International Food Research Journal 24(5): 2153-2160 (October 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Blood glucose reduction of combination of *Andrographis paniculata* (Burm.f) Ness and *Morinda citrifolia* L. ethanolic extract in neonatal streptozotocin-induced Type 2 diabetes mellitus rats

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Article history

Received: 9 July 2016
Received in revised form: 25 August 2016
Accepted: 27 August 2016

Keywords

Andrographis paniculata
Morinda citrifolia
Hypoglycemianeonatal
Streptozotocin
DM type 2

Abstract

Andrographis paniculata (Burm. f.) Ness and *Morinda citrifolia* L. are two medicinal plants that traditionally used by Indonesian people for diabetes treatment. The aim of this study was to evaluate the hypoglycemic effect of *A. paniculata* ethanolic extract (APEE) and *M. citrifolia* ethanolic extract (MCEE) combination in neonatal streptozotocin-induced type 2 diabetes mellitus rats in regard to its pancreatic regeneration effect. The powder of dried *A. paniculata* herbs and *M. citrifolia* fruits were macerated using 70% ethanol. Phytochemical analysis of andrographolide in APEE and scopoletin in MCEE were performed by TLC-densitometry using stationery phase of silica gel 60 F_{254} and mobile phase of n-hexane-chloroform-methanol (6:41:3 v/v/v). Hyperglycemic condition in rats was induced with a single dose injection of 90 mg/kg BW streptozotocin (STZ) intraperitoneally in 2 days neonatal rats. Twelve weeks old neonatal STZ-induced rats were orally administrated with several dosage combination of APEE: MCEE (375:125; 250:250; 125:375 mg/kg BW) for 14 consecutive days. Hypoglycemic effect was evaluated by measuring pre-prandial and post-prandial blood glucose levels and other parameters such as pancreatic islet morphology, density of pancreatic β cells, and pancreatic insulin expression. In the study, concentration of andrographolide in APEE was 13.72% while scopoletin in MCEE was 0.32%. Single treatment of APEE and MCEE exhibited no significant hypoglycemic effect than this of its combination. However, the combination of APEE and MCEE exhibited a better improvements in pancreatic islet morphology and increased insulin pancreatic expression than that of single treatment of APEE or MCEE. Combination of APEE: MCEE in dose 250:250 mg/kg BW exhibited better hypoglycemic effect and pancreatic regeneration than other combinations. Combination of *A. paniculata* and *M. citrifolia* ethanolic extracts is potential to develop as an anti-diabetic agent.

Introduction

Indonesia is the fourth country in the world with the number of diabetic patients. In 2000 there were an estimated 8.4 million people with diabetes mellitus (DM) and will increase to 21.3 million in 2030 (Wild *et al*., 2004). Complex pathogenesis of DM needs multimodal therapeutic approach that requires a combination of several drugs (Tiwari and Rao, 2002). With the increasing severity of diabetes, it takes a combination of two or more oral hypoglycemic agents (OHO) which will increase the risk of drug side effects and increased treatment costs (Ramachandran *et al*., 2010). Complementary treatment for diabetes using medicinal plants has chosen as an alternative solution. The combined use of medicinal plant for the treatment is beneficial because the chemical plant content will give multi-drug multi-target therapeutic effect. Andrographis paniculata herbs and *M. citrifolia* fruit are traditionally used by Indonesian people for the treatment of diabetes.

Hypoglycemic activity of *A. paniculata* has been shown in several in vivo studies using diabetic animal models. Andrographolide is a main diterpene lactone of *A. paniculata* that is responsible for its hypoglycemic activity. Andrographolide succeeded to lower blood glucose levels by increasing glucose utilization and stimulates glucose transporter subtype

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4 (GLUT4) transcriptions (Zhang and Tan, 2000; Yu *et al*., 2003; Nugroho *et al*., 2012). The number of β cells and insulin levels of pancreas in diabetic rats was also reported to increase by presence of andrographolide (Nugroho, Rais, Setiawan *et al*., 2014). Andrographolide also have prevention activity of type 1 DM with homeostatic regulation of Th1/ Th2/ Th17 which will prevent pancreatic β cell death and inhibits T cell infiltration into pancreatic islet (Zhang *et al*., 2013).

Ethanolic extract of *M. citrifolia* showed activity in blood glucose levels decrease in alloxan-induced diabetic rat (Adnyana *et al*., 2004). Treatment of ethanolic extract of *M. citrifolia* fruit in streptozotocin-induced diabetic rats lowers blood glucose levels, glycosylated hemoglobin, blood urea, and serum creatinin (Jin, 2007; Rao and Subramanian, 2009). *Morinda citrifolia* fruit juice can also accelerate wound healing in streptozotocin-induced diabetic rat (Nayak *et al.*, 2007).

The aim of this study was to evaluate the hypoglycemic effect of *A. paniculata* ethanolic extract (APEE) and *M. citrifolia* ethanolic extract (MCEE) combination in neonatal streptozotocin-induced type 2 diabetes mellitus rats in regard to its pancreatic regeneration effect. The combination of both extracts could exhibit better hypoglycemic effect than those single extract administrations. Hypoglycemic effect was evaluated by measuring pre-prandial and post-prandial blood glucose levels and other parameters such as pancreatic islet morphology, amount of pancreatic β cells, and pancreatic insulin expression.

Materials and Methods

Chemicals

Streptozotocin, andrographolide, and scopoletin were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Glucose level was measured using GOD-PAP kit with glucose oxidase 4-aminoantipyrine (DiaSys, Diagnostic Systems GmbH, Holzheim, Germany). Sodium carboxymethyl cellulose, glucose, n-hexane, chloroform, methanol, hematoxylin and eosin were obtained from E. Merck, Darmstadt, Germany. Antibodies for insulin expression determination were primary anti-insulin antibody (Santa Cruz Biotechnologies, California, USA) and secondary Streptavidin-Horse Radish Peroxidase antibody (Invitrogen Carlsbad, CA, USA).

Animals

Male Wistar rats aging 2 days and 3 months old (6-250 g) used in this study were maintained on a constant temperature 25+2°C, relative humidity 45-55%, and controlled 12:12 h light-dark cycle (light on 06.00 p.m.). Rats were fed with a standard laboratory food (Comfeed, Indonesia) and water ad libitum. This experiment has obtained ethical clearance from Ethical Clearance Committee for Pre-clinic Experiment, Integrated Research and Testing Laboratory Universitas Gadjah Mada Indonesia (certificate number: 241/KEC-LPPTIV/2015).

Preparation of ethanolic extract

Andrographis paniculata herbs were obtained from BP2TOOT Tawangmangu, and *M. citrifolia* fruits were collected from Mlati, Sleman, Yogyakarta in December 2014. The plants were authenticated at Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta. The voucher specimen of samples was stored in a herbarium of the department. Dried powder of *A. paniculata* herbs (1.02 kg) and *M. citrifolia* fruits (1.1 kg) was extracted by maceration methods using ethanol 70% for 24 hours, respectively. After 2 times re-maceration, all filtrate was mixed, and evaporated to obtain a thick extract.

Determination of A. paniculata *and* M. citrifolia *marker compounds*

Marker of *Andrographis paniculata* ethanolic extract (APEE) and *Morinda citrifolia* ethanolic extract (MCEE) were determined using thin layer chromatography (TLC) method, with a stationary phase of silica gel 60 F254 and a mobile phase of hexane-chloroform-methanol (6:41:3 v/v/v). Determination of andrographolide in APEE was performed by densitometer at wavelength of 232 nm. Meanwhile, scopoletin in MCEE was determined by densitometer at wavelength of 369 nm.

Induction of diabetes

Male neonatal rats (2 days old) were administered with streptozotocin intraperitoneally at dose 90 mg/ kg BW in citrate buffer (pH 4.3). The animals were weaned at 4 weeks of age. DM was confirmed at 12th week by measurement of pre-prandial and post-prandial blood glucose levels by the glucose oxidase peroxidase (GOD/POD) method.

Experimental design

The diabetic rats were divided into eight groups as follows, i) Positive control, treated with glibenclamide 4.5 mg/kg BW; ii) Negative control, received vehicle solution, 0.5 mL Na-CMC 0,5%; iii) Diabetic treated with single APEE (500 mg/kg BW); iv) Diabetic treated with single MCEE (500 mg/kg

BW); v) Diabetic treated with combination APEE 375 and MCEE 125 mg/kg BW; vi) Diabetic treated with combination APEE 250 and MCEE 250 mg/kg BW; and vii) Diabetic treated with combination APEE 125 and MCEE 375 mg/kg BW. All treatment was given orally, once daily, for 14 consecutive days. At 0; 7; and 14-days of treatment, both pre-prandial (after 12 hours fasting) and post-prandial (2 hours after 1.75 g/kg BW per oral glucose loading) blood glucose levels were determined. Blood glucose level was analyzed with colorimetric method using GOD-PAP reagent. Blood samples from plexus retro orbitalis were incubated at room temperature for 30 minutes. Serum was collected by centrifugation at 5000 rpm for 10 min at 25°C.

Histological observation of pancreatic

At the end of treatment, the rats were sacrificed, and the pancreas was removed and fixed with 4% formalin PBS for 24 hours. Pancreatic tissue in a section slide was stained for hematoxylin eosin (HE) staining and analyzed with immunohistochemistry (IHC) using the primary anti-insulin antibody and secondary Streptavidin-Horse Radish Peroxidase antibody. Another part of the pancreas were fixed with Bouin solution and then made section slide for Victoria Blue (VB) staining. All stained slide was observed with a light microscope (Olympus BX51, Japan) with a 40x objective, and 10x eyepiece magnification.

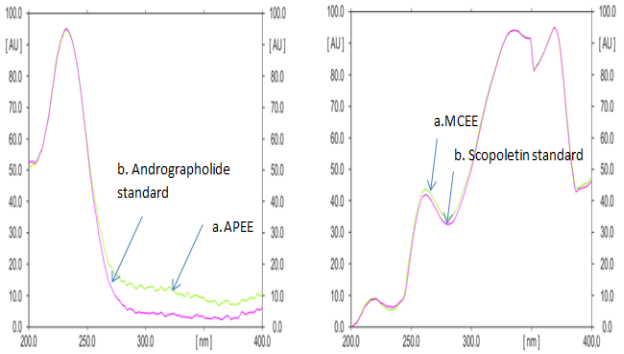
Statistical analysis

All experimental data presented as mean+standard error of mean (SEM). Statistical analysis used one way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc test. Significantly differences showed by P-values less than 0.05.

Results and Discussion

Phytochemical analysis of A. paniculata *and* M. citrifolia

Andrographis paniculata ethanolic extract (APEE) showed positive content of andrographolide based on phytochemical analysis using TLC densitometry method. A same spot with standard andrographolide give same spectra by TLC scanner (Figure 1A). Furthermore, quantitative analysis confirmed the content of andrographolide by 13.7% using TLC scanner at wavelength of 232 nm. Phytochemical analysis of *M. citrifolia* ethanolic extract (MCEE) was performed using scopoletin as a standard. Positive content of scopoletin was



(A) (B)

Figure 1. Spectra of andrographolide of APEE (green) compared with standard (purple) (A), and spectra of scopoletin of MCEE (green) compared with standard (purple) (B). TLC method was performed using a stationary phase of silica gel 60 F254 and a mobile phase of n-hexane:chloroform:methanol (6:41:3 v/v/v)

shown by identical spectra of same spot of MCEE and scopoletin standard by TLC scanner (Figure 1B). Quantitative analysis by TLC scanner at wavelength of 369 nm confirmed that the content of scopoletin in extracts was 0.3%.

Effect of streptozotocin induction

Pre-prandial and post-prandial blood glucose levels were measured at 12 weeks after streptozotocin induction. The result of independent sample T-Test showed that pre-prandial blood glucose level n-STZ rats (163.7+8.01 mg/dL) and normal rats (104.1+10.61 mg/dL) had a significant difference ($P < 0.05$). Post-prandial blood glucose levels of n-STZ rats (182.9+8.13 mg/dL) are also significantly different compared to normal rats (126.2+6.57 mg/dL). The rats experienced a hyperglycemic condition if their blood glucose levels more than 1.5 times to blood glucose levels of normal group. The results showed the blood glucose levels of n-STZ rats more than 1.5 times to the normal rats, so can be concluded if already occurred hypoglycemic condition.

As diabetogenic, streptozotocin was used for the induction of diabetes animal models both of IDDM or NIDDM (Rees and Alcolado, 2005; Lenzen, 2008). Streptozotocin has diabetogenic activity by suppressing the production of insulin (insulinopenia syndrome). Diabetes due to streptozotocin induction is caused by specific necrosis of pancreatic β cells (Lenzen, 2008). Insulin-producing β cells in the n-STZ rats have similar characteristics to patients with type 2 diabetes, resulting in the condition slightly decrease in plasma insulin levels, increased blood glucose levels, and a reduction in pancreatic insulin (Arulmozhi *et al*., 2004).

At day 4 after streptozotocin induction in

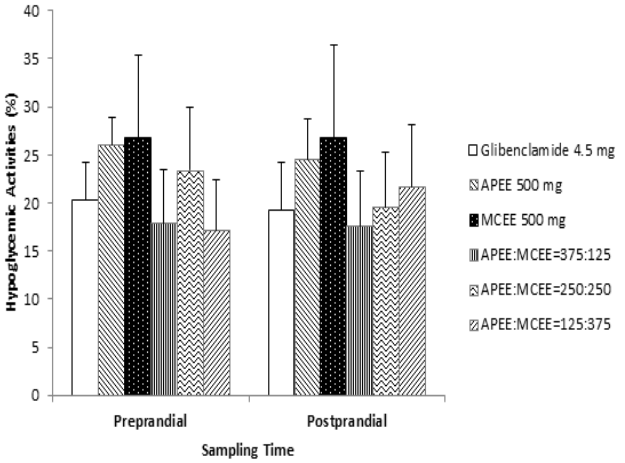


Figure 2. Hypoglycemic activities (%) of treatment groups after 14 consecutive days. Data presented as mean±SEM (n=5). *p<0.05 compared to the value of glibenclamide group

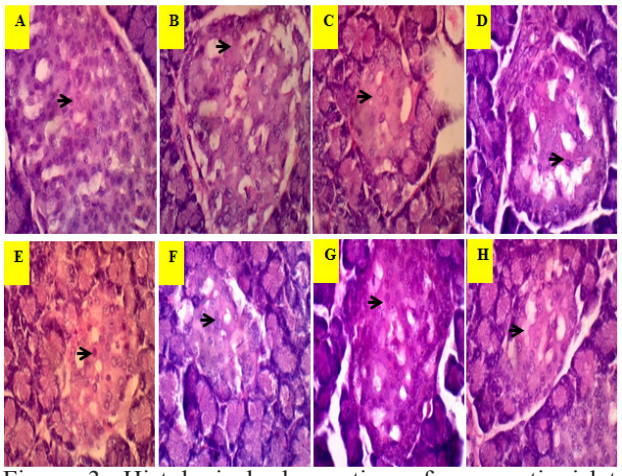


Figure 3. Histological observation of pancreatic islets with HE staining. (A) normal rats; (B) glibenclamide; (C) Na-CMC (negative control); (D) APEE; (E) MCEE; (F) APEE:MCEE=375:125; (G) APEE:MCEE=250:250; (H) APEE:MCEE=125:375. ➔ Showed some cell alteration form (Magnification 10x40 times)

neonatal rats, only 20% of β cells-mass remained in n-STZ rats (Garofano *et al*., 2000). Cells regeneration has already begun on the 10th day, where the number of pancreatic β cells increased to 39.6%. This regeneration was through neogenesis and an increase in cell proliferation mechanisms. Although the pancreatic β cells increased to 48.8% at week 6, hyperglycemia was persists. This situation lasted until the age of 13 weeks. This marks the regeneration of pancreatic β cells are not running perfectly and makes hyperglycemia when the mice aged 3 months (Bonner-Weir, 1981).

Effect of treatment on blood glucose levels

Hypoglycemic activities showed a decrease of blood glucose levels in n-STZ rats were given the test compound treatments compared to the negative control on the 14th day of treatment. Administration of APEE and MCEE for 14 consecutive days both in the form of single treatment or their combination exhibited reduction on pre-prandial and post-prandial blood glucose levels in neonatal streptozotocin-induced type 2 diabetic rats. Pre-prandial and post-prandial hypoglycemic activities of combination of APEE and MCEE were lower than that of their single extract (Figure 2). Statistical analysis results showed no significant difference between groups ($P > 0.05$) both pre-prandial and post-prandial hypoglycemic activities. Thus, the entire test group had a decrease in pre-prandial and post-prandial blood glucose levels that are comparable.

Effect on rats pancreatic islets

Morphological observation of Langerhans islets were observed using HE staining. Some degenerative changes of the Langerhans islets morphology were observed in comparison to normal control rats. Pancreatic islets cells were decreased in number and size. After treatment, an improvement of diabetic rat islets occurred in all treatment groups in comparison to negative control. Combination of 250 mg/kg BW APEE and 250 mg/kg BW MCEE exhibited the best improvement of diabetic rat islets regeneration (Figure 3).

Effect on rats pancreatic β cells

Victoria Blue staining makes pancreatic β cell cytoplasm is blue with red cell nucleus so that the number of pancreatic β cells can be calculated. Streptozotocin induction resulted in the destruction of pancreatic β cells so that the amount of the normal group is very much different than the group receiving streptozotocin induction. Negative control group had a slightly pancreatic Langerhans islet, as well as the number of β cells in the pancreatic Langerhans islets. Administration of glibenclamide and extract treatment will increase the ability of β cell regeneration and decrease cell necrosis, so the number of observed β cells was increased (Figure 4). Combination of 250 mg/kg BW APEE and 250 mg/kg BW MCEE has the highest number of pancreatic β cells, which differ significantly in all treatment groups ($P < 0.05$).

Effect on rat pancreatic insulin expression

Immunohistochemistry staining was conducted to determine the expression of pancreatic insulin qualitatively. Insulin immunoreactive pancreatic β cells will give brown color with staining. Normal group has the most powerful pancreatic insulin

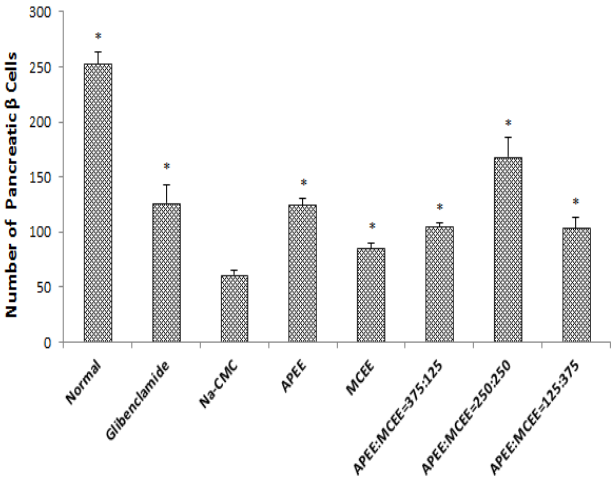


Figure 4. The number of pancreatic β cells/ 5 visual field. Data are presented as mean+SEM (n = 3). *p<0.05 significantly different compared to the negative control Na-CMC.

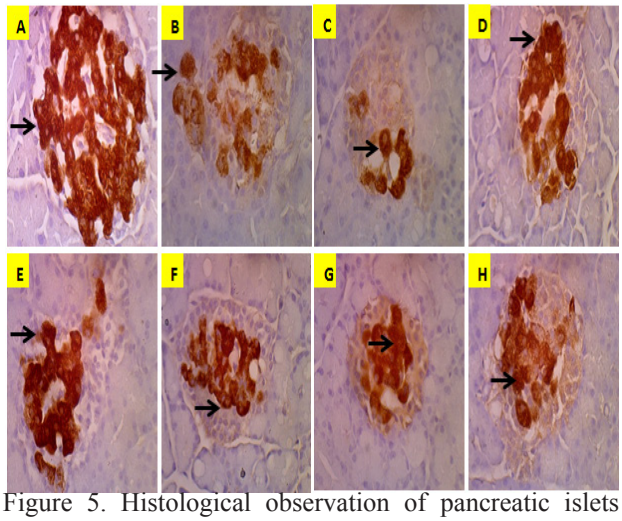


Figure 5. Histological observation of pancreatic islets with immunohistochemistry staining. (A) normal rats; (B) glibenclamide; (C) Na-CMC (negative control); (D) APEE; (E) MCEE; (F) APEE:MCEE=375:125; (G) APEE:MCEE=250:250; (H) APEE:MCEE=125:375. ➔ Showed insulin immunoreactive pancreatic β cells. (Magnification 10x40 times)

expression because insulin production of pancreatic β cells are not disrupted (Figure 5). Insulin expressions in the negative control Na-CMC 0.5% is the lowest and differs significantly in all groups ($P < 0.05$). Glibenclamide and extract treatment give better insulin expression than the negative control group. Combination of 250 mg/kg BW APEE and 250 mg/kg BW MCEE exhibited the best pancreatic insulin expression.

Several studies have reported *A. paniculata* activity in lowering blood glucose levels in various rat models of diabetes i.e. alloxan-induced, streptozotocin-induced, and a high-fructose-fat-fed rat (Zhang and Tan, 2000; Hossain *et al.*, 2007; Ravikumar *et al.*, 2010; Nugroho *et al.*, 2012). *Andrographis paniculata* also reported to have antioxidant activity, renoprotective, and hepatoprotective (Trivedi and Rawal, 2000; Zhang and Tan, 2000; Dandu and Inamdar, 2009; Singh *et al.*, 2009). Andrographolide which is a principal diterpene lactone of *A. paniculata* mainly contributed to its hypoglycemic activity. A decrease of blood glucose levels by andrographolide trough the mechanism of increasing glucose utilization, stimulates transcription of glucose transporter subtype 4 (GLUT4), improved Langerhans islet condition, increase the number of β cells in Langerhans islet, and increase pancreatic insulin levels (Zhang and Tan, 2000; Yu *et al.*, 2003; Nugroho, Andrie, Susilowati *et al.*, 2011; Nugroho *et al.*, 2012; Nugroho, Rais, Setiawan *et al.*, 2014). Andrographolide also prevent pancreatic β cell death and inhibits T cell infiltration into pancreatic islet trough homeostatic regulation of Th1/ Th2/ Th17 (Zhang *et al.*, 2013). In the study, ethanolic extract of *A. paniculata* contained andrographolide by 13.7%.

Reportedly, ethanolic extract of *M. citrifolia* fruit could lower blood glucose levels in streptozotocin-induced diabetic rats and improve kidney function of diabetic nephropathy through the reduction of blood glucose levels, the amount of neutrophils and fibronectin blood glucose in DM rats (Hadijah *et al.*, 2004). Fermented *M. citrifolia* juice reduce levels of glycosylated hemoglobin (HbA1c), improve insulin sensitivity, activate peroxisome proliferator-activated receptor (PPAR-γ) and trigger the uptake of glucose by stimulating AMP-activated protein kinase (AMPK) in cell culture C2C12 (Verma *et al.*, 2013). The content of scopoletin in MCEE which is the identity compound of *M. citrifolia* was 0.3%. Scopoletin has antioxidant properties by catch the superoxide anion in xanthine/ xanthine oxidase reaction system and has hypotensive, antidepressant, hypolipidemic, and hypoglycemic activity (Lee *et al.*, 2012).

Other metabolic compounds in *A. paniculata* and *M. citrifolia* such as flavonoids and phenolic compounds may have contribution in hypoglycemic effect. Flavonoids and phenolic have capability for capture and neutralize free radicals (antioxidant effect). Effect of antioxidant in both extract when combined could more potent than its single extract. Antioxidant could reduce the formation of free radical in diabetic condition, oxidative stress, TNF-α expression, lipid peroxidation, and preventing the pancreatic β cells damage so that insulin can still be produced well (Fenercioglu *et al.,* 2010). Flavonoids also inhibit the carbohydrate hydrolizing enzyme, α-glucosidase and α-amylase so that the flavonoid

effectively lower post-prandial blood glucose levels because of the production and absorption of glucose derived (Gautam *et al*., 2013; Hong *et al*., 2013).

Other medicinal plant often combined with *A. paniculata* in order to investigate anti-diabetic potency in various diabetic animal models. *A. paniculata* was combined with *Centella asiatica* L. in high fructose-fat fed rats (Nugroho, Lindawati, Herlyanti *et al*., 2013), *Azadirachta indica* A. Juss leaves in alloxan-induced diabetic rats (Nugroho, Sari, Sunarwidhi *et al.*, 2014; Ucche *et al*., 2015), *Curcuma xanthorrhiza* rhizome and propolis in high-fructose-fat-fed rats (Nugroho, Kusumaramdani, Widyaningar *et al.*, 2014), and *Gynura procumbens* (Lour.) Merr in alloxan-induced hyperglycemic rat (Sari *et al*., 2015). In this study, APEE and MCEE both in single or its combination have an effect on blood glucose levels reduction and pancreatic islets improvement because of streptozotocin induction. Each herbs extract possessed active compound that reported reduces blood glucose levels. The content of phenolic compounds, flavonoids, scopoletin, and andrographolide are expected to be a synergistic role in pancreas action improvement and blood glucose levels decrease. Further investigation is needed to identify other active compounds in *A. paniculata* and *M. citrifolia.*

These findings support exploration attempts of medicinal plants for the discovery and development of drugs (Nugroho, Riyanto, Sukari *et al.*, 2011a; Harwoko *et al*., 2014). The exploration for drugs (isolated active compounds, medicinal plants, and synthetic drugs) includes pharmacological and toxicological activities, herbal formulation, phytochemical studies, isolation of active compounds etc. (Nugroho, Riyanto, Sukari *et al*., 2011b; Nugroho, Hermawan, Putri *et al*., 2013; Febriansah *et al.*, 2015; Sunarwidhi *et al.*, 2014). To improve the pharmacological activities, some traditional medicinal plants were often combined with other drugs. In the study, *A. paniculata* ethanolic extract exhibited an improvement on its anti-diabetic effect when combined with *M. citrifolia* ethanolic extracts.

Conclusion

Combination of *A. paniculata* and *M. citrifolia* ethanolic extracts showed comparable hypoglycemic effect in comparison to single treatment of both *A. paniculata* and *M. citrifolia* extract. This combination exhibited better improvement in pancreatic islets, increase pancreatic β cells numbers, and improve expression of pancreatic insulin compared to its single extract in neonatal streptozotocin-induced type 2 diabetes mellitus rats.

Acknowledgement

We thank the Ministry of Research, Technology and Higher Education of the Republic of Indonesia, Indonesia through the Research Grant "Penelitian Unggulan Perguruan Tinggi" for financial support of this study.

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