Pancreatic Effect of Andrographolide Isolated from
Andrographis paniculata (Burm. f.) Nees

Agung Endro Nugroho, Ichwan Ridwan Rais, Iwan Setianwan, Pramita Yuli Pratiwi,
Tony Hadibarata, Maulana Tegar and Suwidjyo Pramono

1Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy,
Universitas Gadjah Mada, Yogyakarta, Indonesia, 55281, Indonesia
2Institute of Environmental and Water Resource Management, Universiti Teknologi Malaysia,
Skudai, Johor, Malaysia, 81310, Malaysia
3Department of Pharmacochemistry, Faculty of Pharmacy, Universitas Gadjah Mada,
Yogyakarta, Indonesia, 55281, Indonesia
4Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada,
Yogyakarta, Indonesia, 55281, Indonesia

Abstract: Andrographis paniculata (Burm. f.) Nees is a plant that originates from India and grows widely to Southeast which used for several purposes mainly as treatment of diabetes mellitus so the aim of this study was evaluate andrographolide for its pancreatic effect in neonatal streptozotocin (STZ)-induced diabetic rats, a model of type 2 diabetic rats. Diabetic condition was induced with an intraperitoneal injection of 90 mg kg\(^{-1}\) streptozotocin in two-day-old rats. After three months, the neonatal STZ-induced diabetic rats were treated with andrographolide or andrographolide-enriched extract of A. paniculata (AEEAP) for 8 consecutive days. Pancreatic effect was evaluated by estimating mainly the preprandial and postprandial blood glucose levels and other parameters such as morphology of pancreatic islet, beta cells density and morphology and immunohistochemically pancreatic insulin. Andrographolide significantly (p<0.05) decreased the levels of blood glucose and improved diabetic rat islet and beta cells. However, AEEAP exhibited moderate hypoglycaemic effects on the blood glucose levels. Moderate changes in beta cells were observed after AEEAP treatment. They could restore decreasing of pancreatic insulin contents. Based on these results andrographolide and AEEAP exhibited pancreatic actions in neonatal STZ-induced diabetic rats. The activity of andrographolide was more effective than this of AEEAP.

Key words: Andrographis paniculata (Burm. f.) Nees andrographolide, diabetes, streptozotocin andrographolide-enriched extract

INTRODUCTION

Diabetes Mellitus (DM) is one of dangerous disease caused by metabolic disorders associated with many indications. DM is related to a deficiency of insulin secretion or a decrease in tissues sensitivity such as skeletal muscle, adipose tissue to the presence of insulin (insulin resistance). The condition results in chronic complications including microvascular, macrovascular and neuropathic disorders. Based on exogenous insulin, DM is classified into two categories: type 1 DM related to an absolute deficiency of insulin (insulin-dependent diabetes mellitus, IDDM) and type 2 DM related to insulin resistance with compensatory increase in insulin secretion (non-insulin-dependent DM, NIDDM) (Triplitt \textit{et al.}, 2005; Greenspan and Strewler, 1997). Type 1 DM is caused by a destruction of pancreatic beta cells due to autoimmune processes. Macrophages, beta cell autoantigens, dendritic cells, T lymphocytes and B lymphocytes are kind of pathogenesis caused by autoimmune of DM (Tisch and McDevitt, 1996). Type 2 DM, common in patients over the age of 40, is associated with insulin resistance and/or impaired insulin secretion. Uncontrolled type 2 DM can develop into type 1 DM so that the patients should consider the diet, lifestyle and oral hypoglycaemic drugs (Tisch and McDevitt, 1996; Bastaki, 2005).

\textit{Andrographis paniculata} (Burm. f.) Nees is a plant that originates from India and grows widely to Southeast
Asian. As a traditional medicine, this plant is used for several purposes, primarily preventing DM (Niranjan et al., 2010). Using a model of type 1 DM rats, glucose levels in streptozotocin (STZ)-diabetic rats could decrease the blood by ethanolic extracts of A. paniculata (Burm. f.) Nees, (Zhang and Tan, 2000). In the in vitro study, A. paniculata (Burm. f.) Nees exhibited insulin-releasing actions on BRN-BD11 cells (Wibudi et al., 2008), a pancreatic beta cell line expressing insulin and glucokinase (McClanaghan et al., 1996; McClanaghan, 2007). The mechanism of water soluble extract of A. paniculata (Burm. f.) Nees as antioxidant activity by increasing the activities of superoxide dismutase (SOD) and catalase (Dandu and Inamdar, 2009).

Diterpenoids, flavonoids and polyphenols compounds were mainly contain in A. paniculata (Burm. f.) Nees. Among them andrographolide is a major compound (> 4%) and more active than other compounds (Cheung et al., 2001; Pholphana et al., 2004; Burgos et al., 1997). Reportedly andrographolide exhibited hypoglycemic effects in STZ-diabetes rats. In addition andrographolide could increase both of mRNA and protein levels of GLUT 4, through increasing glucose concentration in blood (Yu et al., 2003). In the previous study andrographolide and an andrographolide-enriched extract of A. paniculata (Burm. f.) Nees (AEEAP) decreased the levels of blood glucose by increasing GLUT-4 expression in high-fat-fructose-fed rats. They also decreased the triglyceride and LDL levels in those rats (Nugroho et al., 2012a). Based on this study, the pancreatic effect of andrographolide isolated from A. paniculata (Burm. f.) Nees in neonatal STZ-induced diabetic rat be evaluated (Goyal et al., 2011). The evaluated-parameters were the level of postprandial and preprandial blood glucose, morphology of Langerhans islets and pancreatic beta cells, beta cells density and immunohistochemically pancreatic insulin.

MATERIALS AND METHODS

Materials: Streptozotocin (STZ) and glibenclamide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Standard andrographolide (Sigma-Aldrich, USA). Glucose level were measured using colorimetric method (GOD-PAP) with glucose oxidase and 4-aminopyridine (DiaSya, Diagnostic Systems GmbH, Holzheim, Germany). Antibodies for determination of insulin expression were primary anti-insulin antibody (Santa Cruz Biotechnologies, California, USA) and secondary chicken anti-goat IgG antibody (Invitrogen Carlsbad, CA, USA). Victoria blue, hematoxylin and eosin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were high-grade qualified materials.

Preparation of andrographolide-enriched extract of A. paniculata leaves: A. paniculata (Burm. f.) Nees was collected in August 2012 from the area around Yogyakarta, Indonesia. The leaves were shade dried, powdered and stored in an airtight container for further use. The plant was authenticated by a botanist at Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia and the voucher specimen was deposited in herbarium of the department.

The dried powder of A. paniculata (Burm. f.) Nees was subjected to extraction using 90% ethanol for 24 h. After filtration, the liquid extract was collected. The sediment was re-extracted using the same solvents for 24 h. This process was done twice. All liquid extracts were collected and concentrated using a rotary vacuum evaporator under reduced pressure. Extract was then fractionated with n-hexane at a ratio of 1:10 (extract:n-hexane) yielding two fractions of hexane soluble fraction and insoluble fraction. The insoluble fraction was collected and then evaporated to obtain viscous extract. The insoluble fraction was then washed with hot water and diluted with ethanol 90% to yield an andrographolide-enriched extract of 1.71%, with andrographolide level of 16.31%. The andrographolide was identified using HPLC-UV compared with a standard andrographolide (Sigma-Aldrich, USA). The quantification of andrographolide was performed using TLC-Densitometry method.

Isolation of andrographolide from A. paniculata leaves: The final extract of A. paniculata (Burm. f.) Nees was subjected to vacuum column chromatography on silica gel 60F254 and eluted with a mixture of solvents consisting of chloroform and methanol (9:1) as the mobile phase. The clear solution was collected and then allowed to stand for 24 h at 4°C to yield an andrographolide sediment. The sediment was recrystallized by washing with cold methanol and then with cold n-hexane, respectively.

Preparation of neonatal STZ-induced type-2 diabetic rats: The animal handling protocols of this study were in accordance with the guidelines of the animal care of the Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. Pregnant Wistar rats were housed in individual cages at a constant temperature (22±2°C) with a constant relative humidity (55±10%) on an automatically controlled 12:12 h light-dark cycle (light on at 7:00 a.m.) and had free access to food and water. They were checked regularly for delivery of pups. Two-day-old neonates were treated with STZ dissolved in citrated buffer (pH 4.5) by
intrapertoneal injection at the dose of 90 mg kg\(^{-1}\) b.wt. (Goyal et al., 2011). Control neonates were injected intraperitoneally with an equivalent amount of buffer. Three months after the injection, male neonatal STZ-induced rats were selected for the study and the preprandial and postprandial glucose levels of that rats were determined. The animal with the blood glucose levels more than 1.5 fold glucose levels of control rats were considered diabetic.

**Experimental design:** The diabetic rats were acclimatized for 1 week and then divided into six groups as follows:

- **Group 1:** The rats received oral saline 10 mL kg\(^{-1}\) BW (control group)
- **Group 2:** The rats received andrographolide dose 1.5 mg kg\(^{-1}\) b.wt orally, twice daily
- **Group 3:** The rats received andrographolide dose 4.5 mg kg\(^{-1}\) b.wt orally, twice daily
- **Group 4:** The rats received andrographolide-enriched extract of *A. paniculata* (Burman f.) Nees (AEEAP) dose 434.6 mg kg\(^{-1}\) b.wt orally, twice daily
- **Group 5:** The rats received AEEAP dose 1303.8 mg kg\(^{-1}\) b.wt orally, twice daily
- **Group 6:** The rats received glibenclamide dose 45 mg kg\(^{-1}\) b.wt orally, twice daily

All treatments (saline andrographolide, AEEAP or glibenclamide) were administered for 8 days. The preprandial blood samples were taken from retro-orbital plexus under light ether anesthesia at days 0 (basal value), 2, 4, and 8 after 10 h fasting. Two hours after feeding, blood was collected again for determination of postprandial blood glucose levels. The sera were separated from blood by centrifugation at 1000 rpm for 10 min. The serum glucose was analyzed spectrophotometrically by glucose oxidase-peroxidase (GOD-POD) method using commercial biochemical diagnostic kits (DiaSys, Holzheim, Germany).

**Histological observation of Langerhans islet and beta cells of pancreas:** The rats were sacrificed at the end of the treatment. The pancreas of control and treated groups were removed and fixed with 4% paraformaldehyde in phosphate buffer for 24 h. The tissues were embedded in paraffin and cut into pieces of desired size (4 µm). The sections were then deparaffinized in xylene and dehydrated a series of alcohol concentrations. The sections were then stained with hematoxylin and eosin (H and E), or 0.1% Victoria blue (pH 0.3) to evaluate the Langerhans islet and beta cells of pancreas. The slides were cleared in xylene and mounted with mounting medium and examined under the light microscope (Olympus BX51, Japan). Pancreatic beta cells were identified and detected in each section for semiquantitative analysis. The detection was performed with a X 40 objective, a X 10 eyepiece and the area observation was randomly photographed four times. The beta cells density per tissue area in each photograph was counted and presented as a percentage of control.

**Immunohistochemistry of pancreatic insulin:** Other parts of pancreas were fixed with 4% formaldehyde in Phosphate-buffered Saline (PBS) for at least two hours. The tissues were dehydrated in a graded concentrations of alcohols. The tissues were cleared using clearing agents (xylol) because the paraffin and ethanol are immiscible. Xylol functions to mix the paraffin and ethanol. The tissue were then embedded in paraffin and 2-3 µm thick sections were obtained using a rotary microtome. Tissue sections were mounted on glass slides. Activity of endogenous peroxidase in the tissue was blocked using 3% H\(_2\)O\(_2\) in methanol for 15 min and then washed with aquadest. Subsequently, the tissue sections were incubated with 20% horse serum for at least 10 min in order to prevent non-specific binding. The sections were incubated at room temperature with primary antibody against rat insulin at a 1:250 dilution for one hour and then with peroxidase-conjugated secondary antibody at a 1:500 dilution for one hour. Expression of pancreatic insulin would be visualized after incubation with a substrate for 15 min. The sections were counterstained with hematoxylin for 30 min, dehydrated with graded alcohol and xylene and then mounted with coverslips. The slides were evaluated under the light microscope (Olympus BX51, Japan).

**Statistical analysis:** All values were expressed as Mean±SEM. One-way analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) test were used for statistical analyses. p-values of less than 0.05 indicated significant differences.

**RESULTS**

**Isolation of andrographolide:** Attempts to provide plant extracts containing high level of its major active compound are being widely developed. Andrographolide was a major and most active compound in *A. paniculata* leaves. In the present study, ethanolic extract of *A. paniculata* was fractionated gradually to provide high level of its active compound andrographolide. n-hexane was used in the step to eliminate chlorophyll, lipid and
other disturbing materials. This insoluble fraction was washed with hot water and diluted with ethanol 90% to provide an AEEAP that contains andrographolide with levels of 16.31%. The AEEAP could be chromatographed on silica gel 60F254 and recrystallized to yield pure andrographolide.

**Neonatal streptozotocin-induced type 2 diabetic rat:**
Intraperitoneal administration of STZ (90 mg kg\(^{-1}\) b.wt) on two-day-old neonates resulted in significant increase of the blood glucose level in comparison to that of control group (normal rats) at the age of three months (Fig. 1). Preprandial and postprandial blood glucose levels of normal rats were 60.55±2.16 and 85.83±2.88 mg dL\(^{-1}\), respectively. Whereas, preprandial and postprandial blood glucose level of neonatal STZ-induced diabetic rats were 113.39±16.38 and 164.85±17.46 mg dL\(^{-1}\), respectively. These facts mean that the blood glucose levels of diabetic rats were two folds higher than those of normal rats.

Treatment of STZ on neonates mildly decreased the body weight in comparison to the control group (normal rats) at the age of three months. The body weight of neonatal STZ-induced diabetic rats was 120.50±2.14 g and that of normal rats was 134.19±1.60 g.

**Effect of andrographolide on blood glucose level:** In the study, administration of andrographolide (4.5 mg kg\(^{-1}\) b.wt) for 8 days decreased the postprandial blood glucose level of neonatal STZ-induced diabetic rats by 30% (Fig. 2). However andrographolide at the dose of 4.5 mg kg\(^{-1}\) b.wt did not influence the preprandial blood glucose level. Indeed andrographolide at lower dose (1.5 mg kg\(^{-1}\) b.wt) did not succeed to decrease both preprandial and postprandial blood glucose levels of neonatal STZ-induced diabetic rats. AEEAP also did not influence both preprandial and postprandial blood glucose levels. Glibenclamide, a sulphonylurea antidiabetic agent, succeeded to decrease postprandial blood glucose levels by 40% (Fig. 2).

**Effect on rat pancreatic islets:** Histopathological observation on Hematoxylin-Eosin stained sections showed that the pancreatic islets of rat without induction of STZ were normal in relation to the size and cell number. The cells uniformly and homogeneously dispersed mainly at the periphery (Fig. 3a). However, the Langerhans islets of neonatal STZ-induced diabetic rats atrophied and some
degenerative changes also occurred in comparison to those of control rats. Some islet cells underwent changes in the size and shrunk. A decrease in the number of islet cells also occurred in comparison to those of control rats (Fig. 3b). An improvement of diabetic rat islets is observed after treatment with andrographolide (4.5 mg kg⁻¹ b.wt) and glibenclamide for 8 days (Fig. 3c and d). However, treatment with andrographolide-enriched extract of *A. paniculata* exhibited moderate improvement of diabetic rat islets (Fig. 3e).

**Effect on beta cells of rat pancreas:** In addition to rat pancreatic islets morphology, we also observed the influence on the morphology of pancreatic beta cells using Victoria blue staining. In the staining, beta cell is represented as a cell with blue-stained cytoplasm. The comparison between pancreatic beta cells in normal rats and in neonatal STZ-induced diabetics rats were showed in Fig. 4a and b. In diabetics rats, histological changes of beta cells have occurred such as extensive degenerative of beta cells and decreased cellular density. In line with previous experiment, shrunken pancreatic islets and beta cells changes were also observed in diabetic rats. Treatment of either andrographolide (4.5 mg kg⁻¹ b.wt) or glibenclamide for 8 days could improve the rat pancreatic beta cells (Fig. 4d). Treatment with andrographolide-enriched extract of *A. paniculata* improved the beta cell of rat pancreas moderately (Fig. 4c and e). Andrographolide 4.5 mg kg⁻¹ b.wt, glibenclamide and AEEAP 1303.8 mg kg⁻¹ b.wt increased the beta cells density by 60, 33 and 64%, respectively (Fig. 5).
Effect on pancreatic insulin expression: In the study, the pancreatic insulin was detected using immunohistochemistry method. The insulin expression represents the content of insulin in the Langerhans islets. Brown area in the islets indicates insulin staining. Figure 6a and 6b showed that the stained-insulin of neonatal STZ-induced rats were less intensive than that of normal rats.

Administration of andrographolide (4.5 mg kg\textsuperscript{-1} b.wt) or glibenclamide for 8 days succeeded to restore the pancreatic insulin content in neonatal STZ-induced diabetics rats in comparison to the control group (Fig. 6c and e). Moderate restoration of pancreatic insulin contents were observed immunohistochemically after the rats treated with AEEAP.
STZ is an antibiotic produced by strain *Streptomyces achromogenes*. The chemical name of STZ is 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) (Rizvi et al., 1986; Szkudelski, 2001). The compound is widely used as a diabetogenic in experimental animals related to both insulin-dependent and non-insulin-dependent diabetes mellitus (type 1 and type 2, respectively) (Arulmozhi et al., 2004; Nugroho, 2006). The compound selectively destroys the beta cells of pancreas by entering the beta cells through a glucose transporter (GLUT2). In beta cells, the compound induces the production of superoxide radicals, hydrogen peroxide and hydroxyl radicals which in turn causes rapid destruction of beta cells. The compound also releases toxic amounts of nitric oxide that inhibits acetylcholinesterase activity and contributes in DNA damage (Szkudelski, 2001; Arulmozhi et al., 2004; Nugroho, 2006).

In the study, streptozotocin was used to induce type 2 DM. The diabetic condition is generated by injecting 90 mg kg\(^{-1}\) streptozotocin in two-day-old rats intraperitoneally (Goyal et al., 2011). After two months, there was partial destruction of the islet cells of pancreas in neonatal STZ-induced rats in comparison to that of normal rats. The blood glucose levels (preprandial and postprandial) of the neonatal streptozotocin-induced rats were two fold higher than those of normal rats.
Eventhough the beta cells are destroyed by streptozotocin at 2-day-old neonatal rats, there is active regeneration of Langerhans islets and beta cells after early injury. However, the capacity of normalization of injured cells and islets are limited. The partial defect of the islet beta cells resulted in hyperglycemia in neonatal STZ-induced rats (Bonnier-Weir et al., 1981; Weir et al., 1981). Other physiological changes in two-day-old neonatal streptozotocin-induced rat include impairment of glucose-induced insulin release (low insulin release in response glucose), glucose intolerance and decrease in pancreatic insulin stores. These conditions were identical to the characteristics observed in patients with type 2 DM (Arulmoozh et al., 2004; Nugroho, 2006).

*A. paniculata* (Burn. f.) Nees originates from South Asian countries and grows widely in many areas in Southeast Asian countries. Traditionally this herb is used as an antidiabetes, antiinflammatory, hepatoprotective, antispasmodic and antioxidant agents (Niranjana et al., 2010). In phytochemical studies, *A. paniculata* (Burn. f.) Nees (whole plant, leaf and stem) contains mainly diterpenoids, flavonoids and polyphenol. The diterpenoid compounds are andrographolide, deoxoandrographolide, 19-O-acetylanhydro-andrographolide, neoandrographolide, 14-deoxy-dihydroandrographolide and homoandrographolide (Rao et al., 2004; Chao and Lin, 2010). The andrographolide is a major and most active compound in *A. paniculata* (Burn. f.) Nees. The compound was reported possessing potent hypoglycemic effects in DM rats models (Yu et al., 2003, 2008; Nugroho et al., 2012a, 2012b; Subramaniam et al., 2008; Zhang et al., 2009). The presence of andrographolide in the extract of *A. paniculata* (Burn. f.) Nees will determine the biological activity of that extract. In the study, ethanolic extract of *A. paniculata* was fractionated gradually to provide high level of andrographolide in AEEAP. The fractionations were used for eliminating chlorophyll, lipid and other disturbing materials.

In the study, administration of andrographolide for 8 days succeeded to decrease the postprandial and preprandial blood glucose levels in comparison to those of the control. An improvement of diabetic rat islets, beta cells (morphology and density) and pancreatic insulin contents were also found after treatment with andrographolide. Glibenclamide, an type 2 antidiabetic drug acting to stimulate insulin-releasing beta cells (pancreatic action), also showed similar effects on the pancreas. Glibenclamide could improve the diabetic rat islets, beta cells and pancreatic insulin contents. In the other side, AEEAP decrease the blood glucose levels mildly. Only moderate changes in Langerhans islets, beta cells and pancreatic insulin were shown after AEEAP treatment. Based on these results, it indicates that andrographolide as a single compound is more effective than in the form of AEEAP in neonatal streptozotocin-induced diabetic rats.

Inline with this finding andrographolide exhibited hypoglycemic effect in type 1 diabetic rats and other type 2 diabetic rats. Reportedly andrographolide decreased the plasma glucose concentrations of STZ-diabetic rats (type 1 diabetic rats) through increased GLUT-4 in soleus muscle for the glucose uptake (glucose utilization) (Yu et al., 2003, 2008). An andrographolide analogue namely andrographolide-lipoic acid conjugate was reported to lower the blood glucose levels through increasing insulin, preventing loss and dysfunction of islet beta cells and stimulating GLUT-4 membrane translocation in soleus muscles of alloxan-diabetic rats (type 1 diabetic rats) (Zhang et al., 2009). Andrographolide also exhibited hypoglycemic effect in high-fructose-fat-fed rats, a model of type 2 DM rats. Andrographolide succeeded to decrease the levels of blood glucose, triglyceride and LDL compared to controls (Nugroho et al., 2012a). The compound also stimulate the soleus muscle GLUT-4 expression in that rats (Nugroho et al., 2012b). However, the compound did not alter the serum cholesterol and rat body weight (Nugroho et al., 2012a). Indeed, the compound can also stimulate the histamine release and also inhibit enzyme alpha-glucosidase and alpha-amylase, enzymes contributing in glucose absorption (Subramaniam et al., 2008). In addition andrographolide, an active compound of *A. paniculata*, decrease the blood glucose levels in neonatal streptozotocin-induced diabetic rats, a model of type 2 DM rats. Andrographolide also could improve the morphology of pancreatic islet, beta cells density and morphology and immunohistochemically pancreatic insulin content. Based on these results andrographolide is potential to develop into as an antidiabetic agent.

**CONCLUSION**

Based on these results andrographolide and AEEAP exhibited pancreatic actions in neonatal STZ-induced diabetic rats. The activity of andrographolide was more effective than this of AEEAP.

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