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Antidiabetic effect of combinations of n-hexane insoluble fraction of ethanolic extract of *Andrographis paniculata* with other traditional medicines

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Abstract

Excessive food consumption regarding to high calorie, obesity, cardiovascular disease, stress, and lack of exercise are risk factors for diabetes mellitus (DM). One alternative therapeutic approach in DM patients is traditional use of herbal medicines such as *Andrographis paniculata* herbs. The aim of the study was to evaluate the antidiabetic effect of a combination of *n*-hexane insoluble fraction of *A. paniculata* herb (HIFA) with other traditional medicines i.e. curcuminoids fraction of *Curcuma xanthorrhiza* rhizome (CFC) or ethyl acetate soluble fraction of propolis (ESP) in high-fructose-fat-fed rats, a model of type 2 diabetes mellitus (DM) rats. These effects were compared to that of the single treatment of HIFA. The DM rats were induced by high-fructose-fat diet containing 36% fructose, 15% lard, and 5% egg yolks in 0.36 g/200 gb.wt for 60 days. The drugs were administered for 10 days, and the blood glucose levels were determined with an enzymatic colorimetric method (GOD-PAP). In the study, all combination exhibited hypoglycaemic effect in the high-fructose-fat-fed rats. Combination of HIFA and CFC showed a higher hypoglycaemic effect than that of the single treatment of HIFA. However, the hypoglycaemic effect of combination of HIFA and ESP was not more effective than that of the single treatment of HIFA. The combination HIFA-CFC is potential to develop as an antidiabetic agent.

Introduction

Therapy of metabolic diseases such as Diabetes Mellitus (DM) takes long periods of time. The use of synthetic drugs are known to cause unwanted effects such as gastrointestinal disturbances, hypoglycaemia, liver disfunction (Rang *et al.*, 2003). Several traditional medicine have been studied and used for treating DM such as *Andrographis paniculata*, *Zingiber officinale*, *Centella asiatica* (Nugroho *et al*., 2012; Mahluji *et al.*, 2013; Nugroho *et al.*, 2013). Ethanolic extract of *A. paniculata* at doses of 500 and 1000 mg/kg BW succeeded to lower the fasting serum glucose levels in type 2 DM rats induced by streptozotosin and nicotinamide (Rammohan, 2010). Its *n*-hexane-insoluble fraction was reported to contain high level of its active compound, andrographolide. Reportedly, the compound exhibited potent hypoglycemic and hypolipidemic effects in high-fat-fructose-fed rat, and pancreatic actions in neonatal streptozotocin-induced diabetic rats (Nugroho *et al.*, 2012; Nugroho *et al.*, 2014). In traditional medicinal practice, combinations of herbal medicines have been used widely to achieve optimal therapeutic benefits. In the study, *n*-hexane-insoluble fraction of ethanolic extract of *A. paniculata* was combined with other traditional medicines in order to increase its antidiabetic effect. Those traditional medicines were curcuminoid fraction of *Curcuma xanthorrhiza* rhizome (CFC) and ethyl acetate soluble fraction of propolis (ESP).

Reportedly, curcuminoids (80 mg/kg BW) isolated from turmeric markedly reduced the blood glucose and increased the blood insulin levels significantly in type 2 DM rats. In addition, curcuminoids at dose of 45 mg/200 g BW suppressed the cholesterol levels (Purbowanti, 2006; Pari and Murugan, 2007). On the other hand, ethanolic extract of propolis at dose of 200 mg/kg BW exhibited a promising therapeutic effect in the prevention of DM and dyslipidemia (El-Sayed *et al*., 2009). In the study, *n*-hexane-insoluble fraction of ethanolic extract of *A. paniculata* was combined with these traditional medicines.

Materials and Methods

Materials

A. paniculata leaves were collected during June 2012 from Imogiri, Bantul, Yogyakarta, Indonesia. Meanwhile, *C. xanthorrhiza* rhizome were collected during July 2012 from Loano, Purworejo, Central Java. The plants were identified by a botanist at Department of Pharmaceutical Biology, Universitas Gadjah Mada, Indonesia. The voucher specimens were stored in the department. Propolis paste 96%

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with batch number 090810006 was obtained from PT. Harmony Dynamic Indonesia. Metformin were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Fructose were obtained from E. Merck, Darmstadt, Germany. Blood glucose levels were measured using a GOD-PAP colorimetric method (DiaSys, Diagnostic Systems GmbH, Holzheim, Germany).

Preparation of n-hexane insoluble fraction of A. paniculata leaves (HIFA)

The dried powdered *A. paniculata* leaves were extracted using 90% ethanol. The filtrate was collected and then evaporated under reduced pressure to provide a viscous ethanolic extract. Fractionation process was carried out by dissolving the viscous ethanolic extract of *A. paniculata* leaves in a *n*-hexane solution. Insoluble fraction of *n*-hexane were collected and then evaporated to get a viscous extract. Dose of HIFA required was equal to 58.55 mg/200 g BW.

Preparation of curcuminoid fraction of C. xanthorrhiza rhizome (CFC)

CFC dried powder was obtained from Prof. Dr. Suwijiyo Pramono. The powder intended for the preparation of suspension solution of CFC. The dose of CFC solution was prepared for the suspension of CFC. The dose used for the solution was 45 mg/200 g BW (Purbowanti, 2006).

Preparation of ethyl acetate soluble fraction of propolis (ESP)

Propolis was extracted using 50% ethanol. The filtrate was collected and then evaporated under reduced pressure to give of viscous ethanolic extract. The ethanolic extract of propolis was then fractionated using ethyl acetate. Fractionation process was conducted by dissolving the viscous ethanolic extract of propolis with ethyl acetate. Soluble fraction of ethyl acetate were collected and then evaporated to provide a viscous extract. The dose of ESPin the studywas 32.15 mg/200 g BW.

Analysis of HIFA

Qualitative analysis of HIFA was performed using a stationary phase of silica gel 60 F254 and a mobile phase of chloroform: methanol (9:1 v/v). The samples elucidated in the study included ethanolic extract of *A. paniculata* (EEA), *n*-hexane soluble fraction of ethanolic extract of *A. paniculata* (HSFA), and *n*-hexane insoluble fraction of ethanolic extract of *A. paniculata* (HIFA). Five milliliters of extracts/ fractionsand standard and rographolide were applied on the plate using a micropipette (5 mL), and then developed in the mobile phase with a distance of 8 cm. The spots were then observed under UV254, UV366, and visible lights. Subsequently, those spots were sprayed with Liebermann Burchard (LB) reagent to detect clusters diterpenes (Wagner and Bladt, 1984). The plat was then preheated at 105°C for 5-10 minutes and observed under UV366 light and visible lights. The spot areas were then measured with a TLC scanner at λ_{max} 232 nm.

Analysis of CFC

Qualitative analysis of CFC was performed using thin layer chromatography (TLC). CFC powder was dissolved in ethanol to the level of 1 mg/mL. The standard curcumin was dissolved in ethanol to obtain a concentration of 0.1 mg/mL. The solution (0.1 mL) was then applied on a plate of silica gel 60 F_{254}. Subsequently, the TLC plate was developed in a mobile phase of chloroform : methanol : glacial acetic acid (97:3:0.5 v/v) with a distance of 8 cm. The spotswere then observed under UV366 and visible lights. Quantitative analysis was performed by measuring the spot areas generated using TLC scanner at λ_{max} 423 nm.

Analysis of ESP

Qualitative analysis of ESP was performed by TLC. The tested samples included ethanolic extract of propolis (EEP), ESP, and ethyl acetate insoluble fraction of propolis (EIP). Each solution (0.1 mL) was applied on a stationary phase of silica gel 60 F_{254} using a micropipette. The TLC plate was then developed in a mobile phase of chloroform : methanol (9:1 v/v). Subsequently, the spots were then observed under UV254, UV366, and visible lights. The spots were then treated by ammonia vapor followed by sitroboratspraying. The spots were heated at a temperature of 105°C for 5-10 minutes and observed under UV_{366} and visible light. On the other hand, determination of total flavonoid content performed by spectrophotometric method (Chang *et al*., 2002).

Antidiabetic assay

Healthy adult male Wistar rats between the age of 2-3 months and weighing 150-200 g were used in the study. They were housed at a constant temperature (22 ± 2°C) with a constant relative humidity (55 ± 10%) on the an automatically controlled 12:12 h light-dark cycle (light on at 7:00 a.m.). They were fed with a standard laboratory food and water as libitum.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.

Hyperglycemia in the animal was induced by high-fructose fat containing 36% fructose, 15% lard, and 5% egg yolks in 0.36 g/200 g BW for 60 days (Nugroho *et al*., 2012). The drugs were administered for 10 days, and the blood glucose levels were determined with an enzymatic colorimetric method (GOD-PAP). The bloods were collected on days 0, 35, 60 and 75 by means of capillary tubes through retro-orbital plexus. The blood samples were allowed to stand at room temperature for 30 minutes, then centrifuged at 4000 rpm for 10 minutes to separate the serum. The serum was removed for determination of glucose levelsusing a GOD-PAP colorimetric method.

Statistical analysis

The results were expressed as mean ± SEM. The data were subjected to the one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test to compare more than two groups. While the unpaired or paired t test was used to compare the mean of two groups. P-values of less than 0.05 were considered significant.

Results and discussion

Analysis of HIFA

Based on the TLC results, both standard andrographolide and sample HIFA did not show any spotunder visible light (data not shown), however elucidation of EEA and HSFA exhibited green spots indicating presence of chlorophyll. Under UV254, elucidation of EEA and HIFA exhibited a spot of andrographolide with hRf value of 50 in comparison to standard and rographolide (Fig. 1a). However, elucidation of HSFA did not showed and rographolide spot. It indicates that the fraction did not contain and rographolide. Under UV366, elucidation of standard andrographolide did not show any spot, however elucidation of other test samples i.e. EEA, HSFA and HIFA exhibited some spots especially a spot with hRf of 30. Chlorophyll spot (hRf 95) was also detected in TLC plate under UV366. Those results indicate that those sample contains other chromophore compound groups. Those results indicate that those samples contains other chromophore compounds.

In the study, the TLC plate was then sprayed with Liebermann-Burchard reagent (LB) and heated at 105°C for 5-10 minutes. That reagent is used especially to detect diterpene lactones or triterpenes. Under visible light and UV366 observation, elucidation of EEA and HIFA exhibited a spot of andrographolide, and HIFA did not show chlorophyll spots.

The level of active metabolite compound

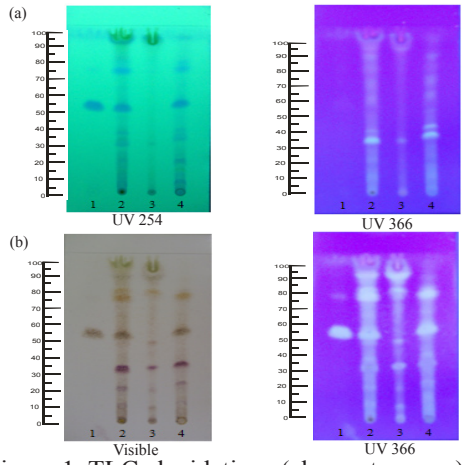

Figure 1. TLC elucidations (chromatograms) of EEA, HSFA, HIFA, and standard and rographolide on a stationary phase of silica gel 60 F_{254} and with a mobile phase of of chloroform: methanol (9:1 v/v), before (a) and after (b) Liebermann-Burchardspraying

Spot : 1:Standard andrographolide, 2:EEA, 3:HSFA, 4:HIFA

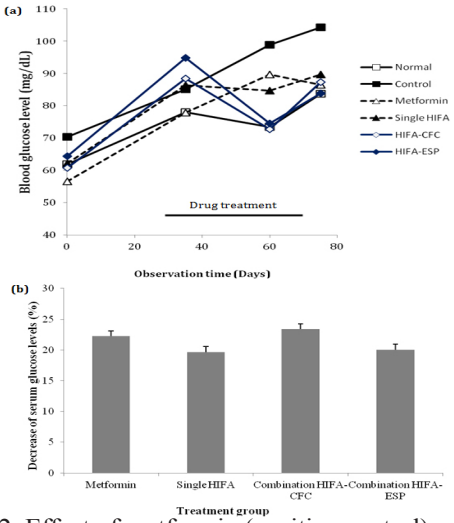

Figure 2. Effect of metformin (positive control), single treatment of HIFA, combination HIFA-CFC, and combination HIFA-ESF on blood glucose levels (mg/dL) in high-fructose-fat-fed rats (a), and the percentage of reduction in blood glucose levels (b).

in the extract was quantitatively detected by a TLC-densitometry method. TLC elucidation of consentration series of HIFA provided a brown spot of andrographolide. The spots areas were measured with a TLC scanner at λ_{max} 232 nm. In the study, the level of andrographolide in HIFA was about 10.27%.

Analysis of CFC

Based on the elucidation, TLC plate observation under visible light showed that there wasa spot at hRf 75 in curcumin and CFC elucidations (Fig. 3). In addition, observation under UV366 found a yellow spot at standard curcumin (hRf 75) and two yellow spots in CFC sample elucidation with hRf values of 52 and 75 indicating the presence of desmethoxy curcumin and curcumin, respectively. Quantitative analysis of curcumin levels in CFC was detected

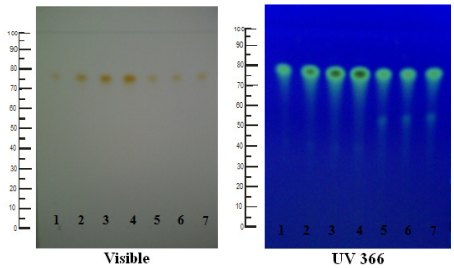

Figure 3. TLC profile (chromatograms) of CFC and standard curcumin on a stationary phase of silica gel 60 F_{254} and with a mobile phase of chloroform: methanol: glacial acetic acid (97:3:0.5 v/v).

Spot:1-4 : Standard curcumin, 5-7 : CFC

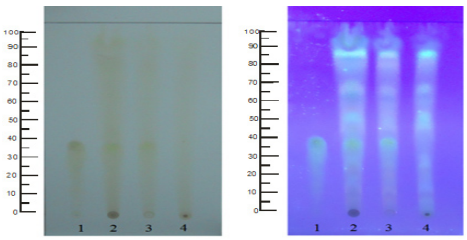

Figure 4. TLC profiles (chromatograms) of EEP, ESP, EIP and standard quercetin on a stationary phase of silica gel 60 F_{254} and with a mobile phase of of chloroform: methanol (9:1 v/v) before sitroborat spraying

by TLC-densitometry method (λ_{max} 423 nm). TLC elucidation of concentration series of CFC provided a yellow spot of curcumin at hRf of 75. In the study, curcumin level contained in CFC was 39.18%.

Analysis of ESP

Qualitative analysis was performed by TLC elucidation. Samples tested in the study included ethanolic extract of propolis (EEP), ESP, and ethyl acetate insoluble fraction of propolis (EIP). The TLC were observed under UV254, UV366, and visible lights, and treated with ammonia vapor then followed by a sitroborat reagent spray. TLC results in visible light showed yellow spots on the TLC profiles of quercetin, EEP and ESP with hRf of 38. Observations on the TLC under UV366 showed glowing yellow spots of quercetin, EES, and ESP at hRf value of 38 (Fig. 4). Elucidation of EIP did not show any spot of quercetin. The presence of flavonoids were detected after the TLC treated with ammonia vapor and sitroborat spray (Fig. 5). Quantitative analysis for determination of total flavonoid level in ESP used a spectrophotometer according to the method of Chang *et al*. (2002).The results showed that the level of total flavonoids contained in ESP sample was 88.71%. Effect on blood glucose levels

In the study, we used male Wistar rats (*Ratus norvegicus*) weighing 150 - 200 grams. The rats were divided into five groups i.e normal control, negative control, positive control, and two extract combinations groups. High fructose-fat diet feeding

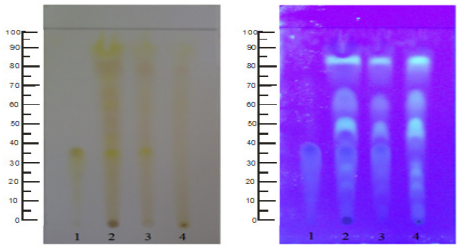

Figure 5. TLC profiles (chromatograms) of EEP, ESP, EIP and standard quercetin on a stationary phase of silica gel 60 F_{254} and with a mobile phase of of chloroform: methanol (9:1 v/v) after sitroborat spraying

Spot : 1:Standard quercetin, 2: EEP, 3:ESP, 4:EIP

was conducted from day 0 to day 75, except the normal control group. The drug were administrated on days 35 and 75. Blood samples of the rats were collected on day 0 as baseline, and then recollected on days 35, 60, and 75 for blood glucose levels. In the study, metformin (positive control), single treatment of HIFA and combination of extracts exhibited obviously hypoglycaemic effects in in high-fructose-fat-fed rats significantly. The average of the percentage reduction in blood glucose levels after treatment of metformin (positive control), single treatment of HIFA and combination of extracts (combination 1 and 2) were 22.24%, 19.67%, 23.38%, and 20,06%, respectively (Fig. 2a and 2b).

Andrographolide is an active compound of *A. paniculata* responsible for the blood glucose level-reducing activity. Andrographolide is able to reduce intestinal absorption of glucose and increase glucose utilization in the body (Yu *et al*., 2003). In muscle tissue, andrographolide can increase glucose uptake, mRNA and protein levels of GLUT4. In hepatocyte cells, andrographolide is able to activate α1-adrenoceptor to increase insulin secretion from β-cells Langerhans and lower the expression of phosphoenolpiruvat carboxykinase (PEPCK) involved the process of preprandial gluconeogenesis. Declination of glucose production will also lower the PEPCK action by hepatocyte cells (Yu *et al.*, 2008). Andrographolide also increases glucose utilization in skeletal muscle tissue. In addition, Andrographolide also able to stimulate GLUT4 translocation and protects β-cell mass of Langerhans (Zhang *et al*., 2009; Rammohan, 2010).

Curcumin in CFC also plays a role in the serum glucose reducing activity in insulin resistant rats. Curcumin is able to improve the regulation of denosin-monophospate-activated-protein kinase (AMPK) expression, which is a major cellular regulator of lipid and glucose mechanism by increasing the glucose utilization of skeletal muscle tissue (Na *et al*., 2011). The possible mechanism of blood glucose reduction activity of curcumin are by increasing the insulin

secreation from pancreatic β cells and by increasing glucose transport into peripheral tissues (Pari and Murugan, 2007).

Meanwhile, serum glucose-lowering activity of ESP is due to the antioxidative activity of the flavonoid compounds. Flavonoids and polyphenols in propolis are the main active compound possessing high antioxidant activity (Moreno *et al.*, 2000). Flavonoid compounds of ESP are mostly in their aglycone form, one of which is quercetion. This aglycone level is thought to be the main factor responsible of its serum glucose lowering activity in insulin resistant rats. Quercetin is a powerful antioxidants that is able to protect the body from reactive oxygen species (Lakhanpal and Rai, 2007). Oxidative stress can cause oxidative damage to cell membranes and alter the function and structure of subcellular organelles. Complications of diabetes mellitus can also be caused by oxidative stress. Flavonoids in propolis have an antioxidants activity and are thought to be the compound that is able to lower serum glucose levels through the inhibition of oxidative stress. In addition, the antioxidants activity of propolis can improve insulin receptor signaling in insulin resistant conditions, therefore the insulin sensitivity can be increased (El Sayed *et al*., 2009; Zhu *et al.*, 2010).

Conclusion

Combination of *n*-hexane insoluble fraction of *A. paniculata* (HIFA) and curcuminoid fraction of Curcuma xanthorrhiza rhizome exhibited a higher hypoglycaemic effect than this of single treatment of HIFA. The combination is potential to develop as an antidiabetic agent.

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