negative control group, the diabetic rats receiving the drug vehicle orally (group 2).

### Effect on rat pancreatic insulin expression

Immunohistochemistry staining was used to observe and determine the pancreatic insulin expression qualitatively. In the methods, the appearance of insulin in pancreatic  $\beta$  cells can be observed with brown colour staining due to interaction between monoclonal anti-insulin antibodies (Rat monoclonal K36AC10, Abcam, USA) with insulin present in the pancreatic  $\beta$  cells (Schacht and Kern, 2015). The non-diabetic group showed the clearest and most obvious expression

of pancreatic insulin. It is because insulin production by pancreatic beta cells is not disrupted (Figure 6). On the other hand, pancreatic insulin expression in diabetic rats was the lowest compared to the other groups due to streptozotocin treatment at two days of age ( $P < 0.05$ ). Treatment of glibenclamide, FECA, and FEAP, both single and combined doses in the *n*-STZ diabetic rats resulted in an improved insulin expression in compared with untreated *n*-STZ diabetic rats.

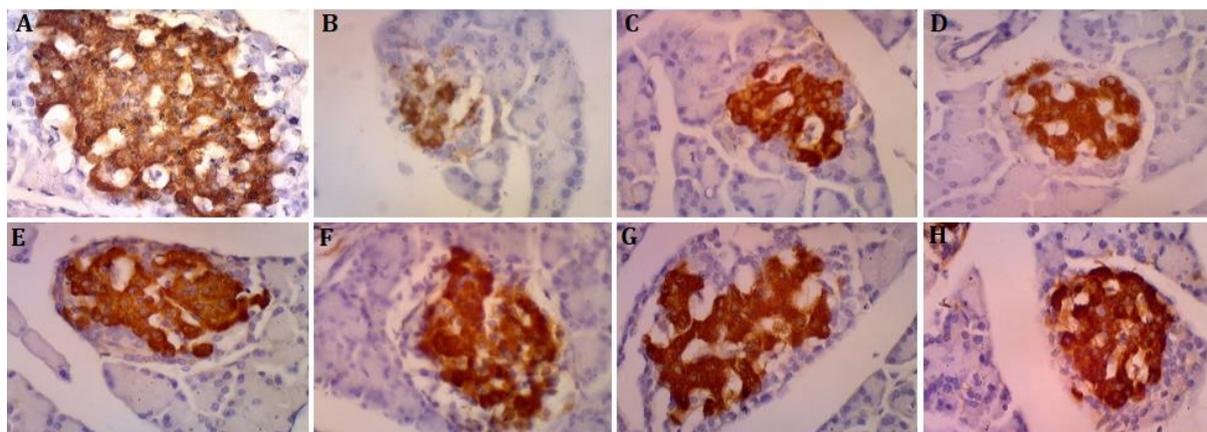


Figure 6. Immunohistochemical-stained sections of insulin rat pancreas observation. Magnification  $\times 400$ . Insulin is stained as a brown colour in the section.

Group A: non-diabetic rats; Group B: the diabetic rats received the drug vehicle orally (negative control); Group C: the diabetic rats received glibenclamide dose  $4.5 \text{ mg.kg}^{-1} \text{ BW}$  orally, once daily; Group D: the diabetic rats received fractionated-extracts of *C. asiatica* (FECA) dose  $1.0 \text{ g.kg}^{-1} \text{ BW}$  orally, once daily; Group E: the diabetic rats received fractionated-extracts of *A. paniculata* (FEAP) dose  $1.3 \text{ g.kg}^{-1} \text{ BW}$  orally, once daily; Group F-H: the diabetic rats received the combination of FECA 0.3, 0.5, 0.7  $\text{g.kg}^{-1} \text{ BW}$  and FEAP 0.9, 0.65, 0.4  $\text{g.kg}^{-1} \text{ BW}$  orally, once daily

Streptozotocin (STZ), or 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is firstly developed as an antibiotic from *Streptomyces achromogenes* (Rizvi RY *et al*, 1986; Szkudelski T, 2001). In its development, STZ tends to be used in experiments and research as a diabetogenic agent. STZ can induce diabetes mellitus in animals either type 1 or 2 diabetes depending on the dose of administration and methods (Arulmozhi DK *et al*, 2004; Nugroho AE, 2006). STZ is able to penetrate pancreatic  $\beta$  cells through trans membrane glucose transporter GLUT-2.

As a diabetogenic agent, STZ stimulates the production of hydroxyl radicals, superoxide radicals, hydrogen peroxide, and nitric oxide in pancreatic beta cells. These substances causes rapid destruction of pancreatic beta cells. Its action is also related to DNA alkylation through transferring of methyl groups from STZ causes DNA methylation resulting in DNA damage causing necrosis and death in pancreatic  $\beta$  cells (Szkudelski T, 2001; Arulmozhi DK *et al*, 2004; Nugroho AE, 2006; Lenzen, 2008).

In the study, we used *n*-STZ diabetic rats as an animal model of insulin-deficient type 2 diabetes mellitus. In the method, STZ ( $90 \text{ mg.kg}^{-1} \text{ BW}$ ) is intraperitoneally injected in two-day-old rats. During its development, there is a recovery process in damaged beta cells, however cannot be perfect or become normal again. At two months of age, beta

cell damage was still partial and blood glucose levels remained higher than this of non diabetic rats as well as glucose intolerance, problem of glucose-induced insulin release, and decrease in pancreatic insulin stores (Bonner-Weir *et al*, 1981; Weir *et al*, 1981; Arulmozhi *et al*, 2004; Nugroho, 2006).

Diabetes mellitus is one of the chronic diseases due to the damage in carbohydrate metabolism resulting in hyperglycemic conditions. In the condition of diabetes mellitus, pancreatic  $\beta$  cells decrease in time due to apoptosis (Butler *et al*, 2003), resulting in insulin deficiency and cause increases blood glucose levels. The condition of hyperglycemia and or elevation of free fatty acid (FFA) levels result in the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that can trigger oxidative stress. This condition can decrease the function of pancreatic beta cells to secrete insulin (Boden and Shulman, 2002). Oxidative stress occurs due to the result of  $\text{H}_2\text{O}_2$  peroxidated lipid metabolism result that can inhibit insulin secretion and glucose oxidation in mitochondria by increasing the production of p21 (inhibitor cyclin-dependent kinase), decreasing cytosolic ATP, insulin mRNA, and calcium flux in cytosol and mitochondria, causing apoptosis (Maechler *et al*, 1999).

Reactive Oxygen Species (ROS) damage cells directly by oxidizing DNA, proteins, and lipids, and

indirectly by activating various intracellular stress-sensitive signaling systems such as NF- $\kappa$ B, p38 MAPK, JNK / SAPK, hexosamine, CCP, AGE / RAGE, sorbitol etc. The activation of this pathway results in increase of many gene products expression that also cause cellular damage and play a major role in the etiology of diabetic complications (Evans *et al.*, 2003). Endogenous enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) have an important role as antioxidant to inhibit the accumulation of free radicals that can initiate degenerative processes and other CNS complications (Ramanathan *et al.*, 1999). Increased levels of uncontrolled blood glucose also can cause complications such as heart disease, stroke, diabetic retinopathy, neuropathy, and renal failure (Centers for Disease Control and Prevention., 2011).

Indonesian herbal natural resources have been widely used as an alternative to treat diabetes mellitus, including *A. paniculata* and *C. asiatica*. Attempts to combine several medicinal plants are often done to get better results. Likewise with the *A. paniculata* research, several studies conducted a combination with other traditional medicines and examined their antidiabetic activity in comparison to their single treatment (Nugroho *et al.*, 2014a; Nugroho *et al.*, 2014b; Sari *et al.*, 2015). In the study, the extract of *A. paniculata* was combined with extract of *C. asiatica*.

Development and research related to the antidiabetic activity of both herbs have been widely practiced. *A. paniculata* and *C. asiatica* proved to reduce blood glucose levels and the protection of pancreatic beta cells, both *in vitro* and *in vivo* tests. The ethanolic extract of *C. asiatica* was able to reduce blood glucose levels, and cholesterol in STZ-induced male rats (Gayathri *et al.*, 2011), besides that, *C. asiatica* was inhibited significant damage to pancreatic  $\beta$  cells in alloxan-induced rats (Chauhan *et al.*, 2010). The purified extract of *A. paniculata* (an andrographolide-enriched extract) and its active compound andrographolide could lower blood glucose levels and increase the number of pancreatic cells in neonatal streptozotocin (STZ)-induced diabetic rats (Nugroho *et al.*, 2013a). Similar studies were conducted by (Zhang *et al.*, 2009), using andrographolide, which is the main active compound in herbal *A. paniculata*. It is also proven to have blood glucose reduction activity and protection against alloxan-induced pancreatic  $\beta$  cells.

Andrographolide and asiaticoside are the main active ingredients of both herbs *A. paniculata* and *C. asiatica*. Andrographolide acts as an antidiabetic agents can prevent diabetes nephropathy by inflammation, inhibiting of Akt / NF- $\kappa$ B signaling pathway and also inhibiting renal oxidative stress in diabetic rats induced streptozotocin (Ji *et al.*, 2016). The hypoglycemic mechanism of andrographolide was reportedly through the inhibitory of alpha-amylase and alpha-glucosidase, enzymes participating absorbed of glucose (Subramanian *et al.*, 2008) and activation of glucose transporter (GLUT-4) membrane translocation in soleus muscles (Zhang *et al.*, 2009). Besides, Asiaticoside has antidiabetic, antioxidant, anti-inflammatory, and modulated PI3K / Akt / GSK-3 pathway effects (Yin *et al.*, 2015). The study showed that the antidiabetic effect of a combination of purified extract of *C. asiatica* and *A. paniculata* by decreasing blood glucose and cholesterol levels in diabetic mellitus type 2 insulin resistant rats in comparison to this of single treatment of each extract (Nugroho *et al.*, 2013b; Lindawati *et al.*, 2014).

In conclusion, the combination of fractionated-extracts of *A. paniculata* and *C. asiatica* exhibited potential antidiabetic effect in relation to its antioxidative effect in type 2 DM rats (STZ-induced diabetic rats) model. The combination succeeded to improve/repair the morphology of pancreatic islet, number of density beta cells pancreatic, and pancreatic insulin content by immunohistochemically. Based on these results, this combination is potential to be developed as antidiabetic agent.

## ACKNOWLEDGMENT

We thank to the Directorate of Research and Community Service, the Directorate General of Strengthening Research and Development, Ministry of Research, Technology and High Education of the Republic of Indonesia (RISTEKDIKTI) through "Penelitian Unggulan Perguruan Tinggi (PUPT) scheme Fiscal Year 2016-2018".

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