Antidiabetic effect of combination of fractionated-extracts of *Andrographis paniculata* and *Centella asiatica*: *In vitro* study

LOM. Fitrawan¹,⁵, R. Ariastuti¹, RR. Tjandrawinata², AE. Nugroho³, S. Pramono⁴

¹Master Program of Pharmaceutical Sciences of Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
²Dexa Laboratories of Biomolecular Sciences, Cikarang 17550, Indonesia
³Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
⁴Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
⁵Faculty of Pharmacy, Universitas Halu Oleo, Kendari 93232, Indonesia

**ABSTRACT**

**Objective:** To examine the effect of combination of *Andrographis paniculata* herb fraction (AHF) and *Centella asiatica* herb fraction (CHF) on PPARγ and GLUT4 mRNA expressions in 3T3-L1 adipocyte, and its effect on insulin-stimulated glucose uptake. **Methods:** 3T3-L1 adipocyte cells were used to investigate gene expression of PPARγ and GLUT4 proteins by reverse transcription-polymerase chain reaction method. The adipocyte cells were differentiated by using insulin, dexamethasone and 3-isobutyl-1-methylxanthine from 3T3-L1 cells. Pioglitazone, AHF, CHF and the combination of both herbs were evaluated on glucose uptake activity, PPARγ and GLUT4 mRNA expressions in 3T3-L1 adipocyte. **Results:** The results showed that combination of AHF at 30 μg/mL and CHF at 10 μg/mL could enhance insulin-stimulated glucose uptake. The combination also increased PPARγ and GLUT4 mRNA expressions significantly in comparison to those of negative control (DMSO). These effects were equal in comparison to those of pioglitazone (0.02 μM) and its single extracts. **Conclusions:** The combination of AHF and CHF can increase glucose uptake and insulin sensitivity through up-regulation of PPARγ and GLUT4 mRNA expressions in 3T3-L1 adipocyte.

1. Introduction

By 2015, there are about 415 millions people living with diabetes mellitus (DM) in the world. In the last three decades, the prevalence of type 2 DM has increased dramatically in developed countries[1]. Nowadays, the treatment of DM continues to be developed by using either single or combination drugs, however, the side effects can harm patient. This issue leads to more and more herbal medicine researches which continued to minimize the...
side effects of drugs. One of the potential herbs to be developed is *Andrographis paniculata* (*A. paniculata*) (Burm. F) Nees. The herbs are widely found in Bangladesh, China, India, Pakistan, Philippines, Indonesia, Malaysia and Thailand[2]. Its main constituent, namely andrographolide, was reported to have the ability to reduce blood glucose levels significantly in type 2 diabetic mice induced with streptozotocin[3,4]. Moreover, the flavonoid-enriched fraction of *A. paniculata* was reported recently to possess its ability to increase GLUT-4 translocation in high fructose fat-fed rats[5]. Another potential herb is *Centella asiatica* (*C. asiatica*) (L.) Urban which is mostly spread in Asia such as India, Sri Lanka, Indonesia, Malaysia and Vietnam[6]. It is reported that the ethanolic and methanolic extracts of *C. asiatica* leaves have their hypoglycemic effect on alloxan induced diabetic rats[7]. The individual effects of *A. paniculata* and *C. asiatica* have been studied that they can reduce blood glucose levels. However, there is no study of the combination of the two plants. It is unclear whether the combination of both fractionated extracts has synergistic effect in enhancing glucose uptake and insulin sensitivity mediated by peroxisome proliferator-activated receptor- γ (PPAR γ) and glucose transporter 4 (GLUT4) expressions. Therefore, our study focused on the mechanism of the combination of fractionated extracts of *A. paniculata* and *C. asiatica* herbs in enhancing glucose uptake and increasing insulin sensitivity by up-regulation of PPAR γ and GLUT4 mRNA.

### 2. Materials and methods

#### 2.1. Material

Pioglitazone was obtained from Sigma Chemical Co. (St. Louis, MO, USA), whereas high glucose of Dulbecco’s Modified Eagle’s Medium (DMEM), bovine serum, fetal bovine serum, and Penicillin-Streptomycin (P/S) were purchased from Gibco (Gaithersburg, USA). Oil red O, insulin, dexamethasone, 3-isobutyl-1-methylxanthine, and antrons were procured from Sigma Aldrich (St. Louis, MO, USA). TRI-zole and the primer were obtained from Invitrogen (CA, USA). Dimethyl sulfoxide (DMSO), 2-propanol, d-glucose, and Lieberman-Burchard reagent were obtained from Merck (Whitehouse Station, NJ). Reverse Transcript-PCR Kit was obtained from TOYOBO (Tokyo, Japan). Nuklease Free Water and GoTaq Green master mix were obtained from Promega (Madison, WI).

#### 2.2. Preparation of fractionated extract of *A. paniculata* herbs

The herbs of *A. paniculata* were collected during April 2017 from Kulonprogo, Special Region of Yogyakarta, Indonesia. The herbs were then dried, powdered and stored in an airtight container. Preparation of fractionated extract was based on the previous method with slight modification[8]. Briefly, the powder of *A. paniculata* was extracted with 95% (v/v) ethanol overnight using maceration method. After two times of re-extractions, collected extract was evaporated under reduced pressure to provide a viscous extract. The viscous ethanolic extract was fractionated with n-hexane and the insoluble fraction was further fractionated with ethyl acetate. The last fraction was named as a fractionated extract of *A. paniculata* herb (AHF).

Five microliters of AHF was applied on a precoated silica gel aluminium plate 60F254 (20 cm × 10 cm; E. Merck, Germany). Chloroform:methanol (9:1) was used as mobile phase. Densitometric scanning was performed triplicate on Camag TLC Scanner 3 at wave length of 230 nm. The andrographolide content was calculated by using calibration curve.

#### 2.3. Preparation of fractionated extract of *C. asiatica* herbs

Collection of *C. asiatica* herbs was done during April 2017 in Wonosari, Special Region of Yogyakarta, Indonesia. The herbs were then dried, powdered and stored in an airtight container. Preparation of fractionated extract of *C. asiatica* was similar to that of *A. paniculata* herb. However, fractionation step with ethyl acetate was not performed, only using n-hexane. The n-hexane-insoluble fraction was named as a fractionated extract of *C. asiatica* herb (CHF).

Briefly, five microliters of CHF were applied on a precoated silica gel aluminium plate 60F254 (20 cm × 10 cm; E. Merck, Germany). The mobile phase consisted of n-butanol: acetic acid: water (3:1:1). To visualize the spots, the plates were sprayed with the Lieberman–Burchard reagent and heated for 10 min at 110 °C. Densitometric scanning was perfomed triplicate on Camag TLC Scanner 3 at wave length mode of 575 nm, operated by CATS software (V1.2.6, Camag). The asiaticoside content was calculated by using calibration curve.

#### 2.4. Extracts administration on cells

AHF and CHF were dissolved in DMSO. Subsequent variations of concentrations of the extracts was provided for the treated cells.

#### 2.5. Cells culture

3T3-L1 fibroblast cells were procured from American Type Culture Collection (Manassas, VA). 3T3-L1 fibroblast cells were cultured in high glucose DMEM supplemented with 10% bovine serum, 1% penicillin/streptomycin and incubated at 37 °C in a condition of humidified atmosphere of 5% CO₂. Cells were then subcultured...
every 2-3 days at confluency of approximately 90%.

2.6. Adipocyte cells differentiation

Differentiation of 3T3-L1 fibroblast to adipocytes in 100 mm culture dishes was performed according to the previous method described by Tjandrawinata et al[9]. Briefly, 3T3-L1 fibroblast cells post 90% confluence, were differentiated via incubation in a medium of high glucose DMEM supplemented with 10% fetal bovine serum, 0.25 μM dexamethasone, 0.25 mM 3-isobutyl-1-methylxanthine and 1 μg/mL insulin, at about 5 × 10⁵ cells/mL in dish for 3 d. On day 3, the medium was changed to a new high glucose DMEM supplemented with 10% fetal bovine serum and 1 μg/mL insulin. Thereafter, cells were maintained by changing the medium every 2-3 days gently. Cell differentiation was visually observed using microscope. The determination of the conditions of cells that have been differentiated was when the cell showed lipid droplets with larger cell size in comparison to that of 3T3-L1 pre-adipocytes.

2.7. Oil red O staining of 3T3–L1 cells

Accumulation of lipids that occurs in differentiated adipocytes can be evaluated by staining neutral fats using the dye oil red O. Adipocytes were fixed in 10% formalin for 1 h or longer. The cells were washed with 60% isopropanol, and the wells were kept until complete dry. The cells were then incubated with oil red O for 10 min. Subsequently, the cells were washed with double distilled H₂O and lipid droplets were visually observed with microscope. The coloured lipid was quantified by adding isopropanol on the cell and measured at 500 nm.

2.8. Induction of insulin resistance

To establish an insulin resistance, adipocyte cells were treated with high level of glucose and insulin for 10 d or more in DMEM medium containing 10% of fetal bovine serum. Incubation was conditioned at 37 °C, 5% of CO₂ and 95% of humidity. Cell medium was replaced every two to three days. Determination of insulin resistance conditions was done by measurement of glucose uptake and GLUT4 expression.

2.9. Analysis of PPARγ and GLUT4 mRNA expressions

RNA Total was extracted from cells by using TRIzol (Invitrogen) reagent according to the manufacturer’s protocol. RNA concentrations were quantified by using NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, MA, USA). The concentration and purity level of RNA (A260: A280) were determined based on the optical density. Preparation of cDNA from 2 μg total RNA was performed by using ReverTra Ace® qPCR RT Master Mix (Toyobo). The reaction was proceeded at 37 °C for 15 min, and then heated to 98 °C. The result of reverse transcription process, cDNA, was stored at -20 °C before further treatment.

Gene expression analysis was carried out at mRNA level. Polymerase chain reaction (PCR) was performed to amplify the specific gene by using primer oligonucleotide which is designed with Primer 3 software as described previously[9]. Gene sequences are derived from genes baseline data. The genes involved in insulin signal transduction (shown in Table 1) were then detected. The gene detection was firstly performed by amplification in mixture of 12.5 μL GoTaq Green master mix, a pair of target genes with the final concentration in 1 μM, a pair of internal controls with final concentration in 0.2 μM, 3 μL cDNA, and nucle free water to total in 25 μL. In general, the PCR process consisted of initial denaturation at 95 °C for 3 min, 30-35 cycles denaturation at 95 °C for 30 s, primer installation at 58-60 °C for 30 s, and elongation at 72 °C for 10 min. Reverse transcription-PCR was performed by using PCR T3000 Thermocycler (Biometra®) machine. Target gene expression was quantified by using Quantity One software. The arbitrary unit was measured by comparing target gene expression with internal control expression using Image J Imanging system software (v 1.24; National Institutes of Health, Bethesda, MD).

### Table 1

| Primer | Forward sequence | Reverse sequence |
|-------|-----------------|-----------------|
| PPARγ | TTCTGAGGTGCCAGTTTC | ACTCTTGGCAACAGGCTCT | |
| GLUT4 | AATGAGACTGATGCGCTCT | TGAGCTGCGTTTACACCCT | |

Abbreviations: PCR, polymerase chain reaction; PPAR γ, peroxisome proliferator-activated receptor gamma; GLUT4, glucose transporter 4.

2.10. Test of glucose uptake activity in vitro

Glucose uptake assay method was performed according to previous method[8]. Glucose uptake assay was performed on both normal and pre-treated adipocyte cells with insulin resistance. Measurement of glucose level was done by calculating the reaction of glucose with antrons in concentrated sulfuric acid. The cells were washed with PBS three times. The cells were incubated in DMEM medium containing 300 μg/mL glucose, with or without 100 nM insulin at 37 °C for 180 min. Subsequently, the cells were then treated with pioglitazone (0.02 μM), AHI, CHF, combination of both fractionated extracts. Combination of fractionated extracts to be tested was one having the biggest effect on PPARγ and GLUT4 expression. During the incubation, the cell would uptake glucose. By determining the glucose level in the medium, the levels of intracellular glucose were
2.11. Statistical analysis

Statistical analyses were performed by using IBM SPSS Statistic for Macintosh version 24, while Shapira Wilk was used to check normality of data. Three independent groups were compared by Kruskal-Wallis and two independent groups were compared by Mann-Whitney with significant levels at $P<0.05$.

3. Results

3.1. Determination of andrographolide and asiaticoside contents in AHF and CHF

The levels of andrographolide contained in the AHF and asiaticoside contained in the CHF were determined by TLC-densitometer using the standard curve equation. In the study, the levels of andrographolide in AHF and asiaticoside in CHF were 17.41% and 11.34%, respectively.

3.2. Expression of PPAR $\gamma$ during the differentiation of 3T3-L1 adipocytes

After being mature adipocytes, we observed the morphology of pre-adipocyte and adipocyte cells and stained them with oil red O. Lipid droplet formation was observed in adipocyte cells markedly (Figure 1). Lipid accumulation was quantified at 500 nm. The increase of lipid accumulation reached 4.8 fold in comparison to that of pre-adipocyte cells. The expression of PPAR $\gamma$ mRNA level was identified to ensure the formation of adipocytes. The quantification results showed that increase of PPAR $\gamma$ expression was 1.3 fold in comparison to that of preadipocyte cells.

3.3. Effects of AHF and CHF combination on PPAR $\gamma$ and GLUT4 expression in insulin-resistant adipocytes

In this study, we evaluated the effect of AHF and CHF combination administered on insulin resistant 3T3-L1 adipocyte cells. Expressions of PPAR $\gamma$ and GLUT4 mRNA level showed up-regulation after cells were exposed with pioglitazone (0.02 μM), AHF (30 μg/mL), CHF (10 μg/mL), and the combination of both in comparison to control (Figure 2). Moreover, AHF and CHF combination at (30 + 10) μg/mL exhibited higher effect than those of its single fractionated extracts and other concentrations of combinations, so this combination was further used in the determination of glucose uptake.

In PPAR $\gamma$ gene expression assay, there was an increase of regulation at the mRNA level after treatment of substances. Single administration of pioglitazone (0.02 μM) and 30 μg/mL of AHF exhibited 1.25 fold increase in comparison to that of control, while treatment of 10 μg/mL of CHF showed 1.18 fold increase. The treatment of AHF and CHF combination with various combinations: (15 + 5), (30 + 10) and (10 + 10) μg/mL succeeded to increase 1.16 fold, 1.35 fold, and 1.17 fold in comparison to that of control. Statistical analysis with Kruskal-Wallis test followed by Mann-Whitney test, showed that the effects of treated groups were significant different ($P<0.05$) compared with control. The combination of AHF and CHF at (30 + 10) μg/mL showed the biggest effect.

In the GLUT4 gene expression assay, there was an up-regulation at the mRNA level of the cell after treatment with the substances. Administration of pioglitazone (0.02 μM) showed 1.29 fold increase compared with that of control. The treatment of 30 μg/mL of AHF and 10 μg/mL of CHF exhibited 1.20 fold and 1.17 fold increase respectively in comparison to that of control. The treatment of combination of AHF and CHF at the concentrations of (30 + 10) μg/mL, (15 + 5) μg/mL, (10 + 10) μg/mL increased the gene expression respectively by 1.41, 1.31 and 1.14 fold. Statistical analysis with Kruskal-Wallis test followed by Mann-Whitney test showed that the effects of treated groups were significant different ($P<0.05$) compared with control (Figure 2). The combination of AHF and CHF at (30 + 10) μg/mL showed the biggest effect, so this combination was used as a sample to be tested on glucose uptake study.

3.4. Effect on glucose uptake in insulin resistant adipose

In the study, the determination of intracellular glucose uptake was performed to evaluate the effect of substances as well as pioglitazone in 3T3-L1 adipocytes cells, a cell culture model of insulin resistance. A series of concentrations of CHF and AHF that were used in this study exhibited no cytotoxicity against the 3T3-
L1 cells (data not shown). This assay was performed for 180 min and the glucose uptake at 30 and 180 min after administration of the substances on insulin resistance adipocytes were compared. Glucose uptake capacity increased over time. At minute 30, CHF showed 33.34% glucose uptake, while AHF showed 35.11% but the effect of substances on the glucose uptake was not observed significantly. However at 180 min, 0.02 μM of pioglitazone increased glucose uptake by 6.75% in comparison to that of control. Treatment with 30 μg/mL of AHF and 10 μg/mL of CHF showed 8.09% and 6.19% of increase respectively in comparison to that of control. Treatment with combination of AHF and CHF [(30 + 10) μg/mL] exhibited 10.18% of increase compared with that of control (Figure 3). The combination of AHF and CHF had potential effect to induce glucose uptake.

**Figure 2.** PPARγ and GLUT4 mRNA level after treatment of the test sample to adipocyte cells (mean ± SD, n=3).

The results are different significantly (P<0.05) compared to the control group.

**Figure 3.** Glucose uptake profile for 180 min after treatment of sample on insulin resistant 3T3-L1 cells.

The results are different significantly (P<0.05) compared to the control group.

4. Discussion

The present study provided information that may be useful to support the exploration of medicinal plants. This effort has an important role for the discovery and development of drugs. The exploration of medicinal plant involved pharmacological test, toxicological test, formulation, phytochemical study, isolation of active compounds etc. To improve the pharmacological activities, some traditional medicine can be combined with other substances[10,11]. Nowadays, the exploration of medicinal plants for the development of antihypertensive, anti-allergy, anticancer and antidiabetic drugs either as a single agent or a combination is increasing[12].

In the study, AHF and CHF were combined and could increase glucose uptake and insulin sensitivity through up-regulation of PPARγ and GLUT4 mRNA levels. The aim of gradual fractionation process in an extract was to provide a high level of active compound-containing extract. In this study, we focused on fractionated extracts of A. paniculata and C. asiatica herbs. Combination of these fractionated extracts indicated antidiabetes that was performed using an in vitro model, a cell culture of insulin resistance, with the aim to optimize the need for screening and determine its effectiveness. This study was performed based on the fact that extracts of these plants were widely used as a medicinal plant, but had lack of scientific evidence regarding to its efficacy.

A. paniculata is a herb that has been empirically used for curing diabetes, dysentery, fever, malaria, inflammation, cancer and as immunostimulant. This plant contains an active compound namely andrographolide. Extract of A. paniculata and andrographolide exhibited hypoglycemic and hypolipidemic effects in diabetic type 2 DM-resistant insulin induced with fructose fat diet[8], while C. asiatica inhibited glucose uptake by impairing disaccharides and α-amylase and by binding of glucose fiber. It can also lower low-density lipoprotein and cholesterol levels, and increase high-density lipoprotein[13].

Thiazolidinediones are a insulin-sensitizing drug. Pioglitazone and rosiglitazone are two widely used drugs in type 2 DM. PPARγ is the molecular target for thiazolidinedione class of antidiabetic drugs. They can increase insulin sensitivity and glucose tolerance. Thiazolidinediones decrease insulin resistance by action as an agonist of PPARγ, a nuclear hormone receptor. PPARγ activation regulates transcription of several genes that are responsible for insulin, in turn can improve the insulin sensitivity in adipocytes tissue, liver and skeletal muscle[14].

Possible mechanisms that cause insulin resistance are reduction mechanism of regulation (lowering amount of insulin), deficiency or genetic polymorphisms of phosphorylation tyrosine insulin receptor, IRS or P3K protein, decreasing translocation of GLUT4 or abnormality function of GLUT4. Decrease of glucose oxidation can cause alteration on glucose uptake, which cannot enter into the cell, subsequently causing hyperglycemia[15]. Insulin resistance is one indication of type 2 DM. Cells occur insulin resistance if there is a significant alteration on genes involved in insulin signal transduction, and one important gene is glucose transporter GLUT4. Expression of GLUT4 will be down-regulated in insulin-resistant cells.

By this study, we can evaluate the antidiabetic mechanisms of A.
paniculata and C. asiatica combination by using adipocyte 3T3-L1 cells. We show that the combination of these two purified extracts can improve glucose uptake and insulin sensitivity by up-regulation of PPAR-γ and GLUT-4 mRNA levels.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgements

We thank to Dexa Laboratories of Biomolecular Sciences for provision of research facilities. Great appreciation is also addressed to the Ministry of Research and Technology and Higher Education of the Republic of Indonesia (RISTEKDIKTI) through “Hibah Unggulan Perguruan Tinggi 2017” for financial support of the research.

Funding

This research is supported by the Ministry of Research and Technology and Higher Education of the Republic of Indonesia (RISTEKDIKTI) through “Hibah Unggulan Perguruan Tinggi 2017”.

References

[1] WHO. WHO diabetes programme [WWW Document]. 2016. [Online]. Available from: http://www.who.int/diabetes/en/ [Accessed on April, 2016].

[2] Hossain MS, Urbi Z, Sule A, Rahman KM. Andrographis paniculata (Burm. f.) Wall. Ex Nees: A review of ethnobotany, phytochemistry and pharmacology. Sci World J 2014: 2014: 274905.

[3] Nugroho AE, Sari KRP, Sunarwindi AL. Blood glucose reduction by combination of Andrographis paniculata (Burm.f.) Ness herbs and Asafoetida indica A. Juss leaves in alloxan induced diabetes rats. J Appl Pharm Sci 2014; 4(9): 30-35.

[4] Subramoniam A. Plants with anti-diabetes mellitus properties. USA: CRC Press; 2016.

[5] Rachmani EPN, Pramono S, Nugroho AE. Effect of flavonoid-enriched fraction of Andrographis paniculata (Burm. f.) Ness increase GLUT-4 translocation in high fructose fat-fed rats. Int J Current Innov Res 2018; 4: 1152-1155.

[6] Suryakala G, Archana G. Genetic transformation of Centella asiatica by Agrobacterium rhizogenes. J Pharmacogn 2012; 3(2): 82-84.

[7] Chauhan PK, Pandey IP, Dhatwalla VK. Evaluation of anti-diabetic effect of ethanolic and methanolic extracts of Centella asiatica leaves extract on alloxan induced diabetic rats. Adv Biol Res 2010; 4: 27-30.

[8] Nugroho AE, Lindawati NY, Herfyanti K, Widyastuti L, Pramono S. Antidiabetic effect of a combination of andrographolide-enriched extract of Andrographis paniculata (Burm. f.) Ness and asiaticoside-enriched extract of Centella asiatica L. in high fructose-fat rats. Indian J Exp Biol 2013; 51(12): 1101-1108.

[9] Tjandrawinata R, Wulan Nailufar F, Sinambela J, Tandrasasmita O. Glucose lowering effect of DLBS3233 is mediated through phosphorylation of tyrosine and upregulation of PPAR-γ and GLUT-4 expression. Int J Gen Med 2011; 4: 345-357.

[10] Nugroho AE, Kusumawardani G, Widyaninggar A, Prasetyo Anggor D, Pramono S. Antidiabetic effect of combination of n-hexane insoluble fraction of ethanolic extract of Andrographis paniculata with other traditional medicines. Int Food Res J 2014; 21(2): 785-789.

[11] Febriansah R, Dyaningtyas DP, Sarmoko, Nurulita NA, Meiyanto E, Nugroho AE. Hesperidin as preventive resistance agent in MCF-7 breast cancer cells line resistance to doxorubicin. Asian Pac J Trop Biomed 2014; 4(3): 228-233.

[12] Harwoko, Pramono S, Nugroho AE. Triterpenoid rich fraction of Centella asiatica leaves and in vivo antihypertensive activity. Int Food Res J 2014; 21(1): 149-154.

[13] Kabir AU, Samad MB, D’Costa NM, Akhter F, Ahmed A, Hanan JMA. Anti-hyperglycemic activity of Centella asiatica is partly mediated by carbohydrate inhibition and glucose-fiber binding. BMC Complement Altern Med 2014; 14: 31.

[14] Whalen K. Lippincott illustrated reviews: Pharmacology. Gainsville, Florida: Wolters Kluwer; 2015, p. 335-346.

[15] Cavan D, Fernandes JR, Makaroff L, Ogurtsova K, Webber S. Diabetes atlas. 7th edition. Brussels: International Diabetes Federation; 2015.