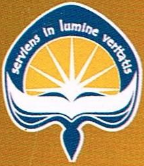

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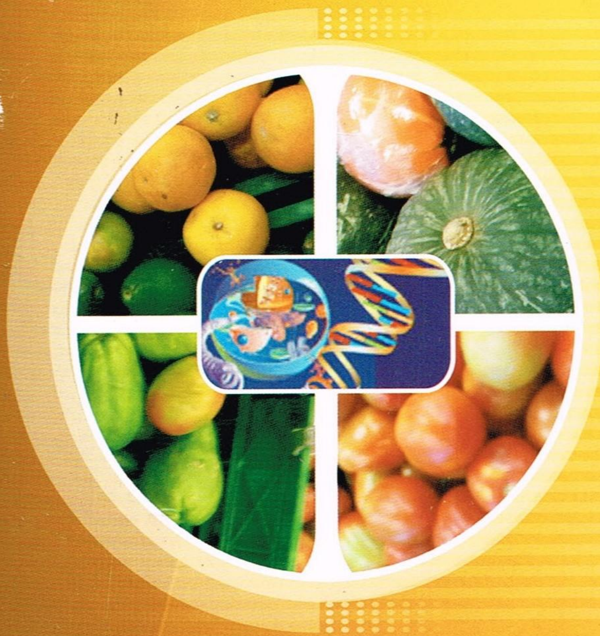

1st International Seminar on

"Natural Resources Biotechnology: From Local to Global"

September 8th – 9th 2015
Faculty of Biotechnology
Universitas Atma Jaya Yogyakarta

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Mixture of Sambiloto (*Andrographis panniculata* Nees.) and Salam (*Syzygium polyanthum* (Wight.) Walp.) Extract to Improve GLUT4 and PPAR-γ Expression in Hyperglicemic Wistar Rats

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Abstract

The increased prevalence of diabetes mellitus stimulates the ongoing study of molecular mechanisms of sambiloto (*Andrographis panniculata* Nees.) and salam (*Syzygium polyanthum* (Wight) Walp.) in relieving the disorder. The molecular study was done by treating hyperglicemia Wistar rats with a combination of sambiloto (AP) and salam (SP) extract. After 14 days of treatment, the rats were sacrificed and the liver organs were collected. The organs were stored in a solution of 10% buffered formalin to be blocked in paraffin afterwards. Subsequent molecular test was performed to identify GLUT4 and PPARγ gene expression by immunohistochemistry method. This study showed that administration of a combination of sambiloto and salam extracts (6:1) in rats was able to increase the expression level of GLUT4 which resembled the expression level of GLUT4 after insulin administration. It was also found that the expression level of PPARγ was increased after rats were treated by combination of AP and SP extracts (6:1). It showed that the increased expression of PPARγ resembles the expression of PPARγ after metformin stimulation. Interestingly, the administration of AP:SP extract (6:1) combination showed a level of GLUT4 expression that was not in line with PPARγ expression.

1. INTRODUCTION

The prevalence of diabetes mellitus (DM) that remains increased stimulates the ongoing study of molecular mechanisms of sambiloto and salam in relieving the disorder. In hyperglycemic condition, ROS (reactive oxygen species) from mitochondria electron transport was occurred. If this condition persisted, cellular organelles may be damaged and enzymes activities may also be disturbed. Thus, increased lipid peroxydation and insulin resistance may ultimately be stimulated. Increased level of ROS can be diminished by induction of PPAR-γ coactivator, PGC-1α (PPAR Gamma Coactivator-1a), MnSOD (manganese superoxide dismutase), and activation of AMPK (adenosine monophosphate activated protein kinase). The mechanism of action is carried out by metformin and pioglitazone. The thiazolidinedione (TZD) is another oral antidiabetic agent that acts as Peroxisome Proliferator Activated Receptorγ (PPARγ) agonis to stimulate insulin sensitivity in cells (Braissant *et al.*, 1996). Increase level of PPARγ may activate GLUT4, the gene

that plays role in regulating transport of glucose to the adipose tissue and skeletal muscle, as a response of increase insulin level in the blood (Watson *et al.*, 2004). It was mentioned that both sambiloto and salam are effective in reducing blood glucose level (Widjajakusuma *et al.*, 2010). Therefore, this molecular study was conducted to explore the efficacy of a mixture of sambiloto and salam extract in increasing PPARγ and GLUT4 in hyperglycemic Wistar rats.

2. METHODS

2.1. Preparation of Extracts

Fresh Andrographis paniculata Nees. herbs (AP) and Syzygium polyanthum Wight. (Walp.) (SP) leaves that obtained from Pandaan, East Java were cleaned, cut, dryed, and water extracted (Kaneria *et al.*, 2012). The water extracts was spray dried in order to obtain the dried extracts.

2.2. General Procedures

Healthy Wistar rats that were aged 2-3 months, weighted 200 – 250 g, blood glucose level of 65-100 mg/dl. The rats were randomly grouped into 8 groups of 6, namely: negative control group (K I); normal control group (K II); positive control group I (K III) – insulin; positive control group II (K IV) - metformin 9 mg/kg BW; treatment group I (K V) - AP extract; treatment group II (K VI) - SP extract; treatment group III - mixture of AP:SP extracts (6:1); treatment group IV (K VIII) – AP:SP mixture extract (2:1); treatment group V (K IX) – AP:SP mixture extract (1:2); treatment group VI (K X) – AP:SP mixture extract (1:6). Dose of extract was 200 mg/kg BW. Prior to the treatment, rats were administered by monohidrate alloxan 150 mg/kg BW through intraperitoneal route in order to reach the hyperglycemic condition (blood glucose level of 300-400 mg/dl). When hyperglycemic condition was achieved, mixture of *AP and SP* extracts was given for 14 days. After 14 days of treatment, the hyperglycemic rats were sacrificed and the liver was collected. The organs were stored in a solution of 10% buffered formalin to be blocked in paraffin afterwards.

2.3. Immunohistochemistry of GLUT 4 and PPARγ

The rats livers were paraffine blocked and sliced into 3-4 µm and incubated overnight 450 °C, then deparaffined with xylene. Subsequently, washed with PBS (phosphate buffer saline) and incubated in 3 % hydrogen peroxide (H_2O_2 in methanol). The antigen retrieval was conducted by incubating the slides in the citrate buffer pH 6. The 0,1% protease was added in 37 °C, digested with 100 µg/ml proteinase K in buffer (0,01 mol/l trish HCl pH 7,8 0,005 mol/l EDTA dan 0,5 % SDS) and incubated in mouse serum. Subsequently, the normal mouse serum was cleaned by primer antibody (1 : 50) of IgG1 anti GLUT 4 and PPARγ monoclonal antibody, in separate. Afterwards, incubation with secondary antibody IgG goat anti rat was conducted, streptavidin-peroxidase was dropped, and incubated in DAB for 5-15 minutes. Counterstain with hematoxylin eosin and rehidration were conducted with addition of absolute ethanol, 95% ethanol, 80% ethanol and xylol. The medium mounting was conducted lastly by adding gliserol gelatin prior to closing by deck glass.

3. RESULTS AND DISCUSSION

3.1. GLUT 4 Expression

This study showed that administration of AP and SP mixture extracts (6:1) in rats was able to increase the expression level of GLUT4 (figure 1 in brown) which resembled the expression level of GLUT4 after insulin administration (table 1). This study indicated that the mixture extracts were able to stimulate glucose transport to the cells by the high level of GLUT4 expression similar as insulin stimulation. This result is in line to the study of Widjayakusuma *et al.,* 2010 who reported the reduction of blood glucose level in rats after administrtion with mixture extracts of Ap and Sp.

Table 1. GLUT4 Expression (n=6) GLUT4 Expression in the liver of hyperglycemic Wistar Rats

	Groups	Means±SD
K I	Negative control	179±11
K II	Normal control	51±18
K III	Insulin	231±15
K IV	Metformin	54±20
K V	Ap (*Andrographis paniculata*)	141±23
K VI	Sp (*Syzygium polyanthum*)	151±23
K VII	Ap:Sp (6:1)	234±11
K VIII	Ap:Sp (2:1)	62±18
K IX	Ap:Sp (1:2)	70±15
K X	Ap:Sp (1:6)	43±18

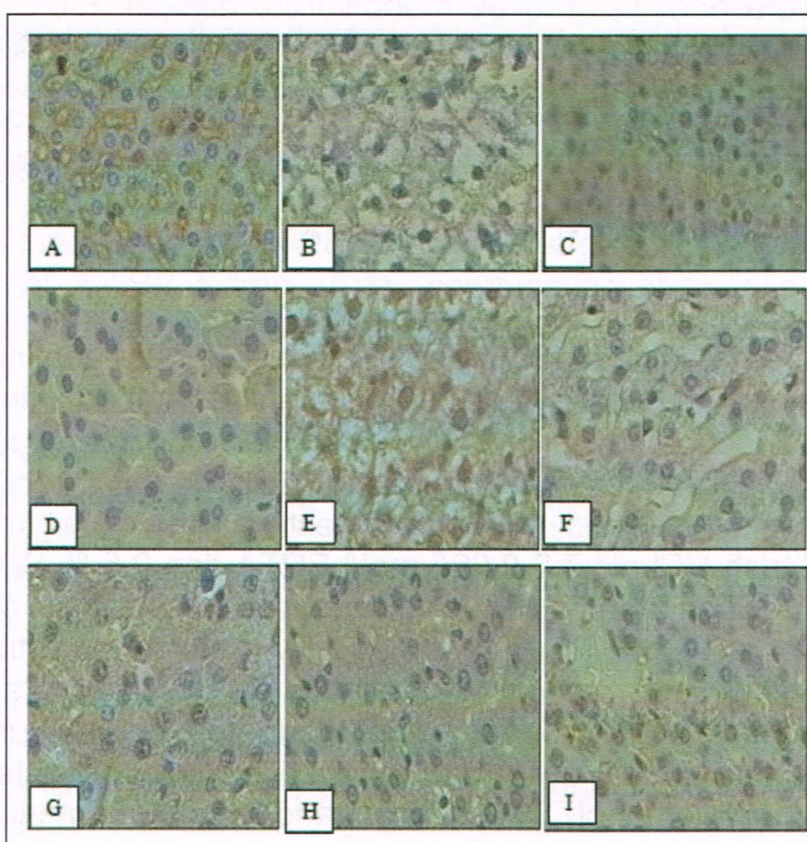

Figure 1. GLUT4 Expression in the liver of hyperglycemic Wistar Rats
(A) Negative control group **(B)** Normal group **(C)** Insulin group **(D)** Metformin group **(E)** Apgroup **(F)** Sp group **(G)** Ap:Sp=6:1 **(H)** Ap:Sp=2:1 **(I)** Ap:Sp=2:1

GLUT4 is a glucose transporter that was exspressed by adipose tissue, muscle, and liver cells (Fukumoto *et al.*, 1989). GLUT4 is activated and facilitates glucose transport to the adipose tissue and muscle when insulin hormone is released. It was proved that insulin was the main regulator of GLUT4 mRNA expression in adipose tissue (Garvey *et al.*, 1989). This study found that GLUT4 was expressed in relatively same level as insulin, thus it was predicted that active compound of AP is able to induce glucose transport by stimulating the activation of GLUT4. The expression of GLUT4 was also increase in high-fat-fructose-fed rats (Nugroho *et al.*, 2011). Moreover, AP was also increase glucose cellular uptake in STZ-diabetics-induced rats by increasing the GLUT4 mRNA and protein level (Zhang *et al.*, 2009). Andrographolide, the active compound of AP also reported its ability to increase glucose uptake into the isolated ileus muscle of STZ-diabetic-induceds rat (Yu *et al.*,, 2008).

3.2. PPARγ expression

This study also showed the increased expression level of PPARγ after rats were treated by mixture of sambiloto and salam extracts (6:1). It showed that the increased expression of PPARγ resembles the expression of PPARγ after metformin stimulation (table 2).

Table 2. PPARγ Expression

Groups		Means±SD
K I	Negative control	164±11
K II	Normal control	169±9
K III	Insulin	183±8
K IV	Metformin	147±24
K V	Ap (*Andrographis paniculata*)	178±13
K VI	Sp (*Syzygium polyanthum*)	150±19
K VII	Ap:Sp (6:1)	141±12

Table 2 showed relative high expression of PPARγ that maybe indication of high reactivity of monoclonal antibody used in the immmunohistochemistry process. Moreover, this result maybe also an indication that PPARγ expression need to be studied in other organs or tissues such as adipose tissue, pancreas, or muscle tissue that are related to glucose transport. In figure 2, the PPARγ expression can be seen brown in colour.

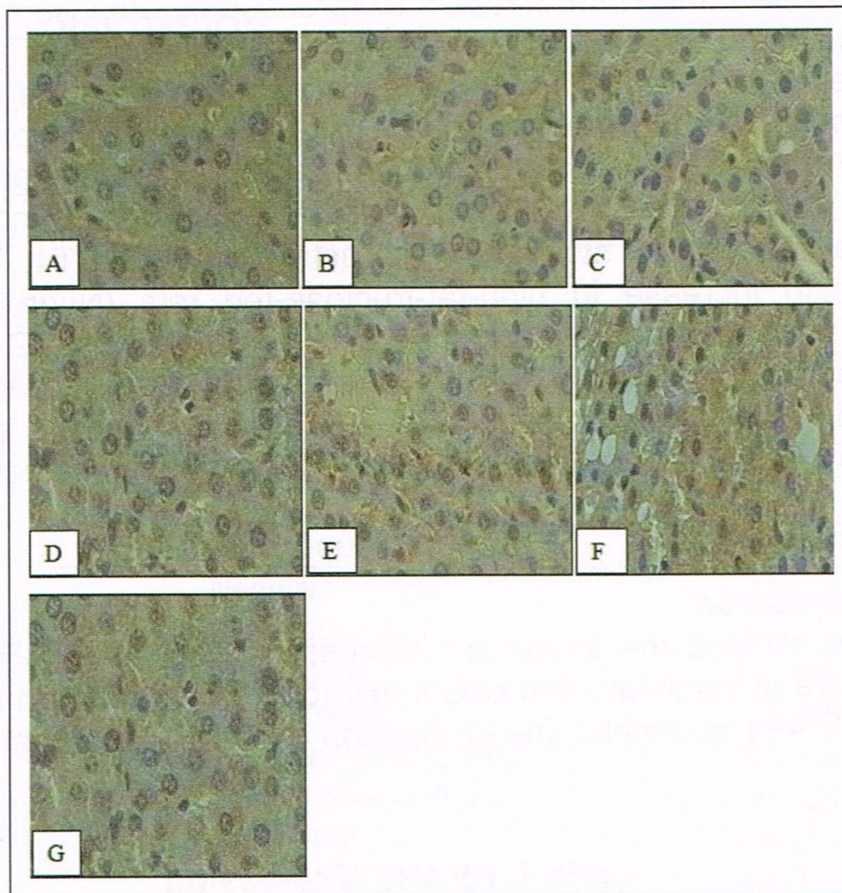

Figure 2. PPAR-γ Expression in the liver of hyperglycemic Wistar Rats
(A) Negative control group **(B)** Normal group **(C)** Insulin group **(D)** Metformin group **(E)** Ap group **(F)** Sp group **(G)** Ap:Sp=6:1

PPARγ expression expressed in adipose and muscle tissue. PPARγ promotes tcel differentiation adipose cells (Braissant *et al.*, 1996). GLUT4 activation during adipogenesis is closely related to the role of PPARγ (Wu Z *et al.*, 1998). The role of PPARγ to GLUT4 expression was shown in a study of wild ginseng in reducing body weight (Ollah *et al.*, 2008). In the study, wild ginseng administration of dose 100 and 200 mg/kg for 4 weeks, revealed the lowering blood glucose level and increasing of PPARγ and GLUT4 expression, as well as insulin receptor in muscle and liver. Active compounds of SP was previously studied and revealed that campest-4-en-3-one, exhibited a significant protein tyrosine phosphatase 1B inhibitory (PTP1B) activity (Syaifudin *et al.*, 2012). PTP1B is an enzyme that found in insulin-targetting tissue (liver, muscle and adipose) that pays a role as a negative regulator in insulin signal transduction (Byon *et al.*, 1998).

Interestingly, the administration of AP and SP mixture extract (6:1) showed a level of GLUT4 expression that was not in line with PPAR-γ expression. The expressed PPAR-γ gene that were stimulated by the mixture extract maybe expressed in the different form as was previously studied, thus no relation with the level of GLUT4 was observed. These results explained that there maybe another gene expression than PPARγ was expressed and induced activation of GLUT4 following the administration of Ap and Sp mixture extract.

4. CONCLUSIONS

Mixture extracts of *Andrographis paniculata* Nees and *Syzygium polyanthum* Wight. (Walp.) able to increase GLUT4 expression in alloxanne-diabetic induced.

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