

Blood Glucose Reduction by Combination of *Andrographis paniculata* (Burm. f.) Ness Herbs and *Azadirachta indica* A. Juss Leaves in Alloxan-Induced Diabetic Rats

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ABSTRACT

Andrographis paniculata (Burm. f.) Ness and *Azadirachta indica* A. Juss are Indonesian local plants that are potentially developed as antihyperglycemic agents. This study was aimed to explore antihyperglycemic effect of herbal extract combination of *A. paniculata* (Burm. f.) Ness and *A. indica* A. Juss in alloxan-induced rats compared to single extract treatment. Diabetic condition was induced with an intraperitoneal injection of 150 mg/kgBW alloxan monohydrate in the rats. After stable diabetic condition, the rats were administered either with single or extract combination for 15 consecutive days. Blood glucose profiles in both preprandial and postprandial were monitored at the day of 5, 10, and 15. Analysis of blood glucose level was performed using colorimetric method of GOD-PAP. In the study, preprandial and postprandial blood glucose levels of alloxan-induced rats could be decreased after administration of the herbal extract combination of *A. paniculata* (Burm. f.) Ness and *A. indica* A. Juss. The extract combination exhibited higher hypoglycemic effects than this of the single extract treatment. In conclusion, the combination of *A. paniculata* (Burm. f.) Ness and *A. indica* A. Juss is potential to develop as an antidiabetic agent.

INTRODUCTION

Diabetes mellitus (DM) is a degenerative disease related to metabolic disorder and marked with hyperglycemic condition that leads to health complications. Each year, number of diabetic patient is increasing and effective therapy to control blood glucose level is required to prevent further complication. Besides, innovation in DM therapy with minimal side effect is interesting due to long-term drug administration. Thus, exploration of new agent that is able to correct hyperglycemic condition with minimal side effect from natural source is urgently needed. One approach for treating DM is use of traditional medicine. To date, the use of single medicinal plant has not shown satisfactory results. Therefore, the attempts to combine several antidiabetic drugs are interesting to be developed. *Andrographis paniculata* (Burm. f.) Ness (known as sambiloto) dan *Azadirachta indica* A. Juss

(known as Mimba) are widely used in Indonesian society for treating various diseases including diabetes melitus (Niranjan *et al.*, 2010; Biswas *et al.*, 2002). Several *in vivo* studies have been done to explore pharmacological activities of *A. paniculata* in controlling blood glucose level using diabetic-induced models. Ethanolic extract of *A. paniculata* was reported to lower blood glucose level in both streptozotocin (STZ)-diabetic rats and high fructose-fat fed rats. The extract also succeeded to increase GLUT-4 level (Yu *et al.*, 2003; Zhang and Tan, 2000; Nugroho *et al.*, 2012). Reportedly, *A. paniculata* exhibited insulin-releasing actions on BRIN-BD11 cells, a pancreatic β cell line expressing insulin and glucokinase (Wibudi *et al.*, 2008). Reportedly, *A. indica* exhibited antidiabetic activities in several diabetic model in rats. The *A. indica* leaves extract inhibited the generation of superoxide anion and hydroxyl free radical in diabetic rats. The oxidative stress has a main role in pathogenesis of diabetes mellitus (Shrivastava *et al.*, 2012). It indicates that *A. indica* is potential to prevent further complications of diabetes. In addition, *A. indica* leaves extract also inhibited the activities of alpha-amylase and alpha-glucosidase, enzymes having important role in glucose absorption (Kazeem *et al.*, 2013).

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The abundance of *A. paniculata* and *A. indica* as natural sources of antihyperglycemic agent become primary consideration for further exploration as a herbal combination agent in reducing blood glucose level. In this study, ethanolic extracts of *A. paniculata* herbs and *A. indica* leaves were combined and evaluated for their anti-diabetic effect in alloxan-induced diabetic rats.

MATERIALS AND METHODS

Materials

Alloxan and glibenclamide were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Glucose level were measured using colorimetric method (GOD-PAP) with glucose oxidase and 4-aminoantipyrine (DiaSys, Diagnostic Systems GmbH, Holzheim, Germany). Sodium carboxymethyl cellulose and glucose were obtained from E. Merck, Darmstadt, Germany. All other reagents were high-grade qualified materials.

Animals

Wistar rats (2-3 month old) weighing 150-200 g used in the 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 *ad libitum*. Ethical clearance for the animal study was obtained from Research Ethics Committee, Integrated Research and Testing Laboratory Universitas Gadjah Mada, Indonesia.

Preparation of ethanolic extract

A. paniculata (Burm. f.) Ness and *A. indica* A. Juss were collected from Sleman, Yogyakarta during October 2013. Plant authentication was performed 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 plant was dried and powdered separately, extracted by maceration using 70% (v/v) ethanol for 24 hours with ratio of 1:10. After twice re-extraction, collected filtrate was evaporated under reduced pressure to get a viscous extract. The extracts of *A. paniculata* and *A. indica* A. Juss were processed into dried extracts, then mixed with ratio by 1:1.

Phytochemical analysis

Analysis of ethanolic extract of *A. paniculata* was performed using a stationary phase of silica gel 60 F254 and a mobile phase of chloroform-ethyl acetate-methanol-acetic acid (7:2:0,5:0,5 v/v). Detection of androgapholide content in spot was performed under UV wavelength 254 nm. Standard androgapholide and *Andrographis paniculata* (Burm. f.) Ness extract were prepared in methanol solvent at concentration of 1 mg/ml and 4 mg/ml, respectively. Quantitative analysis was performed by measuring spot intensity in TLC scanner at wavelength of 230 nm. In the phytochemical analysis of *A. indica*

ethanolic extract, TLC method using stationary phase of silica gel 60 F254 and a mobile phase of *n*-butanol-acetic acid-water (4:1:5 v/v) was used. Rutin was applied as a marker compound and spot detection was performed under UV 254 and UV 366 nm. Quantitative analysis of rutin content in the extract was performed using spot scanning at wavelength of 364 nm.

Induction of hyperglycemia

The rats were induced by an intraperitoneal injection of alloxan monohydrate to achieve hyperglycemic condition at single dose of 150 mg/kgBW. Control group rats were treated with saline solution. Hyperglycemic condition was determined at 72 hours after induction by measuring preprandial and postprandial blood glucose levels. Rats with blood glucose level >216 mg/dL (>12 mmol/L) were categorized as diabetic condition and compared to blood glucose level of normal control group.

Experimental design

The rats were divided into several groups as follows:

- A. Group I (diabetic control) : alloxan-induced diabetic rats received vehicle solution 0.5% CMC-Na;
- B. Group II (positive control) : diabetic rats received oral hypoglycemic drug, glibenclamide dose 4.5 mg/kgBW, orally, once daily;
- C. Group III : diabetic rats received single *A. paniculata* extract 200 mg/kgBW orally, twice daily;
- D. Group IV : diabetic rats received single *A. indica* extract 200 mg/kgBW orally, twice daily;
- E. Group V : diabetic rats received herbal extract combination dose 200 mg/kgBW, orally, twice daily;
- F. Group VI : diabetic rats received herbal extract combination dose 400 mg/kgBW, orally, twice daily;
- G. Group VII : diabetic rats received herbal extract combination dose 800 mg/kgBW, orally, twice daily.

All treatments in each group were administered for 15 consecutive days and blood glucose levels, both preprandial and postprandial were measured at the day of 5, 10, and 15. The preprandial blood sample was collected from retro-orbital plexus after 12 hours fasting. Two hours after glucose administration of 1.75 gr/kgBW, blood was collected for determination of postprandial blood glucose level. Blood sample was incubated at room temperature for 30 minutes. Serum was separated from other blood component by centrifugation at 5000 rpm for 10 min at 25°C . The blood glucose level in the serum was analyzed spectrophotometrically by oxidase-peroxidase (GOD-PAP) method using biochemical diagnostic kit (DiaSys, Holzheim, Germany)

Statistical analysis

Blood glucose levels were presented as mean \pm SEM and percentage of hypoglycemic effect in each experimental group was calculated. The percentage of hypoglycemic effect was analyzed

statistically using ANOVA followed by LSD test. *P*-values less than 0.05 were considered significant.

RESULTS

Phytochemical analysis of *A. paniculata*

Based on phytochemical analysis using TLC-densitometry method, ethanolic extract of *A. paniculata* showed positive content of andrographolide (Fig. 1). Qualitative detection under UV 254 exhibited a same spot with standard andrographolide (hRf 53). Furthermore, quantitative analysis confirmed the content of andrographolide by 16.17% using TLC scanner at wavelength of 230 nm.

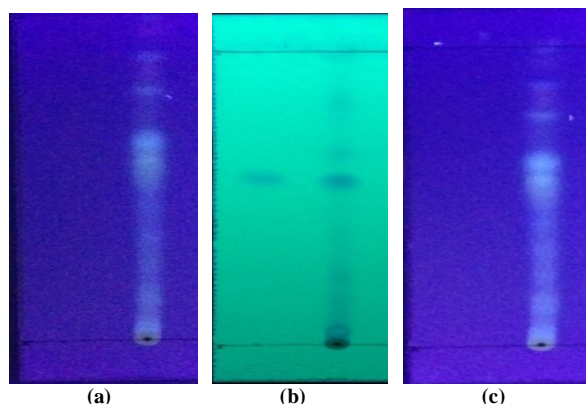


Fig. 1: TLC profile of ethanolic extract *Andrographis paniculata* (Burm. f.) Ness. TLC method was performed using a stationary phase of silica gel 60 F254 and a mobile phase of chloroform-ethyl acetate-methanol-acetic acid (7:2:0.5:0.5 v/v). Spot detection (a) under UV 366nm, (b) under UV 254 after AlCl_3 spraying, and (c) under UV 366 after AlCl_3 spraying

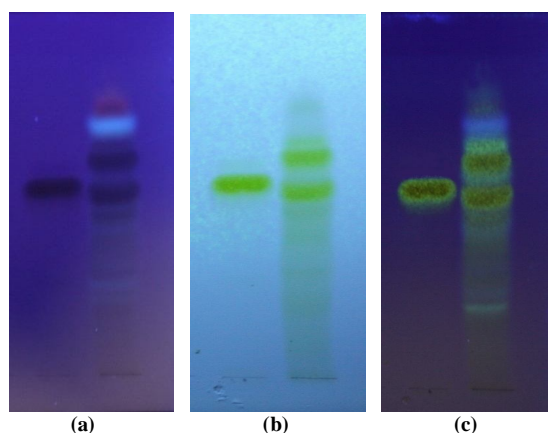


Fig. 2: TLC profile of ethanolic extract *Azadirachta indica* A. Juss. TLC method was performed using a stationary phase of silica gel 60 F254 and a mobile phase of n-butanol-acetic acid-water (4:1:5 v/v). Spot detection (a) under UV 366nm, (b) under UV 254 after AlCl_3 spraying, and (c) under UV 366 after AlCl_3 spraying.

Phytochemical analysis of *A. indica*

Phytochemical analysis of ethanolic extract of *A. indica* was performed using rutin. The content of standard compound was determined based on rutin concentration in *A. indica* extract (Fig. 2). Qualitative detection was observed under UV 254 and UV 366

and showed the presence of rutin in *A. indica* extract. Further analysis using AlCl_3 spray exhibited higher intensity of rutin spot that was detected under UV 254 and UV 366. Quantitative analysis of rutin concentration in extract using TLC scanner at wavelength of 364 nm resulted in rutin level of 2.86%

Effect on blood glucose levels

Both preprandial and postprandial blood glucose levels raised significantly after single dose administration of alloxan (150 mg/kgBW) intraperitoneally in rats. Determination of blood glucose level was performed at 72 hours after alloxan induction. Figure 3 shows that alloxan-induced group had higher blood glucose level in comparison to normal control group.

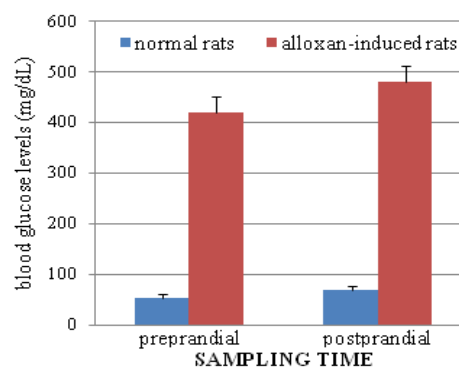


Fig. 3: Profile of preprandial and postprandial blood glucose levels in rats after alloxan induction in comparison to normal control group. Data represent mean \pm SEM, and are five to six independent experiments. **P*<0.05 compared to the control value.

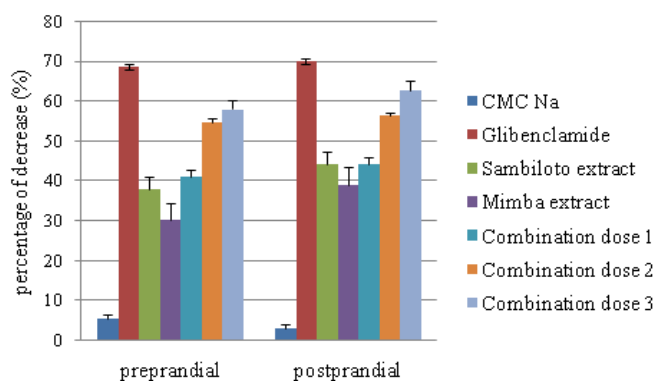


Fig. 4: Hypoglycemic activities (%) of all treatments. Dose 1-3 are 200, 400 and 800 mg/kg BW, respectively. Data represent mean \pm SEM, and are four to five independent experiments. **P*<0.05 compared to the control value.

As a control, there was no significant change in blood glucose level after treatment CMC-Na (fig 4). As shown in table 1-2 and figure 4, administration of combination of *A. paniculata* and *A. indica* for 15 days reduced preprandial and postprandial blood glucose levels in alloxan-induced diabetic rats. In comparison to single ethanolic extract of *A. paniculata* or *A. indica*, the combination exhibited higher activity to reduce blood glucose level. Treatment of diabetic rats with the combination at dose of 200 mg/kgBB demonstrated more reduced blood glucose level in comparison to single extract treatment.

Table 1: The effect of *Andrographis paniculata* (Burm. f.) Ness extract, *Azadirachta indica* A. Juss extract, their combination, and glibenclamide treatments on preprandial blood glucose level. Dose 1-3 are 200, 400 and 800 mg/kg BW, respectively.

Treatment	Blood glucose levels (mg/dL)				
	Baseline (before alloxan administration)	Observation time (after alloxan administration)			
		Day 0	Day 5	Day 10	Day 15
Glibenclamide	43.103 ± 2.31	468.315 ± 1.10	297.267 ± 6.69	229.234 ± 4.75	176.818 ± 3.87
<i>A. paniculata</i> extract	43.916 ± 2.06	468.919 ± 1.02	421.429 ± 9.26	361.613 ± 7.89	306.664 ± 6.45
<i>A. indica</i> extract	46.254 ± 2.10	270.148 ± 1.06	247.938 ± 1.07	223.341 ± 9.9	214.593 ± 8.91
Combination dose 1	28.802 ± 3.99	472.157 ± 8.84	363.156 ± 6.10	321.239 ± 6.03	290.833 ± 5.26
Combination dose 2	49.195 ± 2.17	306.061 ± 9.64	248.725 ± 9.20	205.419 ± 8.1	169.912 ± 5.35
Combination dose 3	54.988 ± 4.47	305.214 ± 8.93	220.734 ± 8.21	196.196 ± 6.81	163.698 ± 4.63

Table 2: The effect of *Andrographis paniculata* (Burm. f.) Ness extract, *Azadirachta indica* A. Juss extract, their combination, and glibenclamide treatments on postprandial blood glucose level. Dose 1-3 are 200, 400 and 800 mg/kg BW, respectively.

Treatment	Blood glucose levels (mg/dL)				
	Baseline (before alloxan administration)	Observation time (after alloxan administration)			
		Day 0	Day 5	Day 10	Day 15
Glibenclamide	70.353 ± 0.86	496.626 ± 1.04	351.104 ± 9.83	309.137 ± 8.73	199.583 ± 3.74
<i>A. paniculata</i> extract	74.975 ± 4.04	542.663 ± 1.06	445.458 ± 9.45	427.956 ± 9.22	337.945 ± 6.25
<i>A. indica</i> extract	70.947 ± 2.01	320.765 ± 1.19	274.407 ± 1.09	247.253 ± 1.05	250.453 ± 1.03
Combination dose 1	82.056 ± 2.38	523.689 ± 9.35	411.340 ± 6.63	368.205 ± 6.46	333.911 ± 5.71
Combination dose 2	74.834 ± 3.02	360.013 ± 1.03	286.535 ± 8.74	241.202 ± 7.41	211.270 ± 6.29
Combination dose 3	76.499 ± 3.57	322.470 ± 9.28	258.808 ± 9.67	224.196 ± 7.76	178.435 ± 5.14

The reduction of blood glucose level was enhanced as increased dose of combination treatment as shown in group VI (combination 400 mg/kgBW) and group VII (combination 800 mg/kgBW). At the highest combination dose, blood glucose level preprandial and postprandial was reduced by 58.121% and 62.839%, respectively. However, the effect of blood glucose reduction at highest combination administration was still lower than this of standard diabetic drug, glibenclamide, that reduced preprandial blood glucose by 68.782% and postprandial blood glucose by 70.25%.

DISCUSSION

Incidence of diabetes mellitus is associated with oxidative stress that leads to production of reactive oxygen species (ROS), peroxide, and disturbance of antioxidant enzyme (Baynes, 1991; Pari and Latta, 2005). Poor healthy lifestyle that followed by high exposure of oxidant compounds may induce metabolic disorder and stress, then unfortunately caused severe diabetes mellitus.

Alloxan (2,4,5,6-tetraoxypyrimidine; 2,4,5,6-pyrimidinetetrone) belongs to derivate compound of oxidized pyrimidine that often used as induction compound in diabetes model (Rohilla and Ali, 2012). It selectively destructs β cells of Langerhans islets. Inside the pancreatic β -cells, alloxan promotes production of superoxide radical and ROS that lead to DNA damage in these cells (Ebelt *et al.*, 2000; Szkudelski, 2001; Das *et al.*, 2012). Alloxan-induced DM resembles clinical DM that is caused by negative impact of high oxidant compound and producing free radical to damage β cells of Langerhans islets. In the study, by an intraperitoneal administration of alloxan monohydrate (150 mg/kgBW), rats developed diabetes likewise in patients as shown by increased preprandial and postprandial blood glucose levels (>100% in comparison to normal control).

Reportedly, *A. paniculata* exhibited potent antidiabetic activities in various diabetic conditions in rats (Zhang *et al.*, 2000;

Yu *et al.*, 2003; Nugroho *et al.*, 2012). Previously, this extract decreased the levels of blood glucose, triglyceride, and LDLin high-fat-fructose-fed rat. However, this extract did not influence the serum cholesterol and rat body weight (Nugroho *et al.*, 2012). Its aqueous leaf extract (400 mg/kg BW, po) also succeeded to decrease the blood glucose levels and increase the activity of superoxide dismutase (SOD) and catalase in STZ-induced diabetic rats (Dandu and Inamdar, 2009). Superoxide dismutase and catalase as well as glutathione peroxidase play a main role as antioxidant enzymes and can protect against free radicals that contribute in oxidative stress. The oxidative stress has a main role in development of diabetes complications (Giacco and Brownlee; 2010).

The antidiabetic activities of *A. paniculata* mainly contributed by its active compounds mainly andrographolide. In addition, andrographolide is a main constituent and abundance in this herb (Cheung *et al.*, 2001; Pholphana *et al.*, 2004). Andrographolide was reported to improve the uptake of glucose in isolated soleus muscle of STZ-diabetic rats. This compound could increase the mRNA and protein levels of GLUT4 (Yu *et al.*, 2003; Yu *et al.*, 2008). In the previous study, andrographolide was able to recover insulal Langerhans, increase density and morphology of β cell thus promote insulin expression that lead to lower blood glucose both preprandial and postprandial in STZ-induced diabetic neonatal rats (Nugroho *et al.*, 2014). In the study, the content of andrographolide in the ethanolic extract of *A. paniculata* was 16.17%.

A. indica was reported to show antidiabetic activities in several diabetic model in rats. The *A. indica* leaf extract succeeded to inhibit the generation of superoxide anion and hydroxyl free radical in diabetic rats (Shailey and Basir, 2012). The oxidative stress contributes in the pathogenesis of diabetes mellitus and its further complications (Shrivastava *et al.*, 2012). *A. indica* leaves extract also inhibited the activities of alpha-amylase and alpha-

glucosidase, enzymes having important role in glucose absorption (Kazeemet *et al.*, 2013).

Besides, active compounds of *A. indica* are also reported to reduce blood glucose levels. There are several active compounds of *A. indica* that are responsible for antidiabetic activity, such as quercetin, rutin, and nimbidin (Biswas *et al.*, 2004). Reportedly, rutin, a glycoside of flavonoid quercetin, was able to reverse islet pancreas morphology, oxidative, and glycemia in diabetic rats. Oral administration of rutin also lowered fasting blood glucose level, glycosylated hemoglobin as well as induced insulin, C-peptide, hemoglobin, and protein (Kamalakkannan and Prince, 2006). In the study, the ethanolic extract of *A. indica* contained rutin as phytochemical marker compound by 2.86%. Moreover, quercetin exhibited potent antioxidant that protects the body from diabetic complication induced by ROS (Lakhanpal and Rai, 2007; Nugroho *et al.*, 2013).

Each ethanolic extract used in this study possessed active compounds that were well-reported to reduce blood glucose level. Furthermore, herbal extract combination showed synergistic effect in lowering blood glucose level in alloxan-induced diabetic rats, even at 200 mg/kgBW (lowest dose in the study). However, the dose used to achieve the optimum effect was obtained at dose of 800 mg/kg BW. Therefore, the next step is to fractionate and purify the extracts so that the dose of the combination can be reduced. Combination of ethanolic extracts of *A. paniculata* and *A. indica* provides various anti-diabetic compounds. 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

In conclusion, combination of *Andrographis paniculata* (Burm. f.) Ness and *Azadirachta indica* A. Juss exhibited higher effect in lowering blood glucose levels, both preprandial and postprandial in comparison to single extract administration in alloxan-induced rat. In conclusion, the combination is potential to develop as an antidiabetic agent.

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