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Original Article

REGULATION OF ETHYL ACETATE FRACTION FROM *MORINGA OLEIFERA* LEAVES TO IMPROVE LIPID METABOLISM AND INSULIN SENSITIVITY IN TYPE 2 DIABETES

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INTRODUCTION

Type 2 diabetes is a progressive disease characterized by hyperglycemia with insulin resistance in early condition. Insulin resistance is a reduction of the response of insulin target cells and tissues to a physiological concentration of insulin. This condition is caused by abnormalities of metabolism such as metabolic stress syndrome, obesity, high blood pressure, high blood lipids, high blood uric acid and diabetes [1]. Type 2 diabetes with insulin-resistant especially marked by a defect in glucose uptake because it associated with regulation of GLUT4 protein. The expression of GLUT4 gene is subject to both tissue-specific and hormonal/ metabolic regulation. GLUT4 plays a critical role in glucose-sensing although only 15% of the blood glucose is absorbed by adipose tissue and the remaining (85%) by muscle in healthy individual [2]. Insulin induces translocation of GLUT4 from intracellular vesicles to the plasma membrane, which permits the facilitated diffusion of circulating glucose down its concentration gradient into muscle cells leading to a rapid increase in the uptake of glucose. Accumulating evidence indicates that either expression deregulation or functional impairment of GLUT4 can cause insulin resistance. Because of its crucial role, GLUT4 has been considered to be a potential therapeutic target for type 2 diabetes [3].

Some plants are shown to have antidiabetic effects, one of which is *Moringa oleifera* leaves [4]. *Moringa oleifera* is a medicinal plant species that has potential as an antidiabetic [5]. *Moringa oleifera* is rich in phytochemical compounds such as flavonoids, alkaloids, and saponins [6]. Flavonoid is one of the biologically active class of secondary metabolite plant compounds that belong to an important part of human diet. The antidiabetic properties of flavonoids are mainly through their effect on a number of molecular targets and regulation of several pathways such as reducing apoptosis, improving proliferation of pancreatic beta cell and promoting insulin secretion, regulation of glucose metabolism in hepatocytes and subsequent improvement of hyperglycemia, decreasing insulin resistant, inflammation and oxidative stress in adipocytes and skeletal myofibers, enhancing glucose uptake in skeletal and adipose tissues [2].

Based on these reviews, *Moringa oleifera* leaves have the potential to be developed as an antidiabetic agent. However, it is not yet known whether the active component has an effect as an antidiabetic and molecular mechanism. Thus, it is important to study the antidiabetic effect of EAFML and molecular mechanism study.

MATERIALS AND METHODS

Material

Streptozotocin (STZ) was purchased from Nacalai tesque, INC (Kyoto, Japan), Nicotinamid (Sigma-Aldrich, USA). Glucose level was measured by using colorimetric metode (GOD-PAP) with glucose oxidase and 4-aminoantipyrine (DiaSys Diagnostic System GmbH, Holzheim, Germany). Lipid profile was measured by enzimatic photometric methods using kit from DiaSys, Diagnostic System GmbH, Holzheim, Germany. Antibodies for determining of GLUT4 expression were a primary anti-GLUT4 antibody (Santa Cruz Biotechnology, California, USA). All other reagents were high-grade qualified material.

Preparation of EAFML

Moringa oleifera leaves were collected from the area of Gunung Kidul, Yogyakarta special region, Indonesia. The leaves were shade dried, powdered and store in airtight container for further use. The plant was authenticated with certificate number 029/lab. Bio/B/III/2017 by a botanist at the Department of Biology Pharmacy, Faculty of Pharmacy, Ahmad Dahlan University, Indonesia. The dried powder of *Moringa oleifera* leaves was subjected to extractor by maceration using 80% ethanol for 3 d and was re-extracted using the same solvent for 4 d. All

liquid extracts were collected and concentrated using a rotary vacuum evaporator under reduced pressure. The extract fractionated with n-hexane and the water-soluble fraction is purified with ethyl acetate and obtained ethyl acetate fraction.

Preparation of type 2 diabetic rat model

The animal model handling protocols of this study were in accordance with the guidelines of the animal care of the Departement of Pharmacology, Faculty of Pharmacy, Ahmad Dahlan University, Indonesia. 30 male wistar rats were acclimatized for 1 w and housed in individual cages at a constant temperature (22±2 °C) with a constant relative humidity (55±10%), controlled lighting 12:12h light-dark cycle (light on at 6:00 a. m) and had free access to food and water ad libitum. The rats were previously exited before being induced fasted with administering nicotinamide 100 mg/kg insulin solution by intraperitoneal (i. p). 15 min followed by injection of STZ in doses of 65 mg/kg (i. p) in 0.1 M buffer citrate, pH 4.5 made new. The control group was injected with a citrate buffer. After 5 d, the animal was measured for their fasting blood glucose level (BGL). BGL ≥ 200 mg/dL fall into the positive category of diabetes. The methodology of this experiment was performed after the approval by Research Ethics Committee Ahmad Dahlan University with ethical approval number 021703005.

Antidiabetic treatment of EAFML

A total of 30 rats were used and divided into 6 groups with 5 rat per group. Group I (control, a healthy rat). Group II (negative control, an untreated diabetic rat). Group III (positive control, a diabetic rat with metformin treatment in doses of 100 mg/kg once daily orally). Group IV, V and VI (treatment groups 1, 2 and 3, a diabetic rat with EAFML treatment in doses of 12.5; 25 and 50 mg/kg once daily orally). Treatment was carried out for 10 d. On the 10th day, all the rats were fasted and measured their BGL and lipid profile.

Histology of skeletal muscle and liver

The rats were sacrificed at the end of the treatment and the skeletal muscle and liver tissue of control and treated groups were taken and fixed with 4% formaldehyde in phosphate buffer. The tissues were embedded in paraffin and cut into pieces of the desired size and then the tissues were deparaffinized in xylane then dehydrated of alcohol concentration. The sections were then stained with Hematoxylin-Eosin (HE) and observed using a light microscope with a magnification of 1000x.

Expression of GLUT4 by immunohistochemistry

Sections of skeletal muscle and liver organ were collected and fixed with 4% formaldehyde solution in the phosphate-saline buffer for more than 2 h. Then the tissue was hydrated with concentrated alcohol, then cleaned using xylol. The tissue was immersed in paraffin and cut with a thickness of 4 µm and placed on a glass slide. Endogenous peroxidase activity in the tissue was blocked with 3% H_2O_2 in methanol for 15 min and washed with aqua dest. The tissue portion was incubated with 20% horse serum for more than 10 min subsequently reincubated with GLUT-4 primary antibody with 1: 250 dilution for one hour at room temperature, followed by incubation peroxidase-conjugate secondary antibody with 1: 500 dilution for 1 hour. Visualization was performed after incubation with the substrate for 15 min, and the section was counterstained with Hematoxylin for 30 min, hydrated with alcohol and xylene. The evaluation of protein expression was calculated based on semiquantitative methods according to the modified Remmele method. Immunoreactive score (IRS) or Rammele scale index is the result of multiplication between the percentage score of immunoreactive cells with color intensity score on immunoreactive cells. The end result obtained ranges from 0-12 points [7].

Statistical analysis

Statistical analysis used SPSS 17.0 with a confidence level of 95%. The date from an examination of blood glucose level and lipid profile was presented in chart table, then it's was normalitis tested by using sapiro-wilk, and the homogenity was tested by using Leven's Test of Homogeneity of Varian test. The average difference of each component was analyzed statistically by one-way ANOVA. Differences of GLUT4 expression between groups were analyzed with the Kruskal-Wallis test and followed by Mann-Whitney test.

RESULTS

Observation of blood glucose level in pre-treatment

On day-1, rats were injected intraperitoneally with nicotinamide in doses of 100 mg/kg in normal saline. After 14 min, the rats were given an intraperitoneal injection with STZ in doses of 65 mg/kg. bw in citrate buffer to induce diabetes. BGL was measured on day-5. Rats with diabetes were those that have BGL>200 mg/dL [2]. The data of BGL before and after induction can be seen in table1.

Tabel 1: Blood glucose level in pre-treatment

Group	Σ	Blood glucose level (mg/dL)	
		H-1	H-5
Control	5	64.7±11.7	105.0±13.5
Diabetes	25	70.1±11.7	493.5±122.9*

*Data of BGL on day-5 significantly different from day-1 ($p<0.05$), Data represent mean±SD

The BGL of induced rats on day-5 increased significantly from 70.1±11.7 mg/dL to 493.5±122.9 mg/dL with $p<0.05$. It showed that the combination of nicotinamide injection and STZ injection was able to improve BGL significantly compared with the control group on day-5.

Observation of BGL in post-treatment

After diabetes induction using the combination of STZ dan nicotinamide, the diabetic rats divided into 5 groups and given treatment for 10 d. On day-11 of post-treatment, the rats' BGL were checked. The result of BGL in post-treatment is presented in table 2.

Tabel 2: Blood glucose level in post-treatment

Groups	Blood glocose level of post-treatment (mg/dL)
Control	84.1±18.7
Diabetes	355.8±83.7*
Diabetes+metformin 100 mg/kg	258.8±175.9*
Diabetes+EAFML 12.5 mg/kg	313.2±19.5*
Diabetes+EAFML 25 mg/kg	337.2±124.2*
Diabetes+EAFML 50 mg/kg	177.5±89.3

*significantly different from the control group with $p<0.05$, Data represent mean±SD

Table 2 shows there are no significant differences between the BGL of diabetic rats with EAFML treatment in doses of 50 mg/kg for 10 d and the BGL of the healthy group. It shows that the administration of EAFML in doses of 50 mg/kg improved BGL significantly.

Observation of lipid profile in post-treatment

Lipid profile observations were performed on day-11 of post-treatment. The lipids that were analyzed included cholesterol, triglycerides, LDL and HDL. Lipid profile data can be seen in table 3.

Table 3: Lipid profile in post-treatment

Groups	Lipid profile (mg/dL)			
	Cholesterol	TG	LDL	HDL
Control	75.0±7.3	62.2±19.6	134.2±11.7	71.7±9.1
Diabetes	105.2±47.4	51.0±22.1	175.6±41.9	80.6±18.0
Diabetes+metformin	72.8±10.6	52.0±9.3	133.7±20.8	71.3±11.6
Diabetes+EAFML 12.5 mg/kg	73.4±23.1	57.2±13.7	124.5±40.5	62.5±15.7
Diabetes+EAFML 25 mg/kg	58.6±6.9 *	65.0±14.9	95.3±8.0 *	49.7±3.0 *
Diabetes+EAFML 50 mg/kg	73.0±13.3	36.4±11.3	134.3±29.3	68.6±15.0

*significantly different from the diabetic group with $p<0.05$, data represent mean±SD

Based on observation of lipid profile in post-treatment, it can be seen that the diabetic group with administration of EAFML in doses of 25 mg/kg is significantly different from the diabetic group. This shows that the administration of EAFML in doses of 25 mg/kg can significantly decrease cholesterol, triglyceride and HDL levels.

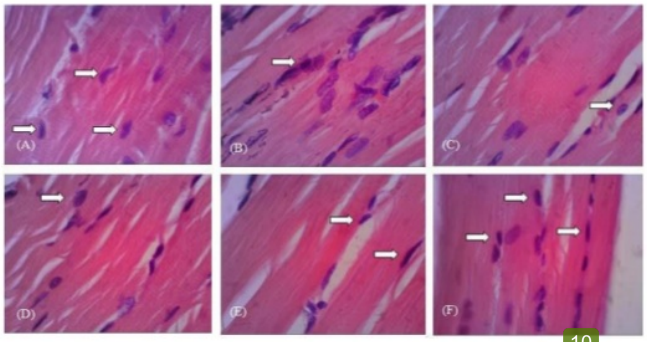

Fig. 1: Cross-section of skeletal muscle tissues with HE staining in 1000x magnification, (A) Control; (B) Diabetes; (C) Diabetes+metformin; (D) Diabetes+EAFML 12.5 mg/kg; (E) Diabetes+EAFML 25 mg/kg; (F) Diabetes+EAFML 50 mg/kg

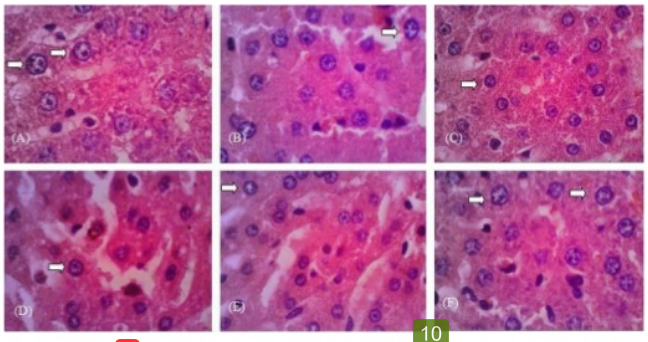

Fig. 2: Cross-section of liver tissues with HE staining at 1000x magnification, (A) Control; (B) Diabetes; (C) Diabetes+metformin; (D) Diabetes+EAFML 12.5 mg/kg; (E) Diabetes+EAFML 25 mg/kg; (F) Diabetes+EAFML 50 mg/kg

Histology of skeletal muscle and liver tissue

Based on histologic observation with HE staining from cross-section of skeletal muscle and liver organs of rats as seen in fig. 1 and fig. 2, it was obtained that in normal rats the cell nuclei and hepatocytes are intact and clear (➩), whereas in diabetic rats the cell nuclei had undergone necrosis which is indicated by less clear and non-intact cell shapes. After administration of EAFML in doses of 12.5 mg/kg; 50 mg/kg and 50 mg/kg, it can be seen that the cell nuclei had improved, in which the number of cell nuclei that were intact and that had clear color increased.

GLUT4 expression in skeletal muscle and liver

Molecular observation had been undergone to see the expression of the GLUT4 protein on skeletal muscle and liver organs using immunohistochemistry method. GLUT4 is one of the parameters of the occurrence of insulin resistance. Changes of GLUT4 protein level which influenced glucose tolerance have been proven on a transgenic animal

with GLUT4 protein deficiency and play roles in insulin resistance condition; thereby, therapy with a target on the increase of GLUT4 protein expression is a new breakthrough in discovering a new antidiabetic medication [8].

The observations were performed under a light microscope with 400x magnification. If in cells there is found GLUT4 protein expression, the brown color will be seen on the cytoplasm. Mayer Hematoxylin was used as a counterstain for examination of GLUT4. Immunohistochemical features for GLUT4 protein expression can be seen in fig. 3 and 4.

The observation result of immunohistochemical of GLUT4 protein in muscle and liver can be seen in table 4.

Table 4: Expression of GLUT4 in skeletal muscle and liver in post-treatment

Groups	GLUT4 expression	
	Skeletal muscle	Liver
Control	3.4±2.3*	4.5±2.3*
Diabetes	0.7±1.0	1.8±1.3
Diabetes+metformin	2.6±1.6*	1.0±0.7
Diabetes+EAFML 12.5 mg/kg	2.2±1.0*	1.3±1.5
Diabetes+EAFML 25 mg/kg	2.2±1.5*	3.6±1.9*
Diabetes+EAFML 50 mg/kg	3.9±1.1*	2.9±1.9*

*significantly different from the diabetic group with $p<0.05$, Data represent mean±SD

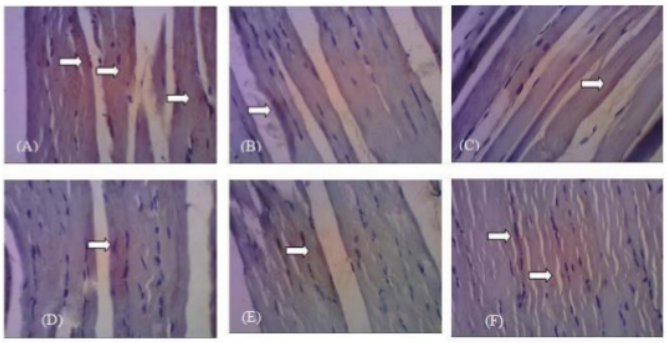

Fig. 3: Immunohistochemical staining results of experimental animals' skeletal muscle tissues using GLUT-4 antibody, (A) Control; (B) Diabetes; (C) Diabetes+metformin; (D) Diabetes+EAFML 12.5 mg/kg; (E) Diabetes+EAFML 25 mg/kg; (F) Diabetes+EAFML 50 mg/kg; Magnification 400x

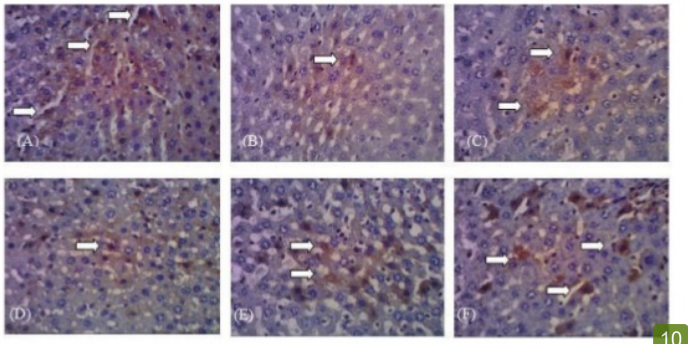

Fig. 4: Immunohistochemical staining results of experimental animals' liver tissues using GLUT-4 antibody, (A) Control; (B) Diabetes; (C) Diabetes+metformin; (D) Diabetes+EAFML 12.5 mg/kg; (E) Diabetes+EAFML 25 mg/kg; (F) Diabetes+EAFML 50 mg/kg; Magnification 400x

Based on fig. 3. Immunohistochemical Results, GLUT4 expression is indicated by dark brown spots (➪. From the pictures can be seen that from the administration of EAFML at the dosage of 12.5 mg/kg; 50 mg/kg and 50 mg/kg, GLUT4 expression increased which was indicated by the increased of the number of brown color and the color is more dense.

DISCUSSION

In this study, type 2 diabetes induction was done to make the combination of STZ and nicotinamide. Induction of STZ and nicotinamide in rat model of type 2 diabetes is based on the protective effects of nicotinamide against β-cytotoxic effects of STZ. This model is as a model for non-obese type 2 diabetes [9].

Based on this study, it showed that the ethyl acetate fraction of *Moringa oleifera* leaves has potential as antidiabetic agent. Several studies related to the effects of *Moringa oleifera* leaves as antidiabetes have been performed. The observation of *in vitro* assay with clonal of pancreatic beta cells of BRIN-BDII showed that acetone extract of *Moringa oleifera* leaves had an insulinotropic effect through K_{ATP} -dependent pathway indicated by the increased

of intracellular Ca^{2+}level [10]. Another *in vivo* study showed that *Moringa oleifera* leaves extract significantly increased the activity of superoxide dismutase (SOD) and glutathione-s-transferase (GST), and decreased lipid peroxidase (LPO), which is a characteristic factor in chronic diabetes [11]. The antidiabetic evaluation of methanol extract of *Moringa oleifera* leaves was proven can decrease blood glucose levels of rat and showed antioxidant activity with the decreased the level of nitric oxide (NO) serum [12]. Another study also suggested that water extract of *Moringa oleifera* leaves had a hypoglycemic effect through normalization of the increase of hepatic pyruvate carboxylase enzyme and regeneration of hepatocyte and pancreatic beta cells damages [13].

The result of phytochemicals *Moringa oleifera* leaves are polyphenols, flavonoids, saponins and alkaloids [14]. Another study suggested that the phytochemical content of *Moringa oleifera* leaves includes: 4-(4'-o-acetyl-alpha-ramnopiranoyloxy) benzyl isothiocyanate, benzyl isothiocyanate [15], total polyphenols, quercetin, kaempferol, and lutein [16], quercetin-3-glycosides (isoquersitrin), quercetin-4-glycosides (spiraeosid), chlorogenic acid [17].

These regulation of flavonoids as antidiabetic in many cases may be direct i.e. mediated by the physical interaction of a receptor (specific enzyme) and the flavonoid compound and may lead to either inhibition or activation of the catalytic function of the enzyme. In addition, natural polyphenols show less specific antioxidant ability of hydroxyl groups linked to aromatic carbon rings [2].

In this study, immunohistochemical tests were performed to see GLUT4 expression. The GLUT4 expression spread to the skeletal muscle, adipose tissue, liver, kidney, and pancreas [18]. In the basal condition, GLUT4 is mostly located in intracellular organelles and in order to move to the surface of the cell membrane, requires a signal from insulin. The expression of some forms of glucose transporter on the surface of the cell plays a role in glucose uptake, signal regeneration, and metabolism to maintain the body's cellular metabolic integrity [19]. This study found that administration of EAFML can increase the expression of GLUT4. This is similar to another study that show curcumin increased expression of GLUT4, in which curcumin is one of the polyphenols similar to flavonoid [20].

The influence of EAFML on lipid profile indicated that this fraction can decrease cholesterol and triglycerides. The mechanism might have a correlation to the ability of flavonoids which are the main secondary metabolites of EAFML bound to pectin. Flavonoid cooperation with pectin can work at the level of intestine and liver to stimulate fat excretion and reduce fat absorption, which augments the direct activity on enzymes, involved in the regulation of carbohydrate and lipid metabolism [21].

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