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Original Research Article

Efficacy of the *Aquilaria Malaccensis* Leaves Active Fraction in Glucose Uptake in Skeletal Muscle on Diabetic Wistar Rats

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ABSTRACT

This study was to know the efficacy of active fraction *Aquilaria malaccensis* leaves (fraction of ethanol water/AFEA and fractions of ethyl acetate/AFEC) in glucose uptake by measuring the levels of glucose transporter 4 (GLUT4) in muscle tissue on Wistar male rats diabetic model. Experimental research design of pre and post-test with control group. AFEA and AFEC was administered to High Fat Diet (5 mL orally)/Dexamethasone (250 µg/kg body weight intraperitoneally)-induced diabetic male Wistar rats at different doses (0.01, 0.1 and 1 g/kg b.w. /day) for 14 days.
GLUT4 level in skeletal muscle tissue was significantly enhanced (p<0.05) after active fraction treatment when compared to the diabetic negative control rats. It is found both fraction in the AFEA and the AFEC start from 0.01 g/kg b.w. with flat dose manner.
It can be concluded that either AFEA or AFEC (0.01 g/kg b.w.) potential to increase level of GLUT4 24.5% and 20.6% respectively in skeletal muscle tissue of diabetic rats model.

Key word: *Aquilaria malaccensis*, GLUT4 skeletal muscle level, in vivo.

INTRODUCTION

The World Health Organization (WHO) has published that Diabetes mellitus (DM) is one of the 10 leading causes of death worldwide. [1] Type 2 diabetes is the most common form of which is 90-95% of all cases of diabetes. [2] Epidemiological studies have shown an increasing trend of the incidence and the prevalence of type 2 DM in many countries in the world; including Indonesia is ranked number 7 of the 10 largest countries of diabetics worldwide. [3]

This disease is characterized by elevated blood sugar levels, which is preceded by insulin resistance or abnormal insulin secretion plays an important role in the onset and progression of disease. [4] Insulin resistance is impaired insulin signaling cascade in target cells to respond normal or elevated circulating insulin to the final cellular effect, such as translocation of vesicles containing GLUT4 glucose transporters, [5] which is the major mediator of glucose removal from the circulation and a key regulator of whole-body glucose homeostasis. GLUT4 is particularly expressed in adipose tissue and skeletal muscle. [6] Insulin resistance in Human type 2 DM has been proposed to connect with functional defect in the muscle glucose transport system. [7]

An important mechanism in the treatment of DM is to stimulate plasma glucose uptake into peripheral tissues including the skeletal muscle and adipose tissue. [8] Skeletal muscle is the primary tissue responsible for glucose use in the

postprandial state. In animal models of diabetes, a decrease in muscle GLUT4 is caused by to provide to the insulin resistance and participates the hyperglycemic state. [9]

A. malaccensis Lamk also known as agar wood, which is one of 15 species of genus *Aquilaria* in the Indomalaya, classified as *Thymelaeaceae* family and class *Magnoliopsida*. Widely distributed in South and Southeast Asia, this plant is found in 10 countries including Bangladesh, Bhutan, India, Indonesia, Myanmar, the Philippines, Singapore and Thailand. [10] Agar wood leaves have been reported have the potential for antidiabetic, with mechanism of action as α glucosidase inhibitor, [11] improves liver insulin resistance via AMP-activated protein kinase (AMPK) activation. [12] Recent study, Pranakhon et al [13] reported *Aquilaria sinensis*, another species of *Aquilaria* could increase glucose uptake in rat adipocytes and we have proved that one mechanism mediated by increased GLUT4 adipose level (not yet published).

It should be well known that adipose tissue accounts less than muscle for percentage of total body glucose metabolism. Muscle is the major tissue responsible for insulin-stimulated glucose uptake mediated by GLUT4. [14] This study aims was to determine the efficacy of active fraction *Aquilaria malaccensis* leaves (AFEA and AFEC) in glucose uptake by measuring the levels of GLUT4 in skeletal muscle of *Rattus norvegicus* male rats induced DM by mixture high-fat diet (HFD) and dexamethasone.

MATERIALS AND METHODS

Materials

The leaves of *Aquilaria malaccensis* were collected from Gaharu Plantation in Gandus District, Palembang, South Sumatera Province, Indonesia, in the month of August-September, identified and authenticated by the Indonesia Science Institute (LIPI). The collected plant material was made free from foreign organic matter. Pioglitazone hydrochloride obtained from Dexa Medica PT in Palembang, South Sumatera Province. Random Access Analyzer Bio System® and Bio System reagent for measuring blood glucose levels in diabetic rats. ELISA reader Bio Rad® was used in ELISA analysis for GLUT4 and insulin, using the GLUT4 *for* Rat ELISA kit from Qayee-Bio and Insulin for Rat ELISA kit from Sun long Biotech. Dexamethasone sodium phosphates were produced by Indofarma®. High fat diet consists of margarine and coconut oil was purchased from the local market, Palembang, Indonesia. All the other chemical used for the experiments were of analytical grade.

Extraction and Fractionation

Dried *A. malaccensis* leaves (1,600 grams) were extracted using soxhlet extraction procedure with ethanol 96% (repeated 2 times a day, for 3 hours each time until 3 days), followed by removal of the solvent on rotary evaporator, to yield a dried ethanol extract (336.6 grams). The ethanol extract were suspended in aquadest and extracted successively with n-hexane and ethyl acetate. It was got fraction n-hexane (22.13 grams), ethyl acetate (78.1 grams) and fraction ethanol aquaest (152.8 grams)

Animals

Male Wistar rats (170-230 grams) purchased with animal health certificate from the veterinarian in the Department of Agriculture, Bandung, and West Java. They were 10 weeks old and have the lowest fasting blood glucose level of 5.6 mmol/L. All of them maintained in an air conditioned room (25±1 ^{o}C), with a 12 h light - 12 h dark cycle and fed with standard diet and water ad libitum. Those were housed in the Animal House Faculty of Medicine, Sriwijaya University (Palembang, Indonesia) for 7 days before starting the experiment. The study which conducted in October-December 2015, approved by Health Research Review Committee of Mohammad Hoesin Central General Hospital and Faculty of Medicine Sriwijaya

University (Ethical approval certificate no. 322/kepkrsmhfkunsri/2015).

Rat diabetes induced by HFD 5 mL and Gc 250 µg/kg b.w./day using modification the method as describe by Sivabalan et al [15] for 2 weeks, and then fasting blood glucose level, collected from the orbital sinus puncture were checked. Rats with fasting blood glucose level over 11.1mmol/L were used. After the experiment, the animals terminated by intraperitoneal injection of ketamin 70 mg/kg b.w.

Experimental design

HFD/Gc rats divided into eight groups with 4 rats in each group as follows: Group I: diabetic control negative rats orally administered with tween 80 0.5%; Group II-IV: diabetic rats orally administered AFEA with 0.01, 0.1 and 1 g/kg b.w./day respectively. Group V-VII: diabetic rats orally administered AFEC with 0.01, 0.1 and 1 g/kg b.w./day respectively. Group VIII: Diabetic control positive rats orally administered with pioglitazone hydrochloride 0.02 g/kg b.w./day. [16] All treatment dissolved in tween 80 0.5% ad 2 mL, for 2 weeks. Fasting blood glucose and body weight measured before and after treatment. On the 15th day of administration, blood sample collected for fasting blood glucose and insulin measurement. During fasting, rats were deprived of food overnight for 12 h but had free access to water. Then, animals terminated. Ractus abdominis muscle (50 mg) collected from all groups for GLUT4 level measurement.

ELISA Assay

This assay used to measure level of fasting insulin from plasma preparation (whole blood collected into tube with anticoagulant-EDTA, incubated at room temperature for 20 minutes, and then centrifugated for 20 minutes at 3.000 rpm, supernatant were collected as plasma samples) and GLUT4 from tissue sample (adipose tissues were placed on a separate micro tube, washed 3 times with PBS 1%, homogenize by hand, add PBS, centrifuge for 20 minutes at the speed of 3000 rpm, supernatant collected) as described in manufacturer's instructions of ELISA kit.

Fasting blood glucose level

Fasting blood glucose level estimated by kits as mentioned by the manufacturer's instructions.

Insulin resistance

Homeostasis model assessment of insulin resistance (HOMA-IR) calculated by the formula:

$$\text{HOMA} - \text{IR} = \frac{\text{FPG(mmol / L)} \times \text{FPI}(\mu\,\text{IU/mL})}{22.5}$$

FPG for fasting plasma glucose and FPI for fasting plasma insulin

Phytochemical Analysis

Specimen from each fraction was examined to check the presence of bioactive compound. Thin layer chromatography (TLC) GF $_{254}$ was used as stationary phase, a mixture of toluene n-hexane, ethyl acetate, formiat acid (6:4:0.2) was used as a mobile phase to examine terpen, flavonoid and phenolic hidroquinon, while test tube was used to examine alkaloid with dragendorff, tannin with $FeCl_3$ 1% and saponin with forth formation test. Chromatograms were visualized under sprayed with anisaldehyde-sulphuric acid, citroborat and FeCl3 5% to examine terpen, flavonoid and phenolic hidroquinon respectively.

Statistical analysis

Statistical analysis was performed using SPSS software package version 18. The values were analysed by paired t test, unpaired t test, one-way analysis of variance (ANOVA) followed by LSD post hoc test. All results were expressed as mean $\pm$ SD. A vallue of $p<0.05$ was considered statistically significant.

RESULTS

Analysis of fractions of A. malaccensis for presence of bioactive phytochemicals

Table 1 shown the main bioactive phytochemicals in fraction ethanol water were flavonoid and tannin, while steroid/terpenoid, flavonoid and tannin were the main bioactive phytochemicals in fraction ethyl acetate.

Table 1. Bioactive Phytochemicals in Fractions

No.	Phytochemical	Fraction	
		Ethanol water	Ethylacetate
1	Alkaloid	-	-
2	Steroid/terpenoid	++	++++
3	Flavonoid	++++	++++
4	Tannin	++++	++++
5	Saponin	-	-
6	Fenol hidroquinon	-	-

Body weight, FPG, FPI, and HOMA-IR

Change in body weight, level of FPG, FPI and HOMA-IR index are summarized in table 2. Negative control group showed no significant reduction in body weights before induced with HFD-Gc. Administration of AFEA (0.01 and 1g/kg/day), AFEC (0.01 and 1 g/kg/day) and pioglitazone for 2 weeks revealed a significant increase in body weight compare negative control with increased 22.5%, 20.1%, 26.7%, 22% and 40.4%, respectively.

The FPG level of HFD-Gc for 2 weeks induce diabetic rats were significant increase ($p<0.001$) more than 11,1 mmol/L, and then treatment in AFEA (0.01, 0.1, and 1), AFEC (0.01, 0.1 and 1) and Pioglitazone for 2 weeks showed a sigificant decrease in FPG compare with the negative control rats with a reduction of 46.47%, 42.54%, 41.65%, 47.81%, 48,26%, 42.24% and 52,56%, respectively.

Similarly, the FPI levels were also increased significantly in the negative control rats induced HFD and Gc ($p<0.001$) compared with the previous condition. Treatment with AFEA (0.01, 0.1 and 1), AFEC (0.1 and 1) and pioglitazone showed significant decline of 39.3%, 33.6%29.1%, 61.2%, 52.7% and 35.3%, respectively.

The insulin resistance (HOMA-IR) was inclined 6-7 fold ($p<0.001$) in the HFD-Gc induced diabetic control negative group compared they had been before. Administration of AFEA (0.01, 0.1 and 1), AFEC (0.01, 0.1 and 1) and pioglitazone for 2 weeks showed a significant decrease in insulin resistance with a percentage decline of 63.1%, 57.2%, 63.1%, 62.1%, 77.4%, 70.2% and 64.8%, respectively, compared with the diabetic negative control rats.

Table 2: The efficacy of AFEA and AFEC on body weight, FPG, FPI and HOMA-IR in diabetic rats for 2 weeks treatment

Group (n=4 each group)	Body weight (g)						FPG (mmol/L)						FPI ((µU/mL))			HOMA-IR Index		
	Day 1			Day 14			Day 1			Day 14			Day 14			Day 14		
Negative control	207	±	5.72	177	±	17.01	11.87	±	0.88	13.47	±	0.95 [a]	11.15	±	0.74	6.7	±	0.91
DM+AFEA 0.01	204	±	11.11	250	±	17.19 [a,c]	11.55	±	0.44	7.21	±	1.67 [a,c]	6.77	±	1.08 [c]	2.11	±	0.23 [c]
DM+AFEA 0.1	198	±	14.90	210	±	8.33 [b,c,d]	11.99	±	0.84	7.74	±	1.19 [a,c]	7.4	±	1 [c]	2.54	±	0.49 [c]
DM+AFEA 1	209	±	9.49	251	±	18.06 [a,c]	15.21	±	0.95	7.86	±	1.83 [a,c]	7.91	±	1.7 [c]	2.78	±	1.01 [c]
DM+AFEC 0.01	215	±	4.76	272.5	±	22.17 [a,c]	14.35	±	1.22	7.03	±	1.5 [a,c]	8.68	±	1.75	2.69	±	0.59 [c]
DM+AFEC 0.1	197	±	13.64	201	±	9.25 [b,c,d]	12.15	±	0.84	6.97	±	1.9 [a,c]	4.33	±	0.98 [c,d]	1.36	±	0.59 [c]
DM+AFEC 1	205	±	15.85	250	±	19.58 [a,c]	12.11	±	1.07	7.78	±	1.5 [a,c]	5.27	±	1 [c]	1.78	±	0.14 [c]
Pioglitazon	192	±	6.78	269.5	±	12.29 [a,c]	12.12	±	0.84	6.39	±	1.27 [a,c]	7.21	±	1.88 [c]	2.12	±	0.9 c

Paired t test, [a] $p<0.05$; Unpaired t test, [b] $p<0.05$ VS pioglitazon; [c] $p<0.05$ VS negative control; Significance level was determined by one way ANOVA followed by LSD post-hoc test, [d] $p<0.05$ VS pioglitazone, [e] $p<0.05$ VS negative control

GLUT4 skeletal muscle

ELISA assay in skeletal muscle performed to determine the efficacy of both the AFEA and AFEC on glucosa up take through GLUT4 protein level. Table 3 shows GLUT4level in diabetic rats induced HFD-Gc as negative control and subjected to AFEA, AFEC and pioglitazone treatment for 2 weeks. Two weeks treatment ethier AFEA (0.01) or AFEC (0.01 and 1)

treatment in diabetic rats significantly increased GLUT4 level (p<0.05) with a percentage increase of 24.5%, 20.6% and 25.7%, respectively, compare with the diabetic negative control rats and showed a more pronounced effect than pioglitazone.

Table 3: Efficacy of AFEA and AFEC on GLUT4 skeletal muscle in diabetic rats for 2 weeks treatment

Group (n=4 each group)	GLUT4 skeletal muscle ((pg/mL))		
Negative control	378.82	±	33.87
DM+AFEA 0.01	471.47	±	19.8 [c, d, e]
DM+AFEA 0.1	386.67	±	16.12
DM+AFEA 1	372.44	±	3.23
DM+AFEC 0.01	456.77	±	28.17 [c, e]
DM+AFEC 0.1	424.41	±	27.08
DM+AFEC 1	476.33	±	16.23 [c, d, e]
Pioglitazon	399.41	±	56.46

Unpaired t test, [b] p<0.05 VS pioglitazon; [c] p<0.05 VS negative control; Significance level was determined by one way ANOVA followed by LSD post-hoc test, [d] p<0.05 VS pioglitazone, [e] p<0.05 VS negative control

Figure 1 demonstrate that both AFEA and AFEC had potentiate to improve GLUT4 level in skeletal muscle, start from 0.01 g/b.w./day with flat dose manner. The potentiation to improved GLUT4 level increased for AFEC, differed from AFEA to the dose enhancement.

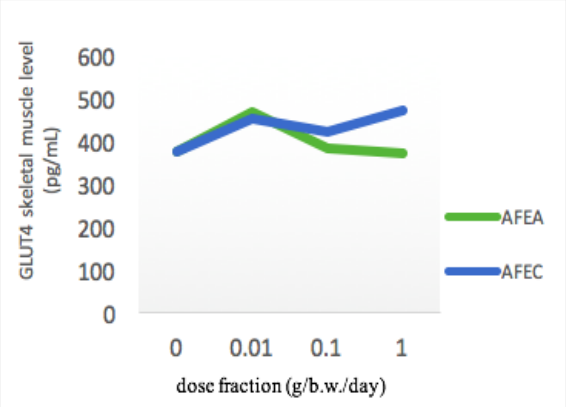

Figure 1: Dose dependent effect of *A. malaccensis* fraction to potentiate GLUT4 level in skeletal muscle diabetic rats

DISCUSSION

In this study, the HFD administration in combination with Gc (250 µg/kg/day) for 2 weeks to rats resulted in pathogenesis of T2DM such as hyperglycemia, hyperinsulinemia and increased HOMA-IR index, similarity Diabetic rats model from Sivabalan *et al* [15] that had shown in negative control. HFD induce insulin resistance in muscle [16] by forming fatty acid intermediates that activate PKC and inhibits the activation of Akt2 thereby preventing translocation GSV, [16] which in turn decreased GLUT4 expression in skeletal muscle. [18] At the same time, glucocorticoids interfere with lipid metabolism leading to the accumulation of lipid outside of adipose tissue such as muscle that to an able the photogenes is of insulin resistance as mentioned before, [19] and increased liver gluconeogenesis. [20]

The results from this study showed that the ethanol water and ethyl acetate had significant activity as an antihyperglycemic, improved insulin resistance in skeletal muscle of rat model induced by HFD-Gc, and increased GLUT4. It is very interesting that 0.01 g/kg b.w./day of the fraction ethyl acetate and the fraction ethanol water exhibited increased GLUT4 level more than pioglitazone. Thus, doses lower than 0.01 g/b.w./day should be tried. However, the increase in GLUT4 level of both fractions was non concentration dependent similar to previous study. [13] At the highest dose of AFEC tested, 1 g/b.w./day, the GLUT4 level tended to increase, differed from AFEA.

GLUT4 has an important role in the homeostasis of glucose contained in the adipose tissue, muscles and heart. [21] The sensitivity of tissues to insulin can be significantly improved through the expression of GLUT4. [22] How does this fraction increase GLUT4 level in skeletal muscle will require further research.

The study showed presence of high level of flavonoid and tannin in both fractions, whereas terpenoids also increased in ethyl acetate fraction. Bioactive compounds which demonstrate these effects requires further investigation.

CONCLUSIONS

A. malaccensis leaves showed the ability to improve glucose uptake by elevated levels of GLUT4 in skeletal muscle. Further research is needed to explore these leaves as a potent antidiabetic.

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