

## Effect of herbal combination of *Andrographis paniculata* (Burm.f) Ness and *Gynura procumbens* (Lour.) Merr ethanolic extracts in alloxan-induced hyperglycemic rats

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### Abstract

Exploration of herbs combination could be an alternative way for developing traditional medicine in order to provide better result or benefits in the therapy of diseases. *Andrographis paniculata* (Burm. f.) Ness and *Gynura procumbens* (Lour.) Merr are two medicinal plants that are already well known for traditional treatment of diabetes mellitus (DM). Combination of potent hypoglycaemic effect of *A. paniculata* and potent antioxidant effect of *G. procumbens* is expected to produce an optimum antidiabetic effect. The aim of this study was to evaluate the antidiabetic effect of their combination in alloxan-induced hyperglycemic rats. Diabetic condition in rats was induced with a single dose of 150 mg/kg BW alloxan intraperitoneally. The diabetic rats were orally administered with these combinations (300:37.5; 200:75; 100:112.5 mg/kg BW) for 15 consecutive days. Diabetic effect was evaluated by measurement of preprandial and postprandial blood glucose levels and other parameters such as morphology of pancreatic islet and pancreatic insulin expression. In the study, the combination of *A. paniculata* and *G. procumbens* significantly decreased the blood glucose level up to 76%. Fifteen days administration of this combination could improve the condition of pancreatic islet due to alloxan. The combination also increased the pancreatic insulin expression. The highest antidiabetic effect of the combination was achieved at the dose of 100:112.5 mg/kg BW. In conclusion, the combination of *A. paniculata* and *G. procumbens* is potential to develop as an antidiabetic agent.

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### Introduction

Diabetes mellitus (DM) is one of degenerative disease that is commonly suffered by many people. In 2008, there are about 13 million diabetics in Indonesia and it is predicted that in 2025 there will be more than 21 million diabetics. This condition can place Indonesia as fourth largest country with DM patient after India, China, and America (Agoes *et al.*, 2009). Therefore, a serious treatment is really needed. In recent DM treatment, complementary system and alternative treatment are widely applied (Eisenberg *et al.*, 1998). There are a lot of medicinal plants in Indonesia often used empirically for treating various diseases (Nugroho *et al.*, 2011a; Nugroho *et al.*, 2011b). Some of them were used as hypoglycemic agents such as *Andrographis paniculata* (Burm. f.) Ness and *Gynura procumbens* (Lour.) Merr.

*A. paniculata* (Sambiloto) and *G. procumbens* (Sambung Nyawa) are traditionally used in treatment

for several diseases especially DM. Zhang and Tan (2000) reported that ethanolic extract of *A. paniculata* showed a reduction effect on the blood glucose levels after 14 days treatment in streptozotocin-induced diabetic rats. The main and active compound of the plant is andrographolide (Cheung *et al.*, 2001; Pholphana *et al.*, 2004). Meanwhile, a significant hypoglycemic effect of *G. procumbens* was reported in streptozotocin-induced diabetic rats (Zhang and Tan, 2000). Its hypoglycemic was related to its antioxidative effect. This plant also increased some antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). However, the blood glucose levels reduction in normal rats were not observed.

The bioactive compounds in *A. paniculata* are flavonoid, polyphenolic and diterpenoid compounds such as andrographolide, neoandrographolide, deoxyandrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide and isoandrographolide

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Table 1. Information of antidiabetic effects of combination of *A. paniculata* with other traditional medicines

No.	Name of medicinal plant (traditional medicine) combined with <i>A. paniculata</i>	Part of the plant	Hypoglycemic activity of the combination (diabetic rat model)	Other related activity of the combination	References
1.	Asiaticoside-enriched extract of <i>Centella asiatica</i> L.	herbs	68% (high-fructose-fat-fed rats)	decrease the serum cholesterol total and increase the serum HDL	Nugroho et al.(2013)
2.	Curcuminoids fraction of <i>Curcuma xanthorrhiza</i>	rhizome	23.38% (high-fructose-fat-fed rats)	-	Nugroho et al.(2014a)
3.	Ethyl acetate soluble fraction of propolis	-	20.06% (high-fructose-fat-fed rats)	-	Nugroho et al.(2014a)
4.	Extract of <i>Centella asiatica</i> L.	herbs	-	Stimulation of GLUT-4 translocation in soleus muscle cells	Lindawati et al. (2014)
5.	<i>Azadirachta indica</i> A. Juss	leaf	58.12% (preprandial blood glucose), 62.84% (postprandial) (alloxan-induced rats)	-	Nugroho et al.(2014c)

(Chao and Lin, 2010). Whereas the bioactive compounds in *G. procumbens* leaves are flavonol and flavonol glycosides including quercetin and kaempferol, and its glycoside form such as quercetin-3-O-rhamnosyl(1-6)glucoside, quercetin-3-O-rhamnosyl(1-6)galactoside, kaempferol-3-O-glucoside and kaempferol-3-O-rhamnosyl (1-6) glucoside (Akowuah et al., 2002).

Combination of potent hypoglycaemic effect of *A. paniculata* and potent antioxidant effect of *G. procumbens* is expected to produce an optimum antidiabetic effect. The aim of this study was to evaluate the antidiabetic effect of their combination in alloxan-induced hyperglycemic rats. Diabetic effect was evaluated by measurement of preprandial and postprandial blood glucose levels and other parameters such as morphology of pancreatic islet and pancreatic insulin expression.

## Material and Methods

### Materials

*A. paniculata* herbs and *G. procumbens* leaves were collected in 2013 from Moyudan, Sleman, Yogyakarta. Materials for assay were alloxan monohydrate, andrographolide and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). GOD-PAP kit with glucose oxidase and 4-aminoantipyrine (DiaSys, Diagnostic Systems GmbH, Holzheim, Germany). Sodium carboxymethyl cellulose, glucose, chloroform, ethyl acetate, methanol, ethanol, acetic acid, hematoxylin and eosin were obtained from E. Merck, Darmstadt, 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).

### Animals

Male Wistar rats aging 2-3 month old (150-200 g) used in this study were maintained on a constant temperature ( $22 \pm 2^\circ\text{C}$ ) and a constant relative humidity ( $55 \pm 10\%$ ) and automatically controlled 12:12 h light-dark cycle (light on at 07:00 a.m.). They were fed with a standard laboratory food and water at libitum. Ethical clearance for the animal study was obtained from Research Ethics Committee, Integrated Research and Testing Laboratory Universitas Gadjah Mada, Indonesia (certificate number of ethic clearance: 141/KEC-LPPT/IV/2014).

### Preparation of *A. paniculata* herbs and *G. procumbens* leaves ethanolic extracts

*A. paniculata* and *G. procumbens* used in this study were authenticated at Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. The voucher specimen was stored in a herbarium of the department. The dried leaves were powdered and then stored in an airtight container for further use. The powder of *A. paniculata* herbs (505.44 g) and *G. procumbens* leaves (413.78 g) were extracted by maceration using ethanol 70% for 24 hours, respectively. After two times of remaceration, filtrate were collected and evaporated to get viscous extracts.

### Induction of diabetic condition

Overnight fasted rats were induced by an intraperitoneal injection of alloxan monohydrate at single dose of 150 mg/kgBW. While control group rats were treated with saline solution. Hyperglycemic condition was determined 72 hours after induction by measuring preprandial and postprandial blood

glucose levels.

#### Experimental design

The diabetic rats were divided into five groups as follows:

- a. Group I (negative control) : alloxan-induced diabetic rats receiving vehicle solution CMC 0.5%;
- b. Group II : alloxan-induced diabetic rats receiving combination of *A. paniculata* 300 mg/kgBW and *G. procumbens* 37.5 mg/kgBW, twice daily, orally;
- c. Group III : alloxan-induced diabetic rats receiving combination of *A. paniculata* 200 mg/kgBW and *G. procumbens* 75 mg/kgBW, twice daily, orally;
- d. Group IV : alloxan-induced diabetic rats receiving combination of *A. paniculata* 100 mg/kgBW and *G. procumbens* 112.5 mg/kgBW twice daily, orally;
- e. Group V (oral hypoglycemic agent) : alloxan-induced diabetic rats receiving glibenclamide dose 4.5 mg/kgBW once daily, orally;

All treatment were administered for 15 consecutive days. Both preprandial and postprandial blood glucose levels were determined at 0; 5; 10; 15-days of treatment. Blood samples were collected from *plexus retro orbitalis*, and then incubated at room temperature for 30 minutes. Serum was collected by centrifugation at 5000 rpm for 10 min at 25°C. Determination of blood glucose level was analyzed with colorimetric method using GOD-PAP reagent.

#### Histological observation of pancreatic

At the end of the experimental period, the rats were sacrificed then the pancreas were removed and fixed with 4% paraformaldehyde in phosphate buffer saline for 24 hours. The tissue were prepared as section slides then stained with hematoxylin and eosin (HE). The slides were analyzed and evaluated under the light microscope (Olympus BX51, Japan). The evaluation was performed with a X 40 objective, a X 10 eyepiece, and the area observation was randomly photographed four times.

#### Immunohistochemistry of pancreatic insulin

Another part of pancreas was fixed with 4% paraformaldehyde in phosphate buffer saline for at least two hours. The tissue were prepared as section slides for Immunohistochemistry (IHC) analysis using primary anti-insulin antibody and secondary chicken anti-goat IgG antibody according to the previous study (Nugroho *et al.*, 2014). The slides were also analyzed and evaluated under the light microscope (Olympus BX51, Japan).

#### Statistical analysis

All experimental data were showed as mean  $\pm$  standard error of 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 and Discussion

#### Effect on blood glucose levels.

Single dose administration of alloxan (150 mg/kgBW) intraperitoneally significantly raised both preprandial and postprandial blood glucose levels in rats. Determination of blood glucose level was performed at 48 hours after alloxan induction. Preprandial blood glucose level of alloxan-treated rats and normal rats were 408.72 $\pm$ 25.52 mg/dL and 53.77 $\pm$ 4.83 mg/dL, respectively. Postprandial blood glucose levels of both groups of rats were 465.12 $\pm$ 13.22 mg/dL and 67.42 $\pm$ 2.47 mg/dL, respectively.

Administration of the combination of *A. paniculata* and *G. procumbens* ethanolic extracts for 15 consecutive days exhibited reduction on preprandial and postprandial blood glucose levels in alloxan-induced diabetic rats. Combination of 100 mg/kgBW *A. paniculata* and 112.5 mg/kgBW *G. procumbens* exhibited the highest hypoglycemic effects in both preprandial and postprandial blood glucose levels by 76.43% and 76.94%, respectively (Figure 1). Glibenclamide, an hypoglycemic oral, reduced the blood glucose levels by 70%.

#### Effect on rat pancreatic islets

Morphological observation of islets Langerhans using HE staining showed that some pancreatic islet cells underwent changes in the size and shrunk in alloxan-diabetic rats. A decrease in the number of islet cells and some degenerative changes of the islets were observed in comparison to that of control rats. An improvement of diabetic rat islets was observed after treatment with the combination in comparison to negative control. Combination of 100 mg/kgBW *A. paniculata* and 112.5 mg/kgBW *G. procumbens* exhibited the best improvement of diabetic rat islets. Fifteen consecutive days of glibenclamide showed mild improvement of diabetic rat islets (Figure 2).

#### The effect on pancreatic insulin expression

Pancreatic insulin expression was detected using immunohistochemistry method. Based on Figure 3, the stained- insulin of alloxan-induced rats were less intensive than that of normal rats. The similar improvement results were also observed in pancreatic insulin expression after 15 consecutive days of herbal combination treatment. All treatment groups



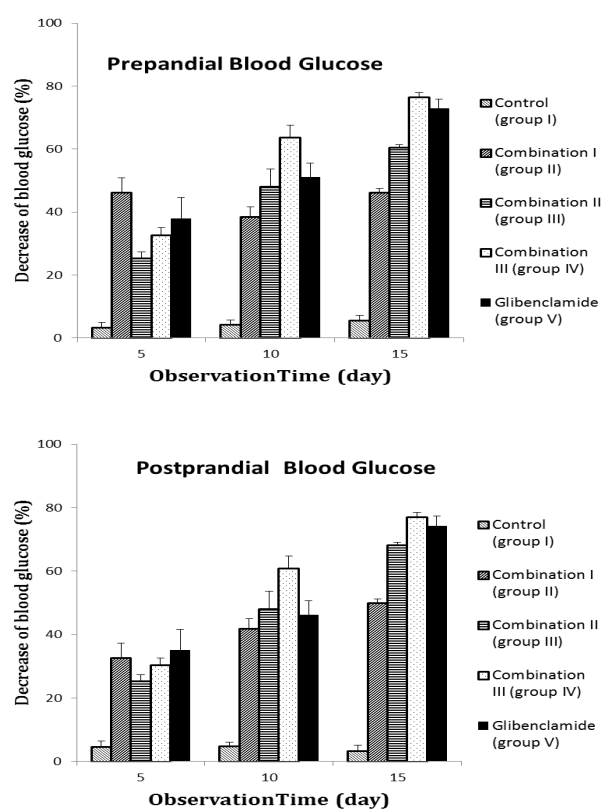


Figure 1. Hypoglycemic activities (%) of drugs treatment after 15 consecutive days. Data represent mean $\pm$ SEM from five independent experiments. \* $P < 0.05$  compared to the value of negative control. # $P < 0.05$  compared to the value of glibenclamide group. The value of decrease of blood glucose level is calculated based on the basal value (blood glucose level at day 0)

exhibited better insulin expression in comparison to negative control (Figure 3). Combination of 100 mg/kgBW *A. paniculata* and 112.5 mg/kgBW *G. procumbens* exhibited the best improvement of pancreatic insulin expression. Moderate restoration of pancreatic insulin expression was observed after glibenclamide treatment (Fig 3).

One of the substance often used as an diabetogenic is Alloxan (2,4,5,6-tetraoxypyrimidine; 2,4,5,6-pyrimidinetetrone). That substance belongs to derivate compound of oxidized pyrimidine and selectively destructs  $\beta$  cells of Langerhans islets by promotes production of superoxide radical and ROS that lead to DNA damage in pancreatic  $\beta$ -cells (Ebelt *et al.*, 2000; Szkuldelski, 2001; Das *et al.*, 2012).

*A. paniculata* that contains several bioactive compound such as flavonoids, phenolic compounds and andrographolide is already well-known for its antidiabetic effect. Reportedly, *A. paniculata* exhibited potent antidiabetic activities in various diabetic conditions in rats (Zhang and Tan, 2000; Yu *et al.*, 2003; Nugroho *et al.*, 2012). The aqueous extract of leaves at dose of 400 mg/kg BW (p.o.) succeeded to decrease the blood glucose levels and increase the

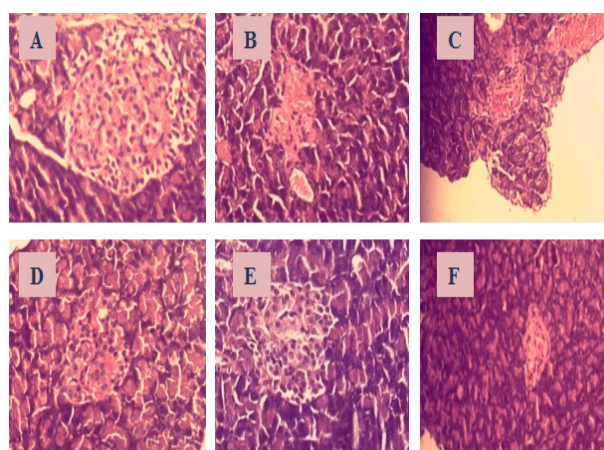


Figure 2. Histological observation of pancreatic islets with HE staining. (A) normal rats; (B) negative control (CMC Na)(group 1); (C) combination I (group 2); (D) combination II (group 3); (E) combination III (group 4); (F) glibenclamide (group 5) (magnification 400x)

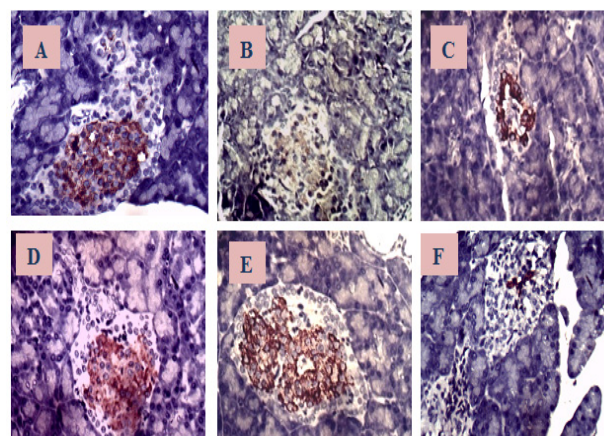


Figure 3. Immunohistochemical-stained sections of insulin rat pancreas observation. (A) normal rats; (B) negative control (CMC Na; group 1); (C) combination I (group 2); (D) combination II (group 3); (E) combination III (group 4); (F) glibenclamide (group 5) (magnification 400x). Brown area indicates insulin staining

activity of superoxide dismutase (SOD) and catalase in STZ-induced diabetic rats (Dandu and Inamdar, 2009). The antidiabetic activities of *A. paniculata* was mainly contributed by its active compounds namely andrographolide, which is known as main constituent and abundance in this herb (Cheung *et al.*, 2001; Pholphana *et al.*, 2004; Rafi *et al.*, 2014).

Andrographolide was known for its activity in lowering blood glucose level through several mechanisms such as increased GLUT-4 protein in soleus muscle for glucose uptake, improved pancreatic islet, increase of beta cell density, increase of pancreatic insulin content (Yu *et al.*, 2003; Nugroho *et al.*, 2014b). Andrographolide also obstructs the  $\beta$ -cell pancreas death through Nf-kB activation blocking (Zhang *et al.*, 2009). Moreover, the capability of andrographolide in reducing ROS, stimulating mRNA and GLUT-4 expression can

contribute in downstream effect of blood glucose reduction (Shen *et al.*, 2002; Yu *et al.*, 2003). Its bioavailability after administration of herb infusion in healthy animal has been studied. This compound was rapidly absorbed, distributed in the circulation system and then metabolized mainly in the liver (Levita *et al.*, 2014).

Meanwhile, the hypoglycemic effect of *G. procumbens* was reported by Zhang and Tan (2000) in streptozotocin-induced diabetic rats. The plant extract showed significant effects in increasing antioxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT) but the blood glucose levels reduction in normal rats was not observed. The bioactive compounds in *G. procumbens* leaves such as kaempferol, quercetin and astragalins were reported to reduce blood glucose levels (Chattopadhyay, 2003; Hassan *et al.*, 2010; Zhang *et al.*, 2010). Moreover, quercetin was reported to be potent antioxidant that protects the body from diabetic complication induced by ROS (Lakhanpal and Rai, 2007; Nugroho *et al.*, 2014a).

Metabolic compound contained in *A. paniculata* and *G. procumbens* such as andrografolide and quercetin may contribute in their hypoglycemic effect. Moreover, other compounds in both extracts such as phenolic and flavonoid compounds may also have contribution in their hypoglycemic effect. The phenolic and flavonoid compounds detected in each extract could give more potent antioxidant effect when both extracts were combined. Antioxidant was known for its ability to reduce the formation of free radical in diabetic condition, oxidative stress and TNF- $\alpha$  expression (Widowati, 2010). Coskun *et al.* (2004) also reported that flavonoid have an important role in blood glucose levels reduction and increase of improvement of  $\beta$ -cell pancreas distribution based on its ability in superoxide binding. Quercetin also has an effect for blocking the nitric oxide synthase activity in beta cell pancreas so that the beta cell destruction caused by radical nitric oxide formation can be blocked (Rifaai *et al.*, 2012).

Extract of *A. paniculata* was often combined with other medicinal plants for investigation of their potency of antidiabetic effect in various diabetic conditions in rats. The medicinal plants or traditional medicines have been combined with *A. paniculata* are *Centella asiatica* L., *Curcuma xanthorrhiza*, propolis, *Azadirachta indica* A. Juss (Nugroho *et al.*, 2013; Nugroho *et al.*, 2014a; Nugroho *et al.*, 2014c) (table 1). In the study, the extracts combination of *A. paniculata* and *G. procumbens* have an effect in pancreatic islet improvement and in line with it the pancreatic insulin content was rising. In advance,

hypoglycemic condition could be achieved. The diversity of active compounds in the herbal extract combination may synergistically increase antihyperglycemic effect and may give an alternative in diabetes therapy. However, exploration related to the mechanism of action of each active compound from ethanolic extract remains to be elucidated.

## Conclusion

Combination of *A. paniculata* and *G. procumbens* ethanolic extracts exhibited potent antidiabetic effect by parameters of decrease of preprandial and postprandial blood glucose levels, improvement in pancreatic islets, and restoration of pancreatic insulin expression in alloxan-induced diabetic rat. The herbal combination is potential to develop as an antidiabetic agent.

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