Indonesian Journal of Pharmacy

VOL 33 (4) 2022: 630–640 | RESEARCH ARTICLE

Efficacy of the *Hibiscus surattensis* L. Leaves Active Fraction in Reducing the Levels of HbA1c, AGEs, and Glucose Uptake in Muscle Cells of Diabetic Type 2 Model Rat

Yuliet Susanto[1]*, Elin Yulinah Sukandar[2], I Ketut Adnyana[2] and Joni Tandi[3]

1. Prodi Farmasi Universitas Tadulako Palu, Jl. Universitas Tadulako, Kota Palu, Sulawesi Tengah 94148
2. Department of Pharmacology and Toxicology, School of Pharmacy, Bandung Institute of Technology Jl. Ganesa No.10, Kota Bandung, Jawa Barat 40132
3. Department of Pharmacy, Sekolah Tinggi Ilmu Farmasi Pelita Mas, Jl. Wolter Monginsidi No.106 A, Kota Palu, Sulawesi Tengah 94111

Submitted: 25-03-2021
Revised: 06-06-2022
Accepted: 16-11-2022

*Corresponding author
Yuliet Susanto

Email:
yuliet_susanto@yahoo.com

ABSTRACT

Hibiscus surattensis L. is a traditional medicinal plant often used for diabetes treatment in Indonesia, especially in Central Sulawesi. The leaves contain mainly kaempferol, morine, and trifolin which have various pharmacological effects, including high antioxidant properties and the potential to increase insulin secretion. Therefore, this study aims to investigate the efficacy of the *Hibiscus surattensis* L. leaves (HSL) active fraction in reducing the levels of HbA1c, AGEs, and GLUT-4 expression in the muscle tissue of rat model with type 2 diabetes mellitus using a high-fat and fructose diet of 1.8 g/kg BW (HFD/HF) for 8 weeks. A total of 20 male rats were randomly assigned into four groups namely (1) normal control diet with standard rat chow; (2) negative control with HFD/HF diet; (3) treatment group with metformin 100 mg/kg BW as standard; (4) treatment group given HSL active fraction (FEA) 50 mg/kg BW. All treatments were given orally for 21 days and the data obtained were analyzed by ANOVA and Duncan Post-Hoc test. HFD/HF induction significantly increased the HbA1c and AGEs levels 3.5 times and 1.88 times higher than the normal control group. Moreover, the treatments significantly reduced ($p<0.05$) HbA1c and AGEs levels compared to the negative control on day 21. Blood HbA1C levels with FEA treatment decreased by 56.4%, while AGEs reduced by 54.7%. GLUT 4 expression in the muscle tissue was significantly different from the negative control with $p<0.05$. The results also indicate that the administration of FEA had an antidiabetic effect by reducing the levels of HbA1C and AGEs along with other potentially beneficial effects on the treatment of type 2 DM with the probable mechanism of targeting GLUT4 glucose transporter by increasing its translocation and expression.

Keywords*:* AGEs, HbA1C, *Hibiscus surattensis* L, Glut-4.

INTRODUCTION

Diabetes mellitus (DM), simply recognized as diabetes is a non-communicable disease which has become a significant health problem due to its increasing number of cases and prevalence. It is estimated that 11.3% of the world's population will suffer from DM in 2030. Meanwhile, Indonesia is one of the countries with the highest number of DM sufferers globally, specifically, it ranks 5th out of 10 countries with the largest prevalence (International Diabetes Federation, 2021) and is predicted to increase every year. The development of DM occurs due to changes in lifestyle, lack of physical activity, and increased obesity. It is characterized by elevated blood glucose levels due to disturbances in the body's metabolic system (Kyrou *et al*., 2020). Continuous or prolonged high blood glucose levels also cause complications of diabetes. Various complications occur when DM treatment is not optimal, such as microvascular and macrovascular which affect the quality of life and can cause death. Furthermore, obesity is a risk factor for type 2 DM and the relationship between both can be explained through 2 mechanisms

namely resistance to obesity-associated insulin and pancreatic beta-cell dysfunction. Obesity and metabolic disorders can cause beta cell decompensation, but when the condition is prolonged, this leads to hyperglycemia and hyperinsulinemia. An abnormality in the body's metabolism which leads to a decreased response or sensitivity to insulin action is called insulin resistance (Al-Goblan *et al*., 2014).

Glucose uptake into cells is regulated by insulin and occurs through a specific protein associated with the plasma membrane and facilitated by the glucose transporter. GLUT 4 is an important glucose transporter for maintaining glucose homeostasis in the blood. It is an insulin-responsive glucose transporter in the skeletal muscle, brain, heart, and adipose tissue. GLUT 4 is translocated to the plasma membrane in response to insulin secreted by beta cells as a glucose sensor to reduce postprandial blood glucose levels. Besides, insulin resistance in type 2 diabetes will cause disturbances molecules in the insulin-releasing system of the heart. After the release by pancreatic beta cells, insulin then induces the uptake of glucose into skeletal muscle through the binding to insulin receptors on the cell surface. Insulin resistance causes a reduction and impairment in GLUT4 translocation, leading to impaired glucose uptake and a high amount of circulating glucose (hyperglycemia) (Richter & Hargreaves, 2013; (Pereira *et al*., 2017).

Long-term high blood glucose levels in DM patients can trigger lipid and protein glycation, which increases advanced glycation end products (AGE). Meanwhile, AGE plays a significant role in the occurrence of various complications in diabetes. An increase can also stimulate the production of advanced glycation end product (RAGE) receptors. The interaction of AGE with RAGE can affect intracellular signal transduction, gene expression, the release of pro-inflammatory molecules, and the production of reactive oxygen species (ROS) that contribute to the pathology of diabetic vascular complications. The initial product of this reaction is called Schiff base, which will spontaneously form the Amadori product in diabetes known as HbA1c (Mendez, 2003; Peppa *et al*., 2003). Long-term glycemic status monitoring of DM patients can be carried out by measuring HbA1c to identify the quality of blood glucose control between 2-3 months. Normal HbA1c ranges from 4-6%, and extremely high levels potentially cause complications (Sherwani *et al*., 2016). Every 1% rise in HbA1c will increase the risk of microvascular and macrovascular complications by 37% and 14% respectively as well as cause a 21% increase in the risk of death from diabetes, hence, a decrease in HBA1c is beneficial for DM patients (Jiang *et al*., 2019).

Diabetes treatment using insulin and oral hypoglycemic drugs is still a problem due to the accompanying side effects, including hypoglycemia, and the inability to prevent complications in ROS-mediated DM. Consequently, alternative therapies are needed to reduce certain side effects, treatment costs, as well as for the prevention and treatment of vascular complications (Chaudhury *et al*., 2017; Al Kury *et al*., 2022). Meanwhile, the World Health Organization (WHO) recommends using herbs to treat DM when access to conventional medicine is inadequate. This condition can increase the use of natural products that have antidiabetic activity (Kasole *et al*., 2019).

One of the plants with antidiabetic effects is *Hibiscus surattensis* L. (HSL) from familia Malvaceae. The leaves have traditionally been used to treat various diseases (Anoopa John L *et al*, 2020) such as DM. Previous studies showed that the active fraction (FEA) of HSL leaves had antioxidant and antidiabetic effects. Moreover, this plant reportedly contains several secondary metabolites such as alkaloids, tannins, triterpenoids, and flavonoids including kaempferol, morin, and trifolin (Yuliet *et al*., 2020).

The compounds contained in the active fraction of HSL can also be used to treat diabetes and prevent damage or complications. Therefore, this study aims to examine the efficacy of HSL active fractions on HbA1c and AGEs levels, as well as GLUT 4 translocation in the skeletal muscles in diabetic rats, fed a high-fat diet and fructose.

MATERIAL AND METHODS

The materials used include Metformin (Glucophage 500®), CMC Na, standard rat feed (Rat Bio/CitraFeed)), fructose (Krystar®), rat HbA1c ELISA kit, rat Advanced Glycation End Products ELISA kit (Bioassay Technology Laboratory), insulin (Novarapid), rotary evaporator (Buchi rotavapor R-124, Buchi water bath B480), glucometer One Touch Horizon (Life Scan Johnson & Johnson), and microplate reader (Infinite M200 Pro). The high-fat feed consisted of 5% chicken liver, 10% beef fat, 10% goat fat, 10% quail eggs, 5% duck egg yolks, 20% corn flour, 20% fish meal, and 20% mung bean flour. Meanwhile, the test animals were male Wistar strain rats which were 3-

4 months old with body weight ranging from 150-200 grams and healthy physical condition with no defects or abnormalities. The study protocol was approved by Ethical Committee, Padjajaran University (No.51/UN6.KEP/EC/2019). The active fractions of ethyl acetate fraction/FEA used were made according to a previous study (Yuliet *et al*., 2020).

Experimental animals used were white male rats where they were first acclimatized for 1 week before testing, adjusted to the conditions, and their bodyweight was weighed. A total of 20 rats were used with 4 test groups, each consisting of 5 rats. The grouping was divided into two categories, namely the normal and the hyperglycemic induced rat group. Hyperglycemia induction was performed by giving fructose at a dose of 1.8 g/kg BW and a high-fat diet for 60 days. This was carried out based on several previous studies that used a high-fat and fructose diet in a rat model of insulin-resistant DM (Haroun *et al*., 2011; Lozano *et al*., 2016). Standard feed in the normal and high-fat feed in the induced animal group was given every day up to 20 g/rat. Before and after induction, fasting blood glucose levels were measured, while the Lee index and insulin tolerance test were calculated.

Hyperglycemic rats were divided into three groups (n=5) and one control which was not induced by diabetes. The normal and negative controls were given 0.5% Na CMC, the comparison group was given metformin 100 mg/kg BW, while the others were treated with FEA at a dose of 50 mg/kg BW. The treatment was given orally once a day for 21 days. Subsequently, the fasting blood glucose and KITT namely glucose decay constant percentage levels of test animals in all groups were measured on the 21st day after treatment. On day 81, the test animals were sacrificed, then blood was taken to measure insulin, HbA1c, and AGEs levels. Skeletal muscle tissue was also taken at the gastrocnemius part of each group at the end of the study, while the immunohistochemical examination was carried out using the GLUT4 polyclonal antibody. In addition, an immunohistopathological examination of the heart muscle tissue was conducted. The measurement of insulin, HbA1c, and AGEs levels was carried out using the ELISA kit.

Insulin resistance test

In the animal model, an insulin resistance or sensitivity test was performed using the insulin tolerance method. This was carried out by measuring the rat blood glucose levels every 15 minutes for 1 hour after insulin administration up to 0.5 IU/kg BW intraperitoneally. Insulin sensitivity is expressed in the KITT value, namely the gradient or slope of the curve multiplied by 100 from the linear regression curve of the natural logarithm of blood glucose levels to time. The K value represents insulin receptor sensitivity, and the low value indicates poor sensitivity (Ratwita *et al*., 2017).

Measurement of insulin, HbA1c levels and AGEs with ELISA method

Insulin levels were determined by the enzyme-linked immunosorbent assay method (ELISA). Tests are carried out according to the procedure for using the Bioassay Technology Laboratory® Rat Insulin ELISA kit. The HbA1c and AGEs levels were measured according to the kit instructions. Specifically, HbA1c level was measured with the standard solution available in the reagent ELISA Rat Hemoglobin A1c with a 240 ng/mL. Standard solution series were prepared using a diluent to obtain serial concentrations of 7.5, 15, 30, 60, 120, and 240 ng/mL. Meanwhile, AGEs levels were assessed with a standard solution available in Rat Advanced Glycation End Products ELISA reagent with a concentration of 1920 ng/mL. The standard solution series was formulated using a diluent to obtain serial concentrations of 60, 120, 240, 480, 960, and 1920 ng/mL, while a standard diluent was used as zero control (0 ng/mL).

Measurement of HbA1c and AGEs levels was carried out by preparing tools and material. Pre-coated ELISA microplate was washed with buffer 2 times, then solution sample containing test animal blood serum of 40 µL, standard solution and zero control 50 µL each, was then inserted into the wells. The mixture was added with 10 µL anti-HbA1c antibody for testing HbA1c levels and 10 µL anti-AGEs antibody for testing AGEs levels. Afterward, the sample and standard solutions were added with 50 µL of streptavidin-HRP each, covered with a plastic plate sealer, and incubated for 60 minutes at a temperature of 37°C. The solution was removed by inverting the microplate, then 350 µL of wash buffer was added to each well. The wash buffer was later removed, and the microplate was tapped against the tissue paper several times. The sample was washed and dried 5 times until there were no more droplets on the surface well. Next, 50 µL of substrate A and 50 µL of B were added to each well, covered with a plastic plate sealer, and incubated for 10 minutes at 37°C, a blue color will be formed according to concentration.

Subsequently, 50 µL stop solution was added to each well, shaken for 5 seconds, a color change with the appearance of yellow color was observed, and the absorbance was read at a wavelength of 450 nm with a microplate reader for 30 min. The absorbance was processed by linear regression, while the HbA1c and AGEs levels were obtained in units of ng/mL using the curve standard respectively.

Glucose transporter 4 (GLUT-4) protein translocation analysis with the immunohistochemical method

Immunohistochemical analysis of GLUT-4 was conducted according to Feitosa *et al*., (2018). Observations were made under a microscope with 400 times magnification. The results of gastrocnemius muscle tissue preparations with GLUT-4 antibody produced a color whose intensity was measured to determine the amount of GLUT-4 protein which reacts with these antibodies, the greater the reaction, the higher the intensity and brighter the color. Observations were carried out by taking photos of the preparations from a light microscope with 400x magnification in 5 fields of view. Gastrocnemius muscle cells containing the reacted GLUT-4 protein appear in the form of a brown-colored spot expressed in the cytoplasm. Quantification of GLUT-4 protein translocation used parameter extent, while the intensity was assessed using the Image J program with the IHC Profiler plug-in (Varghese *et al*., 2014).

RESULT AND DISCUSSION

An animal model of insulin resistance was formed through induction using a high-fat diet and fructose of 1.8 g/kg BW orally for 60 days The parameters measured were insulin tolerance test constants (KITT), insulin levels, HBA1c, AGEs, and GLUT-4 immunohistochemistry in the gastrocnemius muscle. Moreover, the comparison drug used was metformin which has a mechanism of increasing insulin sensitivity and is one of the oral antidiabetic drugs often prescribed for obese type 2 DM patients. The mechanism of action is to reduce glucose production in the liver, improve the uptake from tissues, and enhance the muscles and fat cells to absorb excess glucose from the bloodstream to lower blood sugar levels (Rena *et al*., 2017).

To induce obesity in the rats, the feed must contain fats ranging from 30-50% of the total calories. According to Kucera & Cervinkova, 2014, the standard feed contains less than 10% fat of the total calories, while high and very high-fat feeds contain 30-50% and more than 50% respectively. The administration of a high-fat and fructose feed at a dose of 1.8 g/kg BW for 60 days induced obesity, hyperglycemia, and insulin resistance. One of the causes of insulin resistance is obesity through increased production of free fatty acids which accumulate in the tissues, especially in the liver and muscles. Insulin resistance causes reduced lipase inhibition in fat cells, leading to increased lipolysis and production of free fatty acids which are then transported to the liver causing elevated VLDL production, apolipoprotein B, and triglyceride secretion (Choi & Ginsberg, 2011).

The provision of high-fat and fructose feed is expected to improve obesity conditions, which can trigger insulin resistance conditions, especially in muscles and the liver. A high-fat diet can increase the fat cell size and number, the increase in cell size through lipogenesis elevates the number of preadipocytes through proliferation and differentiation into mature adipocytes. It is regulated by the transcription factor sterol regulatory element-binding protein (SREBP)-1 (Pereira, Botezelli, *et al*., 2017). With an excessive intake of carbohydrates and saturated fatty acids (SFA), SREBP-1 will increase the synthesis of fats such as fatty acids, triglycerides, and phospholipids. Furthermore, a high-fat diet causes hyperinsulinemia which potentially increases the work of SREBP-1 adipocytes leading to hypertrophy of the cells. New adipocytes are then formed repeatedly by insulin growth factor-1 (IGF1) which leads to hyperplasia and an increase in free fatty acids (FFA). This condition affects insulin action, as well as decreases glucose uptake, glycolysis, and glycogen synthesis. Prolonged intake of a high-fat diet, especially for approximately 60 days can cause insulin resistance due to visceral fat accumulation. An increase in visceral fat mass can elevate the amount of free fatty acids (FFA) in the liver, as well as the circulating triglycerides and the rate of hepatic glucose production. Meanwhile, one of the causes of insulin resistance is abdominal obesity. There is a direct relationship between the consumption of a diet rich in fat and insulin sensitivity. This does not depend on the body weight but the quality of fat consumption, namely foods high in fatty acids (McArdle *et al*., 2013).

Table I. The mean Lee index of insulin resistance diabetic rats before and after induction, as well as after treatment

Groups (n=5 each group)	Lee index		
	Day 0 (before treatment)	Day 60 (after induction)	Day 81 (after treatment)
Normal control	275.3 ± 4.6	288.3 ± 3.3	286.2 ± 3.8#
Negative control	270.2 ± 2.8	376.1 ± 9.0*	416.6 ± 9.0
Metformin	276.7 ± 5.2	346.5 ± 7.1*	296.8 ± 7.4#
FEA 50	267.8 ± 3.1	354.7 ± 5.2*	287.7 ± 3.9#

Values are expressed in mean±SEM values, n=5, *: shows a significant difference with the normal group ($p<0.05$), #: shows a significant difference with the negative control ($p<0.05$)

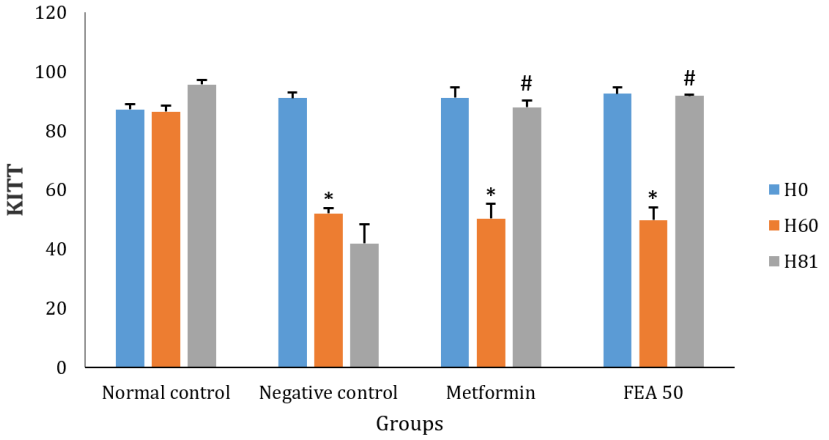

Figure 1. Insulin tolerance test

Notes: Values are expressed in terms of mean±SEM, n=5, *: significantly different from normal control ($p<0.05$); #: significantly different from the negative control group ($p<0.05$)

Obesity was determined based on the Lee index in which the rats were declared obese when the index value is >300 (Table I.) Compared with the normal value, the body mass index value on day 60 in the induced group was obese (Malafaia *et al*., 2013). Table 1 shows that the induction with a high-fat and fructose diet significantly increased the body mass index value, the rats fed for 60 days had a Lee index value ranging from 342.99-376.05. At the end of the treatment, after 21 days, all treatment groups except the negative control had a significantly lower Lee index value ($p<0.05$). This is caused by a reduction in the size and number of adipocytes, leading to a decrease in fat tissue mass as evidenced by a decline in body weight. This shows that the administration of metformin extract and the active fraction of *H. surattensis* L. leaves can reduce the body weight of insulin resistance test animals.

An insulin tolerance test is used to observe the sensitivity of the tissue to insulin. The results are often in the form of blood glucose levels, hence, it needs to be converted into a constant value of the insulin tolerance test (KITT). Insulin resistance refers to the sensitivity of tissues such as skeletal muscle, liver, kidneys, and adipocytes to insulin, leading to reduced utilization of blood glucose for metabolism, causing hyperglycemia. The obtained KITT value indicates insulin sensitivity, hence, an increase in the value leads to a proportional rise in insulin sensitivity. The induction with a high-fat and fructose diet in the test animals for 60 days caused insulin resistance characterized by a significant decrease in KITT as shown in Figure 2 compared to the normal group which had a value ranging from 40-42%. On the 81st days after treatment, a significant increase in KITT was observed in the groups treated with metformin and FEA with values amounting to 2,1 and 2,2 times respectively compared to the negative control. These results indicate that FEA increased sensitivity in insulin-resistant diabetic rats.

Table II. Mean insulin levels, HOMA-IR index, HbA1c and AGEs levels of insulin resistance after the end of treatment

Groups (n=5 each group)	Plasma insulin level (mIU)/L)	HOMA-IR	HbA1c (ng/mL)	AGEs (ng/mL)
Normal control	8.8±0.6$^{\#}$	1.60±0.02$^{\#}$	36.5±2.2$^{\#}$	327.0±15.3$^{\#}$
Negative control	14.2±0.4*	2.16±0.06	127.8±11.3*	614.3±29.4*
Metformin	13.1±0.5$^{*,\#}$	1.64±0.10$^{\#}$	58.0±2.3$^{*,\#}$	426.5±12.3$^{*,\#}$
FEA 50	11.5±0.9$^{*,\#}$	1.48±0.12$^{\#}$	55.7±1.5$^{*,\#}$	278.3±32.7$^{\#}$

Values are expressed in mean ± SEM, n=5, *: significantly different from the normal group ($p<0.05$); #: significantly different from the negative control ($p<0.05$)

The results of insulin, HbA1c, and AGEs levels (Table II) show that the negative control group had high insulin and fasting blood glucose levels. This indicates that insulin secreted by pancreatic cells did not reduce fasting blood glucose levels, which means that insulin resistance occurred. Furthermore, the HOMA-IR level parameter was used to determine the condition of insulin resistance, the higher the level, the greater the insulin resistance and vice versa. In the negative control group, the HOMA-IR level had the highest value indicating high insulin resistance, while the levels in the treatment group were lower. These results indicate that the administration of metformin and the active fraction of *H. surattensis* L. (FEA) increased insulin sensitivity. Based on the reference, a HOMA-IR value of 1.9 is indicated as an early symptom, while values > 2.9 represent high-risk insulin resistance (Table II).

The HbA1c levels increase in the negative control group compared to the normal (Table II). This phenomenon is related to the high glycation process of free radicals formed due to chronic hyperglycemia with hemoglobin lysine. When the blood glucose level is high, the non-enzymatic glycation process increases. The glycation also elevates the concentration of free radicals, which triggers an increase in HbA1c levels. High levels of HbA1c might be associated with more interactions between free radicals mainly malondialdehyde/MDA and hemoglobin lysine. The MDA aldehyde group reacts with lysine to form N-(h)-lysine amino acrolein (h-LAA). This radical then interacts with the glucose aldehyde to form HbA1c (Astari *et al*., 2017; Sharma *et al*., 2017).

The active fraction of *H. surattensis* L. leaves (FEA) reduced HbA1c levels in insulin-resistant diabetic rats by approximately twofold (Table II). This decrease was due to the chemical contents contained in FEA, which potentially prevent glycation and ward off free radicals. The inhibition of hemoglobin glycation by polyphenols is based on the binding to the reactive side of glucose in the form of a carbonyl by a polyphenol hydroxy group. Moreover, the decrease in MDA by polyphenolic compounds can prevent the reaction of these radicals with hemoglobin lysine. The low acrolein formed affects the reaction with the glucose aldehyde leading to a reduction in HbA1c formation (Sadowska-Bartosz & Bartosz, 2015). The decrease in HbA1c indicates decreasing blood glucose levels with increased insulin secretion.

The measurement results of the mean AGEs levels using the ELISA method (Table II). Based on the results, the administration of FEA inhibited the formation of AGEs which were significantly different compared to the negative control group. The ethyl acetate fraction showed the best potential in inhibiting the formation of AGEs. Long-term high blood glucose levels in DM patients trigger the process of lipid and protein glycation, which increases advanced glycation end products (AGE). Meanwhile, AGEs play a significant role in the occurrence of various complications in diabetes. They are a group of highly oxidized compounds implicated in the development of diabetes and several other chronic diseases (Sanchis *et al*., 2018). Besides, they are complex biochemicals formed by non-enzymatic glycation reactions between glucose and amino acids. The formation process known as the Maillard or Browning reaction occurs through a non-enzymatic reaction between the carbonyl group of reduced glucose, proteins, lipids, and nucleic acids. An increase in AGE can also stimulate the production of advanced glycation end product (RAGE) receptors. The interaction of AGE with RAGE potentially affects intracellular signal transduction, gene expression, the release of pro-inflammatory molecules, and the production of reactive oxygen species (ROS) that contribute to the pathology of diabetic vascular complications.

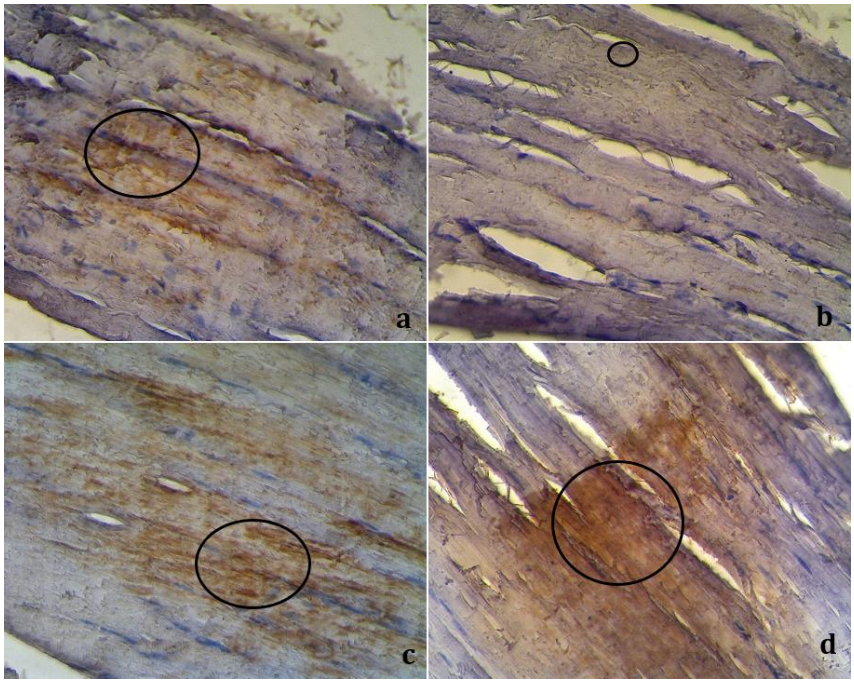

Figure 2. Immunohistochemical staining of normal control (a), negative control (b), metformin (c) and FEA (d). ◯ = GLUT-4 expression

AGEs can induce oxidative stress and inflammation through various bonds, which in turn cause pathological effects associated with long-term damage and dysfunction of several organs of the body, especially the eyes, kidneys, nerves, heart, blood vessels, as well as various complications, including atherosclerosis, neuropathy, kidney failure, and retinopathy (Moldogazieva *et al*., 2019; Cepas *et al*., 2020). Ramasamy *et al*., 2011 suggested that elevated expression of RAGE in diabetes is associated with increased glycosylation of hemoglobin. Glycosylated hemoglobin species are precursors of AGEs or ligands of RAGE as demonstrated in the correlation between HbA1c and AGEs of 0.772, which indicates a strong correlation. Moreover, decreased hyperglycemia contributes to a reduction in HbA1c levels. This was observed in the relationship between blood glucose level and HbA1c of 0.845, which shows a significant correlation.

One of the prominent features of type 2 DM is a decrease in the ability of insulin to act on peripheral target tissues, especially muscle and liver, which is known as insulin resistance. This condition can impair glucose utilization by insulin-sensitive tissues and increase hepatic glucose output leading to a reduction in the synthesis and translocation of glucose transporter 4 (GLUT-4) to cell membranes. GLUT-4 is present in several tissues, especially in the muscle and adipose tissue where translocation requires insulin and high glucose concentrations. Therefore, a decrease in GLUT-4 translocated to cell membranes will reduce the amount of glucose entering the cells. This leads to an increase in circulating blood glucose levels (hyperglycemia) because only a small amount of glucose can be transported into the tissues (Chadt & Al-Hasani, 2020).

GLUT-4 is a major glucose transporter located mainly in muscle and fat cells. Meanwhile, insulin binds to an insulin receptor substrate (IRS) located on the cell membrane causing the production of a valuable signal for regulating glucose metabolism in muscle and fat cells. After binding, signal transduction plays a role in increasing the quantity of GLUT-4 to occupy a position in the cell membrane. The process of synthesis and translocation of GLUT-4 potentiates the entry of glucose from the extracellular to the intracellular space for further metabolism (Abdul-Ghani & Defronzo, 2010).

The GLUT-4 protein was examined by counting the amount in gastrocnemius muscle cells stained by immunohistochemistry. The immunohistochemical technique used was the indirect method of forming antigen-antibody bonds (Figure 2). The antigen is the GLUT-4 protein present in skeletal muscle cells, while the primary antibodies are polyclonal and anti-GLUT-4. Besides, the secondary antibody is biotinylated and was labeled to visualize the antigen-binding (Jafri *et al*., 2019). The muscle cells observed were considered representative with unfolded cells, no overlapping cells, and no thick slices.

Staining was performed using DAB (Diaminobenzidine) to make the muscle tissue that contains GLUT-4 appear as dark-brown color. This indicates that some GLUT-4 are translocated to activated cell membranes and bound to antibodies. In DM patients, spots on muscle tissue will appear silvery-gray compared to dark-brown. This is due to the delivery of the PI-3 kinase signal, which leads to reduced GLUT-4 translocation to the cell membrane. This occurs in muscle tissue with insulin resistance diabetes mellitus. Furthermore, muscle cells containing the protein GLUT-4 have a blue nucleus surrounded by brown cytoplasm. The observation results of GLUT-4 protein expression in the gastrocnemius muscle tissue of insulin-resistant rats after treatment for 21 days (Figure 2) show that the negative control group has a fainter and thinner color intensity in the cell membrane than the normal group. This means that GLUT-4 expression in negative control is weaker compared to the normal group. Based on the results, long-term induced feeding of high fat and fructose diet can cause desensitization and downregulation of insulin receptors to interfere with intracellular GLUT-4 translocation to the plasma membrane of skeletal muscle cells leading to hyperglycemia. Meanwhile, the metformin and the active fraction of H. surattensis L. leaves showed a higher brown color intensity than the negative control. This implies that the metformin group and FEA increased the amount of GLUT-4 translocated to activated cell membranes.

The amount of GLUT-4 protein was examined using a light microscope with 400x magnification for each muscle slice (Figure 3).

Based on the results, the calculation showed that GLUT-4 protein in the negative control was significantly different from the normal group. This indicates that the hyperglycemic state causes a decrease in the amount of GLUT-4 protein in the gastrocnemius muscle of rats. Furthermore, the amount of the transport protein in the normal group, as well as metformin, and FEA showed almost the same high value, and there was no significant difference, indicating that the FEA can increase the amount of GLUT-4 protein translocation. There was an increase in the expression of the transport protein in the active fraction of *H. surattensis* L. leaves due to the presence of flavonoids such as kaempferol, morine, and trifolin which increase the secretion of the hormone glucagon-like peptide-1 (GLP-1) by L cells in the human small intestine. Glucagon-like peptide-1 activates phosphoinositide 3-kinase (PI3K) in skeletal muscle cells, which can increase GLUT 4 expression and glucose entry. Meanwhile, skeletal muscle is the main site for the use of consumed glucose transferred from the blood by GLUT-4. Disruption of this activity will inhibit the use of glucose in skeletal muscle leading to hyperglycemia, hence, one of the therapeutic targets is to increase the expression of GLUT-4 to reduce blood glucose levels (Hajiaghaalipour *et al*., 2015; Fang *et al*., 2019).

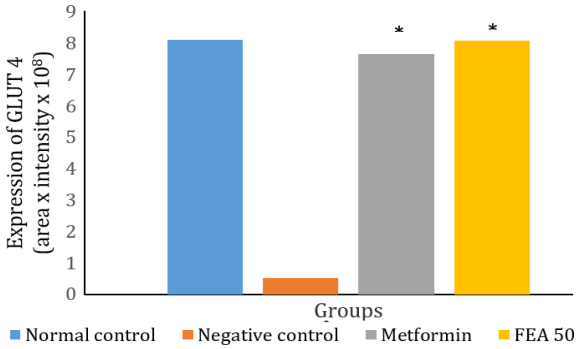

Figure 3. GLUT-4 protein expression of rat gastrocnemius muscle cells insulin resistance *: significantly different from negative control ($p<0.05$)

Based on the statistical correlation analysis, it can be concluded that the decrease in blood glucose levels was influenced by the increase in the amount of GLUT-4 protein in rats with insulin resistance DM. The probability value (p) was smaller than the 5% level of significance ($0.00 < 0.05$), meaning that there is a relationship between the decrease in blood glucose levels and the expression of GLUT-4. Moreover, the Pearson Correlation (r) test showed a value of -0.851, indicating that the relationship between decreasing blood glucose levels and GLUT-4 expression is strong. The negative value obtained implies that

there is an inverse relationship between a decrease in blood glucose levels and the amount of GLUT-4, hence, a decrease in blood glucose levels will proportionally increase the amount of GLUT-4 protein translocated to the gastrocnemius muscle cell membrane.

CONCLUSION

This study proves that the active fraction of *H. surattensis* L. (FEA) leaves can increase insulin sensitivity, reduce HbA1c and AGEs levels, as well as increase GLUT4 translocation as a complementary therapy in type 2 diabetes.

ACKNOWLEDGMENT

The authors are grateful to the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia, as well as the Indonesia Endowment Fund for Education (LPDP) for funding this study through the BUDI DN Scholarship.

REFERENCES

Abdul-Ghani, M. A., & Defronzo, R. A. (2010). Pathogenesis of insulin resistance in skeletal muscle. *Journal of Biomedicine and Biotechnology*, *2010*. https://doi.org/10.1155/2010/476279.

Al-Goblan, A. S., Al-Alfi, M. A., & Khan, M. Z. (2014). Mechanism linking diabetes mellitus and obesity. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, *7*, 587-591. https://doi.org/10.2147/DMSO.S67400.

Al Kury, L. T., Abdoh, A., Ikbariah, K., Sadek, B., & Mahgoub, M. (2022). In vitro and in vivo antidiabetic potential of monoterpenoids: An update. *Molecules*, *27*(1), 1-29. https://doi.org/10.3390/molecules27010182.

Anoopa John, L., Kannappan, N., & Manojkumar, P. (2020). Evaluation of hepatoprotective activity in methanolic extract of Aerial parts of *Hibiscus surattensis*. *Research J. Pharm. and Tech*, *13*(10), 4635-4640. https://doi.org/10.5958/0974-360X.2020.00816.1.

Astari, L., Cahyono, H., & Widjajanto, E. (2017). Correlation of interleukin-10, superoxide dismutase (SOD), and malondialdehyde (MDA) levels with HbA1c in pediatric type 1 diabetes mellitus. *Journal of Tropical Life Science*, *7*(3), 286-292. https://doi.org/10.11594/jtls.07.03.15.

Cepas, V., Collino, M., Mayo, J. C., & Sainz, R. M. (2020). Redox signaling and advanced glycation endproducts (AGEs) in diet-related diseases. *Antioxidants*, *9*(2), 1-20. https://doi.org/10.3390/antiox9020142.

Chadt, A., & Al-Hasani, H. (2020). Glucose transporters in adipose tissue, liver, and skeletal muscle in metabolic health and disease. *Pflugers Archiv European Journal of Physiology*, *472*(9), 1273-1298. https://doi.org/10.1007/s00424-020-02417-x.

Chaudhury, A., Duvoor, C., Reddy Dendi, V. S., Kraleti, S., Chada, A., Ravilla, R., Marco, A., Shekhawat, N. S., Montales, M. T., Kuriakose, K., Sasapu, A., Beebe, A., Patil, N., Musham, C. K., Lohani, G. P., & Mirza, W. (2017). Clinical Review of Antidiabetic Drugs: Implications for type 2 diabetes mellitus management. *Frontiers in Endocrinology*, *8*(January). https://doi.org/10.3389/fendo.2017.00006.

Choi, S. H., & Ginsberg, H. N. (2011). Increased very low density lipoprotein (VLDL) secretion, hepatic steatosis, and insulin resistance. *Trends in Endocrinology and Metabolism*, *22*(9), 353-363. https://doi.org/10.1016/j.tem.2011.04.007.

Fang, J. Y., Lin, C. H., Huang, T. H., & Chuang, S. Y. (2019). In vivo rodent models of type 2 diabetes and their usefulness for evaluating flavonoid bioactivity. *Nutrients*, *11*(3). https://doi.org/10.3390/nu11030530.

Feitosa, S. G., Viana, K. F., Luna, E. C. M., Costa, F. W. G., Cavalcante, R. B., Chaves, F. N., Chaves, H. V., & Pereira, K. M. A. (2018). Immunohistochemical evaluation of GLUT-3 and GLUT-4 in oral epithelial dysplasia and oral squamous cell carcinoma. *Asian Pacific Journal of Cancer Prevention*, *19*(7), 1779-1783. https://doi.org/10.22034/APJCP.2018.19.7.1779.

Hajiaghaalipour, F., Khalilpourfarshbafi, M., & Arya, A. (2015). Modulation of glucose transporter protein by dietary flavonoids in type 2 diabetes mellitus. *International Journal of Biological Sciences*, *11*(5), 508-524. https://doi.org/10.7150/ijbs.11241.

Haroun, M. A., Elsayed, L. A., Rashed, L. A., & Mohammed, M. A. (2011). The effect of high fat diet and high fructose intake on insulin resistance and GLP-1 in experimental animals. *Medical Journal of Cairo University*, *79*(2), 23-32. www.medicaljournalofcairouniversity.com.

International Diabetes Federation. (2021). *IDF*

Diabetes Atlas 10 th edition. www.diabetesatlas.org.

Jafri, A. A., Sharma, S. B., Khurana, N., M, M., Singh, U. R., & Luthra, K. (2019). Herbal anti-hyperglycemic compound increases expression of glucose transporter molecules in diabetic rats. *Journal of Diabetes & Metabolism*, *10*(4), 4-10. https://doi.org/10.35248/2155-6156.19.10.824.

Jiang, Z., Wang, J., Zhao, P., Zhang, L., & Guo, Y. (2019). HbA1c: High in acute cerebral infarction and low in brain trauma. *Progress in Molecular Biology and Translational Science* (1st ed., Vol. 162). Elsevier Inc. https://doi.org/10.1016/bs.pmbts.2019.01.008.

Kasole, R., Martin, H. D., & Kimiywe, J. (2019). Traditional medicine and its role in the management of diabetes mellitus: "patients'' and herbalists' perspectives"." *Evidence-Based Complementary and Alternative Medicine*, *2019*. https://doi.org/10.1155/2019/2835691.

Kucera, O., & Cervinkova, Z. (2014). Experimental models of non-alcoholic fatty liver disease in rats. *World Journal of Gastroenterology*, *20*(26), 8364-8376. https://doi.org/10.3748/wjg.v20.i26.8364.

Kyrou, I., Tsigos, C., Mavrogianni, C., Cardon, G., Van Stappen, V., Latomme, J., Kivelä, J., Wikström, K., Tsochev, K., Nanasi, A., Semanova, C., Mateo-Gallego, R., Lamiquiz-Moneo, I., Dafoulas, G., Timpel, P., Schwarz, P. E. H., Iotova, V., Tankova, T., Makrilakis, K., & Manios, Y. (2020). Sociodemographic and lifestyle-related risk factors for identifying vulnerable groups for type 2 diabetes: A narrative review with emphasis on data from Europe. *BMC Endocrine Disorders*, *20*(Suppl 1), 1-13. https://doi.org/10.1186/s12902-019-0463-3.

Lozano, I., Van Der Werf, R., Bietiger, W., Seyfritz, E., Peronet, C., Pinget, M., Jeandidier, N., Maillard, E., Marchioni, E., Sigrist, S., & Dal, S. (2016). High-fructose and high-fat diet-induced disorders in rats: Impact on diabetes risk, hepatic and vascular complications. *Nutrition and Metabolism*, *13*(1), 1-13. https://doi.org/10.1186/s12986-016-0074-1.

Malafaia, A. B., Nassif, P. A. N., Ribas, C. A. P. M., Ariede, B. L., Sue, K. N., & Cruz, M. A. (2013). Obesity induction with high fat sucrose in rats. *Arquivos Brasileiros de Cirurgia Digestiva : ABCD = Brazilian Archives of Digestive Surgery*, *26 Suppl 1*(Suplemento 1), 17-21.

McArdle, M. A., Finucane, O. M., Connaughton, R. M., McMorrow, A. M., & Roche, H. M. (2013). Mechanisms of obesity-induced inflammation and insulin resistance: Insights into the emerging role of nutritional strategies. *Frontiers in Endocrinology*, *4*(MAY), 1-23. https://doi.org/10.3389/fendo.2013.00052.

Moldogazieva, N. T., Mokhosoev, I. M., Mel'Nikova, T. I., Porozov, Y. B., & Terentiev, A. A. (2019). Oxidative stress and advanced lipoxidation and glycation end products (ALEs and AGEs) in aging and age-related diseases. *Oxidative Medicine and Cellular Longevity*, *2019*. https://doi.org/10.1155/2019/3085756.

Pereira, R. M., Botezelli, J. D., da Cruz Rodrigues, K. C., Mekary, R. A., Cintra, D. E., Pauli, J. R., da Silva, A. S. R., Ropelle, E. R., & de Moura, L. P. (2017). Fructose consumption in the development of obesity and the effects of different protocols of physical exercise on the hepatic metabolism. *Nutrients*, *9*(4), 1-21. https://doi.org/10.3390/nu9040405.

Pereira, R. M., De Moura, L. P., Muñoz, V. R., Da Silva, A. S. R., Gaspar, R. S., Ropelle, E. R., & Pauli, J. R. (2017). Molecular mechanisms of glucose uptake in skeletal muscle at rest and in response to exercise. *Motriz. Revista de Educacao Fisica*, *23*, 1-8. https://doi.org/10.1590/S1980-6574201700SI0004.

Ramasamy, R., Yan, S. F., & Schmidt, A. M. (2011). Receptor for AGE (RAGE): Signaling mechanisms in the pathogenesis of diabetes and its complications. *Annals of the New York Academy of Sciences*, *1243*(1), 88-102. https://doi.org/10.1111/j.1749-6632.2011.06320.x.

Ratwita, W., Sukandar, E. Y., Adnyana, I. K., & Kurniati, N. F. (2017). Alpha mangostin and xanthone from Mangosteen (*Garcinia mangostana* L.) role on glucose tolerance and glucose transporter-4 in diabetes mellitus. *International Journal of Pharmacognosy and Phytochemical Research*, *9*(9), 1206-1212. https://doi.org/10.25258/phyto.v9i09.10307.

Rena, G., Hardie, D. G., & Pearson, E. R. (2017). The mechanisms of action of metformin. *Diabetologia*, *60*(9), 1577-1585. https://doi.org/10.1007/s00125-017-4342-

z.

Richter, E. A., & Hargreaves, M. (2013). Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiological Reviews*, *93*(3), 993-1017. https://doi.org/10.1152/physrev.00038.2012.

Sadowska-Bartosz, I., & Bartosz, G. (2015). Prevention of protein glycation by natural compounds. *Molecules*, *20*(2), 3309-3334. https://doi.org/10.3390/molecules20023309.

Sanchis, P., Rivera, R., Berga, F., Fortuny, R., Adrover, M., Costa-Bauza, A., Grases, F., & Masmiquel, L. (2018). Phytate decreases formation of advanced glycation end-products in patients with type II diabetes: randomized crossover trial. *Scientific Reports*, *8*(1), 1-13. https://doi.org/10.1038/s41598-018-27853-9.

Sharma, M., Arora, M., Mustafa, I., Kumar, S., Mittal, A., & Soam, S. S. (2017). Correlation of glycated hemoglobin with oxidative stress and erythrocyte fragility in type-2 diabetes mellitus. *International Journal of Contemporany Medical Research*, *4*(9), 1909-1911.

Sherwani, S. I., Khan, H. A., Ekhzaimy, A., Masood, A., & Sakharkar, M. K. (2016). Significance of HbA1c test in diagnosis and prognosis of diabetic patients. *Biomarker Insights*, *11*, 95-104. https://doi.org/10.4137/Bmi.s38440.

Varghese, F., Bukhari, A. B., Malhotra, R., & De, A. (2014). IHC profiler: An open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PLoS ONE*, *9*(5). https://doi.org/10.1371/journal.pone.0096801.

Yuliet, Sukandar, E. Y., & Adnyana, I. K. (2020). Active subfractions, phytochemical constituents, dipeptidyl peptidase-IV inhibitory activity and antioxidant of leaf extract from *Hibiscus surattensis* L. *The Natural Products Journal: Vol. 10(4)* (pp. 400-410). https://doi.org/10.2174/2210315509666190626125330.