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Antioxidant Effect of Snakehead Fish (*Channa Striata*) Albumin Extract on Diabetic Rats (*Rattus Norvegicus*)

Nurlita Abdulgani[1,*], Dwi Winarni[2], Win Darmanto[2], Dewi Hidayati[1] and Muhammad Zamharir Rojafi[1]

[1]*Department of Biology, Faculty of Science and Data Analytics, Sepuluh Nopember Institute of Technology, Surabaya 60115, Indonesia*
[2]*Department of Biology, Faculty of Science and Technology, Airlangga University, Surabaya 60115, Indonesia*

([*]Corresponding author's e-mail: nurlita@bio.its.ac.id)

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Abstract

Snakehead fish extract (SHE) has been traditionally used for diabetes therapy due to its antioxidant properties, yet its effects in high-fat diet (HFD) and streptozotocin (STZ)-induced diabetic models remain underexplored. This study evaluated the antioxidant and antidiabetic effects of SHE in diabetic rats by measuring malondialdehyde (MDA), glutathione (GSH), GLUT-4 density and HOMA-IR index. Thirty Wistar rats were divided into five groups: Healthy control (C−), diabetic control (C+) and diabetic rats treated with low (LD: 2 mL/day), medium (MD: 3 mL/day) and high (HD: 4 mL/day) doses of SHE for 28 days. SHE was administered orally following HFD-STZ induction. SHE significantly reduced MDA levels in diabetic rats to levels comparable with healthy controls (p-value < 0.05), though GSH levels showed no consistent improvement. GLUT-4 density in striated muscle membranes increased significantly in all SHE-treated groups (p-value < 0.05) and fasting blood glucose levels decreased, though not to normoglycemic levels. The HOMA-IR index was also significantly reduced in MD and HD groups, indicating improved insulin sensitivity. Antioxidant activity was confirmed via the ABTS assay, with 50% radical inhibition at 125 µg/mL. In conclusion, SHE demonstrates dose-dependent antioxidant and antidiabetic effects by lowering MDA levels, enhancing GLUT-4 density, reducing blood glucose and improving insulin resistance. However, this study has limitations, including the use of blood-based biomarkers for MDA and GSH, which may not fully reflect tissue-specific oxidative stress. Additionally, the total protein analysis did not identify the specific albumin content or amino acid composition of SHE, which should be addressed in future studies to better understand its bioactive components and mechanisms.

Keywords: Antioxidant, Diabetes, *Channa striata*, GLUT-4, GSH, HOMA-IR, MDA

Introduction

Diabetes mellitus (DM) is a chronic metabolic condition characterized by persistently high blood sugar levels that is becoming more and more common [1]. DM is typically categorized as type 1 diabetes, also known as insulin-dependent diabetes mellitus; type 2 diabetes, referred to as non-insulin-dependent diabetes mellitus; various forms of diabetes mellitus; and gestational diabetes mellitus [2,3]. Estimated that 642 million people worldwide are thought to have diabetes mellitus (DM) and by 2,045, that figure is predicted to rise to 800 million. Whereas type 2 diabetes mellitus (T2DM) impacts approximately 463 million adults aged 20 to 79 years and is anticipated to affect 578 million individuals by 2,030 [4].

T2DM is a metabolic condition defined by high blood sugar levels caused by insulin resistance and/or insufficiency. If individuals with T2DM do not adequately regulate their blood sugar levels, they may rise and fall in an unpredictable way, leading to difficulties [2]. Insulin resistance medically

characterized as a condition of decreased responsiveness in insulin-targeting tissues to high physiological insulin levels. Insulin levels rise to satisfy normal insulin needs in prediabetes, which causes chronic hyperinsulinemia, β-cell failure from hyperglycemia and ultimately T2DM [5]. This condition of impaired insulin-stimulated glucose (insulin resistance) can reduce the delivery of glucose transporter type 4 (GLUT-4) to the cell surface [6]. GLUT-4 is an intracellular protein that requires a stimulus (insulin or exercise) to migrate to the plasma membrane and promote glucose absorption. In unstimulated circumstances, glucose transport is restricted by the intracellular localization of GLUT-4 [7]. GLUT-4 expression can also be suppressed by oxidative stress (OS), which is commonly elevated in T2DM patients. The increase in blood glucose levels is without a doubt the main contributor to OS. The accumulation of glycolysis intermediates, the activation of the polyol pathway, the generation of advanced glycosylation end products (AGEs), the activation of Protein Kinase C (PKC) and the activation of the hexosamine pathway are some of the complicated interactions that lead to OS in diabetes [8]. The harmful effects of OS lead to β-cell malfunction and apoptosis, which further impairs insulin secretion and synthesis [9]. OS in the body can be seen through the examination of Malondialdehyde (MDA) concentration, which is an end product of lipid peroxidation that has been an oxidative stress biomarker in various biological samples [10]. While the calculation of insulin resistance formula based on serum glucose and insulin levels such as homeostasis model assessment-insulin resistance (HOMA-IR) that have been proven [11] and commonly used [12].

The suggested initial strategy to T2DM management is a combination of effective lifestyle changes and the use of pharmacological medications. In recent years, numerous orally given and injectable medications have been developed to treat T2DM patients. However, none of these synthetic medications are free of harmful effects [13]. Commonly used medications in clinical practice have the potential to disrupt glucose homeostasis, resulting in impaired glucose tolerance, hyperglycemia, or newly diagnosed diabetes mellitus, or they may exacerbate glycemic control in diabetics [14]. This makes natural therapies preferable to traditional anti-diabetic medicines since they are safer for the body, less expensive and have fewer potential side effects [15].

Snakehead fish (*Channa striata*) is a significant freshwater fish species in various Asian countries. In Indonesia, it is mostly found on Sumatra, Java and Borneo [16]. In addition to being used as fish food and as ornamental fish, snakeheads are also used to make pharmaceutical products like albumin [17]. Preliminary research by [18] has proven that snakehead fish extract (SHE) has the potential to become a diabetes drug, as shown by an increase in the size of the diameter of the islets of Langerhans, which indicates an improvement in pancreatic tissue by 68.78% and is able to reduce blood glucose levels by 34.42%. When compared to other animal protein sources, snakehead fish have an extremely high albumin proportion of protein, reaching more than 50% [19]. The albumin fraction of the snakehead fish makes up about 64.61% of its total protein and is of higher quality than the albumin found in egg whites [20]. Snakehead fish possess the highest amounts of glutamic acid (32.39%), glycine (9.60%) and cysteine (6.61%), respectively [21]. According to [22], the three amino acids are precursors to the formation of the endogenous antioxidant glutathione (GSH). Therefore, there is an interaction between the three amino acids to increase the formation of GSH as an antioxidant, which can ultimately reduce oxidative stress in diabetics, as indicated by a decrease in blood MDA concentration. The decrease in oxidative stress will affect the decrease in the level of tissue damage and it is expected that there is an improvement in pancreatic β-cells and insulin resistance in insulin-sensitive muscle cells, which is indicated by a decrease in the HOMA-IR Index. Reports on the effect of SHE on insulin sensitivity, GLUT-4 density in skeletal muscle and oxidative stress in high-fat diet (HFD)/streptozotocin (STZ)-induced type 2 diabetic rats are not available in the literature. Therefore, this study was conducted to determine the effect of SHE on HFD/STZ-induced type 2 diabetic rats.

Materials and methods

Extraction of snakehead fish

Snakehead fish used for this extract is cultivated from Blitar Regency and previously has been identified in Laboratory of Zoology, Department of Biology,

Sepuluh Nopember Institute of Technology. The extraction of snakehead fish refers from [18,23,24]. 10 kg fish aged one year were cleaned (removed scales, gills, entrails and head) then washed until clean and free of blood and mucus. The fish were filleted and extracted under vacuum at 70 °C for 3 h. The net weight of the fillets was approximately 50% about 5 kg. After extraction, about 1 L of extract is obtained with a yield of 3.5 - 4 kg.

Total protein test in SHE

Measurement of total protein content was carried out with the Bradford Test modified from [25]. In this test, Coomassie Brilliant Blue (CBB) dye is used, which binds to proteins and will produces a bluish color. Absorbance is measured using spectrophotometry at a wavelength of 465 - 595 nm. The Bradford reagent was prepared using 0.05 g of CBB G-250 as much as 0.05 g, 95% ethanol as much as 25 mL and phosphoric acid (H_3PO_3) as much as 50 mL. The three were then homogenized and H_2O was added to 500 mL. After that, it was stored in a dark bottle at 4 °C. Before the test, BSA standard solution was prepared with concentrations of 0 mg/mL, 20 mg/mL, 40 mg/mL, 60 mg/mL, 80 mg/mL and 100 mg/mL. Each solution that has been allowed to stand at room temperature for 15 min is measured for absorbance using a spectrophotometer with a wavelength of 595 nm. Blanko was made from 0.4 mL of H_2O, which was added with 4 mL of Bradford reagent; the absorbance was then measured using a spectrophotometer at a wavelength of 595 nm. Sample measurements were made by adding 0.2 mL of sample (SHE) with Bradford reagent as much as 2 mL. After being homogenized with a vortex, it was then allowed to stand for 15 min at room temperature. Then read the absorbance with a spectrophotometer with a wavelength of 595 nm. After that, the protein content was calculated using the formula y = ax + b, where y = sample absorbance, a = slope, b = intercept and x = protein content in the sample.

Antioxidant activity in SHE with ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid))

This method was performed to measure the inhibitory activity of ABTS radicals. The antioxidant test with the ABTS method is based on the loss of blue color due to the reduction of ABTS by antioxidants. This test was carried out using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) with ten replications and the method used was done by [26]. The solution used in this method is a mixture of 7 mM ABTS solution and 12 mM potassium persulfate ($K_2S_2O_8$) solution. The mixed solution was incubated in dark conditions for 24 h at room temperature.

Prior to the measurement of free radical activity with ABTS solution, SHE samples were made into a series of concentrations of 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL and 1,000 µg/mL, respectively. The samples then mixed with 100 µg/L of ABTS working solution and incubated at room temperature for 60 min. After incubation, the absorbance was determined using spectrophotometry with a wavelength of 745 nm. The scavenging activity was calculated using the following formula:

$$\%\ Attenuation\ activity = (Abs\ control - sample\ Abs) / Abs\ control\ x\ 100 \quad (1)$$

The lower the absorbance, the higher the free radical scavenging activity. The IC50 value is the concentration of extract required to inhibit 50% of ABTS radicals.

Experimental animals

The total of 30 Wistar rats within age two until three months and weighing between 200 - 250 g were used for the experiment. The animals were placed in a cage with controlled environment and had free access to water and were provided daily with standard pelleted chow diet *ad libitum*. This research was approved by the ethics research committee, Animal Care and Use Committee (ACUC), Airlangga University, Indonesia, with a grant number No.2. KE.026.02.2018. The experimental animals were grouped into five groups with two control groups and three treatment groups, as shown in **Table 1**.

Table 1 Group assignment of experimental animals.

Groups	Treatment
C–	Healthy rats control with no SHE treatment
C+	Diabetic rats control with no SHE treatment
LD (Low Dose)	Diabetic rats with low dose SHE treatment in 2 mL/day
MD (Mid Dose)	Diabetic rats with intermediate dose SHE treatment in 3 mL/day
HD (High Dose)	Diabetic rats with high dose SHE treatment in 4 mL/day

Induction of diabetes

Rats were conditioned on a HFD before STZ induction by orally administering 2 mL lard for 28 days [27]. After being treated with a HFD for 28 days, rats blood glucose levels were measured before induction of T2DM with 30 mg/kg BW streptozotocin (STZ) in citrate buffer with pH 4.5 intraperitoneally [27]. One week after induction, blood glucose levels were measured again with Glucotest. If the fasting blood glucose level exceeds 200 mg/dL, then the rats can be confirmed in hyperglycemia condition and can be used as diabetic experimental model.

Treatment with SHE

SHE treatment was given orally every day for 28 days. The dose of SHE given to hyperglycemia rats refers to research conducted by Abdulgani [18]. The terms LD, MD and HD refer to the provisions of the drug dose calculation. The treatment was carried out for four weeks (28 days) and during the treatment, blood glucose levels and body weight were measured weekly. On day 29 after the end of the study, rats were fasted for 12 h. Rats were anesthetized using 90 mg/kg BW ketamine and 5 mg/kg BW xylazine prior to dissection for an intracardiac blood collection for fasting blood glucose, MDA and GSH levels analysis. Organ harvesting is done after blood collection and in a state where the rats are still anesthetized. Then confirmation of the death of experimental animals is done by cutting the aorta. Intracardiac blood samples obtained were centrifuged at 3,000 rpm for 10 min. Then the plasma was stored at –20°C for further analysis of MDA, GSH and insulin levels.

Blood sugar measurement

Measurement of blood glucose levels was performed weekly during maintenance and treatment. Blood sampling was done from the tip of the rat's tail. Rat blood glucose and fasting blood glucose level test were performed weekly using the ACCU-Chek® Active Kit (Roche Diagnostics, Mannheim, Germany).

Plasma insulin and HOMA-IR measurement

Plasma insulin concentration was measured using the Rat Insulin ELISA Kit (Elabscience®, Catalog No: E-EL-R2466 96T). Meanwhile, the calculation of the insulin resistance indicator (HOMA-IR) was performed using the formula HOMA-IR = fasting blood glucose (mg/dL)×fasting insulin (ng/mL)/22.5. The formula was taken and elaborated from the HOMA-IR formula written by [28] with the Eq. (2):

$$HOMA - IR = fasting\ blood\ glucose\ (mmol/L) \times fasting\ insulin\ (\mu U/mL)\ /\ 22.5 \quad (2)$$

where fasting blood glucose conversion unit 1 mmol/L = 18.018018 mg/dL and insulin unit 1 μU/mL = 0.04 ng/mL).

MDA analysis in diabetic rats after SHE treatment

Malondialdehyde (MDA) levels in blood plasma were analyzed using the MDA ELISA Kit from Elabscience® (Catalog No. E-EL-0060 96T). MDA assay was performed according to the kit procedures and the optical density was measured at 450 nm.

GSH analysis in diabetic rats after SHE treatment

The measurement of glutathione (GSH) levels in SHE was conducted using the Reduced Glutathione Kit

(Elabscience® Assay, Catalog No. E-BC-K030) to determining GSH concentration in blood plasma. This is followed by centrifugation at 3,500 rpm for 10 min, after which the supernatant is collected for analysis. After mixing the supernatant thoroughly and allowing it to stand for 5 min, the optical density of each well is measured at a wavelength of 405 nm. The GSH content in serum (plasma) is then calculated using the formula:

$$GSH\ \left(\frac{\mu mol}{L}\right) = \frac{(OD\ sample - OD\ blank)}{(OD\ standard - OD\ blank)} \times Concentration\ of\ standard \times Dilution\ factor\ of\ sample\ pretreatment \quad (3)$$

where the concentration of standard is 20μmol/L.

GLUT-4 analysis in striated muscle cells

Striated muscle slides preparation

Using immunohistochemical staining to preparing the striated muscle from the processing stage to sectioning is based on Bancroft's (1982), with some changes made to fit the needs of the Histology Laboratory at Airlangga University's Department of Biology. The rat tongue that has been fixed using neutral buffer formalin (NBF) 10% is cut and washed for further hydration, clearing and embedding in paraffin blocks. The muscle then cut with a thickness of 4 μm, which is then placed on a poly-L-lysine-coated slide for further deparaffinization stage. The next step involves placing the slides containing samples in a container containing sodium citrate buffer, boiling them for 20 min and then removing them for 10 min. The muscle tissue is then stained with immunohistochemistry, left to sit overnight in anti-GLUT-4 and washed with TBS. Finally, the specimen was dehydrated in ethanol solutions and xylene. Entellan was added and the specimen was covered with a cover glass. Samples were then observed using the Optilab® system attached to a light microscope with 100×10 magnification, while ImageRaster® was used to analyze the samples images.

GLUT-4 density measurement

GLUT-4 density was observed semi quantitatively by observing the presence of GLUT-4 on the cell surface in tongue muscle histology preparations stained by immohistochemical staining with anti-GLUT-4 using DAB chromogen and hematoxylin counterstain. GLUT-4 density data was obtained by counting the number of GLUT-4 located in the striated muscle cell membrane that was sliced transversely. In addition, cell diameter measurements were made to determine the circumference of striated muscle fiber. Based on [29], GLUT-4 staining resulted in the formation of brown granules. Each GLUT-4 granule, whether clustered or not, was counted as a single unit. Calculation of GLUT-4 density using a binocular microscope equipped with an ocular micrometer with a magnification of 100×10. The formula to determine the density of GLUT-4 in the striated muscle membrane is as follows:

$$GLUT-4\ density\ in\ membrane = \frac{Number\ of\ GLUT-4}{Fiber\ circumference} \quad (4)$$

Data analysis

Statistical analyses of MDA levels, GSH levels, blood glucose levels, HOMA-IR index and GLUT-4 density in striated muscle membrane were performed using IBM SPSS Statistics 21 software while the image analyses were performed using ImageRaster®. Data are presented as mean ± standard deviation (SD). Normality of data distribution was assessed using the Kolmogorov-Smirnov test and homogeneity of variances was evaluated with Levene's test. If the data were normally distributed and variances were homogeneous, a one-way analysis of variance (ANOVA) was conducted, followed by Duncan's multiple range test to identify significant differences (p-value < 0.05) between treatments. In cases where variance homogeneity was not met, the Brown–Forsythe test was applied. Comparisons between two treatment groups were made using independent t-tests. A significance level of α = 0.05 was used throughout, with a 95% confidence interval (CI).

Results and discussion

Snakehead fish extract (SHE) antioxidant activity

Prior to the antioxidant activity test, the total protein content of SHE was tested. Protein levels in snakehead fish extract were quantitated using the Bradford method with BSA (bovine serum albumin) as the standard. As a standard, BSA was used in linear regression analysis in the quantitation of protein content and the linear regression equation obtained was

$y = 0.0097x + 0.938$ with $R^2 = 0.9836$. The results of the analysis of protein content in snakehead fish extract showed that the total protein content was 41.151 mg/mL. The results of the protein content test on snakehead fish extract show that the protein content equivalent to the standard, namely BSA, is quite large. With a large enough protein content, this supports that the protein from snakehead fish extract is responsible for antioxidant activity, especially the amino acids that make up the protein in snakehead fish extract are allegedly responsible for its bioactivity in reducing free radicals.

The antioxidant activity test in this study used ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), with the results shown in **Table 2**. Based on the **Table 2**, it is known that the antioxidant activity test with ABTS shows results with a trend of increasing concentration and increasing percent inhibition.

Table 2 Antioxidant activity of snakehead fish extract (SHE) with ABTS.

SHE Concentration (µg/mL)	ABTS Inhibition (%)
31.25	13 ± 0.39
62.5	33 ± 5.25
125	50 ± 0.33
250	76 ± 0.3
500	81 ± 0.39
1,000	85 ± 0.63

The antioxidant test with the ABTS method is based on the loss of blue color due to the reduction of ABTS by antioxidants. The ABTS assay measures antioxidant activity based on the decolorization of the radical cation 2, 20-azino-bis(3-ethylbenzotiazoline-6-sulphonicacid), reducing blue color intensity [30]. The ability of SHE to reduce ABTS free radicals is evidence that the test sample has antioxidant activity, which can be proven by the percentage value of inhibition in the test sample (SHE). In **Table 2** and **Figure 1**, it can be seen that at a concentration of 125 µg/mL, SHE is able to reduce ABTS radicals above 50% and the percentage of inhibition increases with increasing SHE concentration.

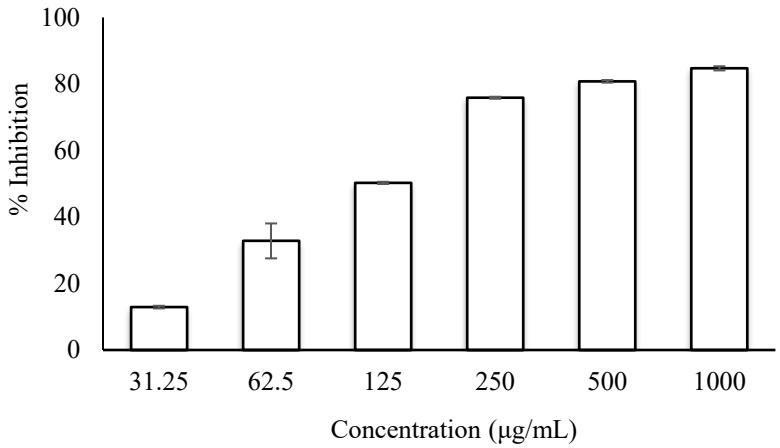

Figure 1 Inhibition activity of snakehead fish extract (SHE) by ABTS method with ten replications.

The inhibition percentage of ABTS radicals by snakehead fish extract is significantly high. The SHE concentration of 125 µg/mL is able to inhibit ABTS radicals above 50%. SHE is rich in protein, with

albumin as its predominant fraction. If we look at the content of amino acid in albumin, there are lysine, methionine, cysteine and arginine [31]. These amino acids possess functional groups such as thiol, amine, or thioether, which contribute to their antioxidant activity [32]. The main site on albumin involved in antioxidant activity is Cysteine (Cys34), which contains a sulfhydryl group and through reduced Cys34, albumin can reduce hydroxyl radicals. Sulfhydryl groups (also called "thiol groups") consist of a sulfur atom with two free electron pairs, bonded to hydrogen. Groups that have sulfhydryl groups, such as cysteine, have an important role in the extracellular antioxidant defense system against oxidative stress. It is reported that under oxidative stress conditions, protein sulfhydryl groups (-SH) will disappear, as thiol groups can undergo oxidation to form disulfide bonds [30]. Albumin is also involved in metal binding to proteins (chelating) [32]. The results of the protein content test on snakehead fish extract show that the protein content equivalent to the standard, namely BSA, is quite large. With a large enough protein content, this supports that the protein from snakehead fish extract is responsible for antioxidant activity, especially being able to reduce ABTS free radicals. The amino acids that make up the protein in snakehead fish extract are allegedly responsible for its bioactivity in reducing ABTS free radicals.

Previous research [33] stated that the antioxidant capacity of SHE is higher when compared to the antioxidant capacity of sea bass (0.43 μM) and rohu fish (0.025 μM). In addition, the antioxidant capacity of snakehead fish is high with a percentage inhibition of 56.7 ± 1.21% per 10 mg crude protein concentrate [34]. One of the amino acids that make up albumin in SHE is the amino acid cysteine (Cys) with a thiol group (–SH) at the end, which interacts directly with free radicals. Albumin is known to consist of 17 disulfide bonds to stabilize protein secondary structure [35]. The true mechanism of peptide antioxidant activity is not yet fully understood but various studies have shown peptides can act as; lipid peroxidation inhibitors, free radical scavengers and chelators [36]. However, the analysis of total protein content did not show the albumin content and amino acid composition contained in SHE and this is a limitation that should be addressed in future studies on SHE.

Diabetic induction with High-fat diet Low-dose streptozotocin (HFD-LD STZ)

Induction of diabetic model rats with high-fat diet (HFD) treatment or high-fat food with lard and low-dose streptozotocin injection (LD STZ) of 30 mg/BB aims to get diabetic DM 2 model rats [37]. This method was also used in the several studies [38-40]. This model involves the combination of a high-fat diet to induce insulin resistance, which is one of the core hallmarks of T2DM. The followed STZ treatment afterwards can result in a β-cell malfunction in animals. Thus, the Rat models fed a high-fat diet in conjunction with STZ were used for studies that closely matched the natural course of illness episodes and metabolic features of human T2DM [41].

The indicator used to measure the success of HFD treatment is indicated by an increase in body weight [37]. The results of weight gain of the rat groups without and with HFD treatment groups are presented in **Table 3**. Based on this table, rats treated with HFD for 5 weeks had an average weight gain of 17.3 g, while the untreated group had a lower average weight gain of 4 g.

Table 3 Mean body weight (BW) of rats after HFD treatment for 5 weeks.

Group	n	Initial BW (g)	Final BW (g)	Weight gain (g)
Without HFD	5	234.8	238.8	4
With HFD Treatment	20	223.2	240.5	17.3

Rats that had been treated with HFD for 5 weeks and had experienced weight gain were then treated with LD STZ induction with a 30 mg/BB STZ injection. STZ injection will cause damage to pancreatic β cells that have diabetogenic action, which will cause an increase in blood glucose levels

(hyperglycemia) [42] and weight loss [43]. **Figure 2** shows that after STZ injection at week 5 (M5), there was a decrease in body weight in the C+, LD, MD and HD groups and after SHE treatment at week 6 (M6), the body weight of the LD, MD and LD treatment groups increased. The C+ group of diabetic rats that were not treated with SHE tended to decrease in body weight until week 10.

HFD-STZ induction in the C+, LD, MD and HD groups also caused a decrease in body weight at week 6, which is 1 week after STZ injection. Streptozotocin is a common tool to induce diabetes in animal models that affects the weight loss of rats [43]. With reduced insulin due to pancreatic damage, the body will respond by producing energy through glycogenolysis, gluconeogenesis and lipolysis. The destruction of protein and body fat results in weight loss in HFD-STZ-induced DM2 rats.

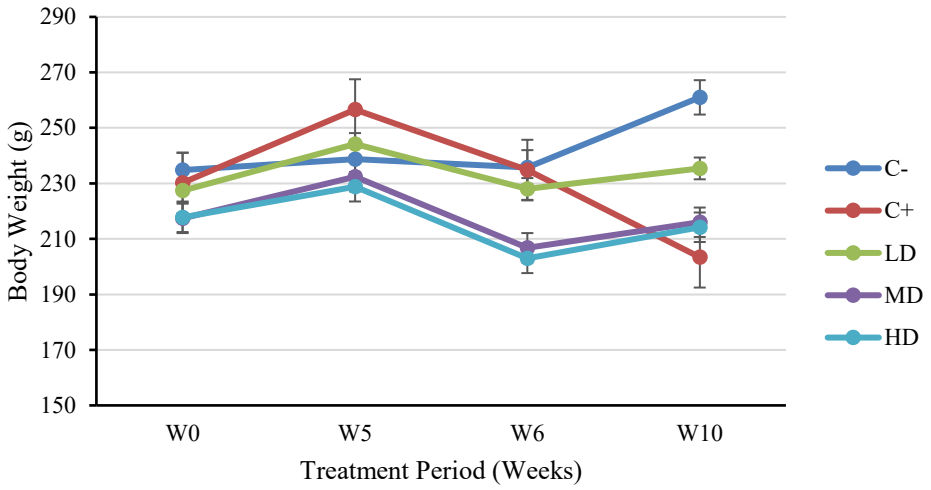

Figure 2 Graph of average body weight in rats from the beginning of treatment to the end of treatment Description: M0 = Week 0, beginning of HFD treatment; M5 = Week 5, end of HFD treatment, STZ injection; M6 = Week 6, beginning of SHE treatment after HFD-STZ induction; M10 = Week 19, end of SHE treatment.

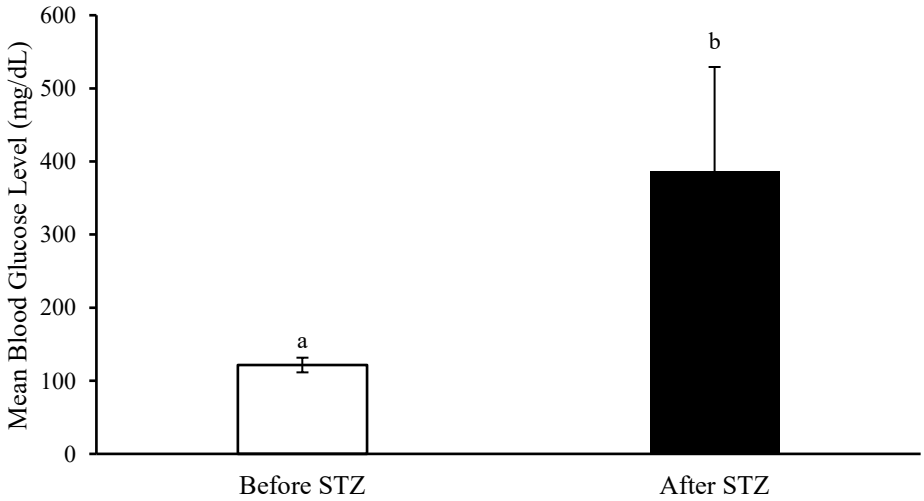

Figure 3 Graph of mean blood glucose levels before and after STZ injection. Different letter notation indicates significant difference in t-test (p-value < 0.05).

To ensure that the model rats have experienced hyperglycemia, blood glucose levels were measured at week 6, which is 1 week after STZ injection and expected blood glucose levels of test animals > 200 mg/dL. To determine the increase in blood glucose levels after STZ injection, a T-test was conducted to distinguish blood glucose levels before and after STZ injection. The Kolmogorov-Smirnov test results showed that the blood glucose level data were normally distributed so that further tests could be carried out for comparison between the two treatment groups using the T-test. The results of this test showed a significant difference (p-value < *0.05*) in blood glucose levels before and after LD STZ injection. One week after STZ injection, the mean blood glucose level was 386.24 ± 142.97 mg/dL, which was higher than the rats before STZ treatment, which was 121.56 ± 10.02 mg/dL (**Figure 3**). Thus, it can be confirmed that all STZ-injected rats have experienced hyperglycemia, with blood glucose levels > 200 mg/dL.

Before SHE treatment, all diabetic rats that had been induced by HFD-LD STZ were tested for homogeneity of blood glucose levels (GL) and body weight (BW). This is done so that the initial condition of the test animals is homogeneous so that it can be used as the initial condition of treatment before SHE administration. The initial BW data of each group was tested using the Kolmogorov-Smirnov test results on the initial BW, obtaining a probability value of 0.135 (p-value > *0.05*), which indicates that the distribution of data obtained is normally distributed. Based on the results of the homogeneity test (Levene's test), the significance of 0.650 (p-value > *0.05*) was obtained, which indicates that the variance obtained is homogeneous and so the data is tested using the ANOVA test. Based on the ANOVA test, p-value = 0.245 (p-value > *0.05*). This indicates that there is no difference in initial BW in all groups of rats (C+, LD, MD and HD) that will be treated with SHE, as shown in **Figure 4**.

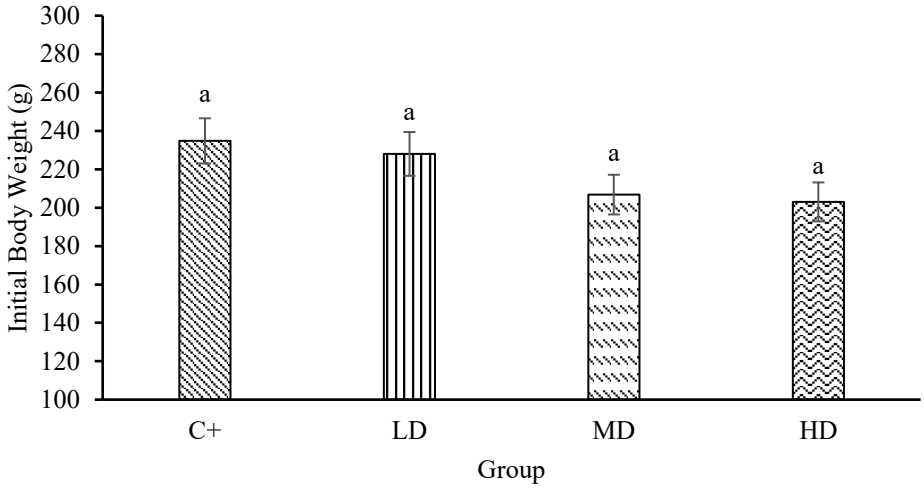

Figure 4 Mean initial BW of all groups of HFD-LD STZ-induced rats Same letter notation indicates no significant difference from ANOVA test (p-value > *0.05*).

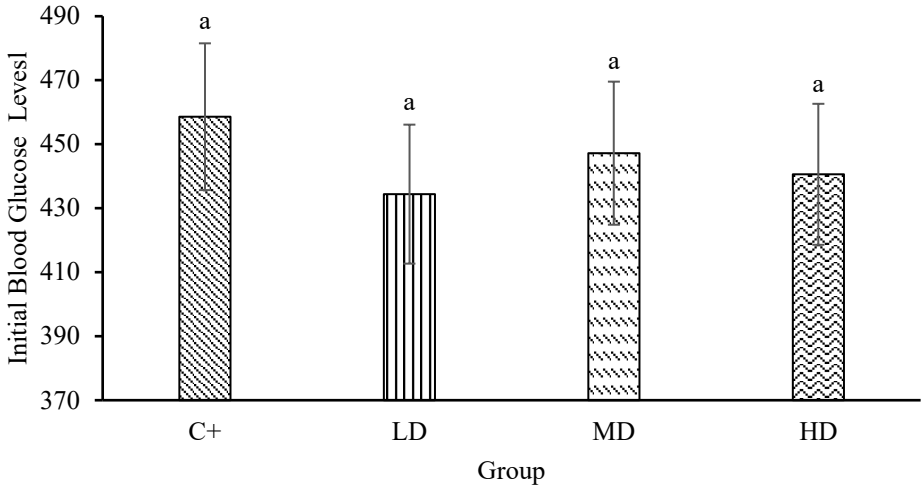

Figure 5 Mean initial blood glucose (BG) levels of all groups of HFD-LD STZ-induced rats. The same letter notation indicates no significant difference from Brown Forsythe test (*p*-value > *0.05*).

Similarly, the GL of the diabetic rat group at the beginning of treatment was also analyzed for homogeneity. The Kolmogorov-Smirnov test results showed that the distribution of data obtained was normally distributed and based on the results of the homogeneity test (Levene's test), a significance of 0.003 (*p*-value < *0.05*) was obtained, so the data could not be tested using the ANOVA test. The Brown-Forsythe test performed showed a value of *p*-value < *0.05*, indicating no significant difference in initial GL levels in all groups that had been induced with HFD-LD STZ (**Figure 5**). All groups of rats that have been induced with HFD-LD STZ show homogeneous body weight and blood glucose levels (not different, *p*-value > *0.05*), so it can be said that the initial condition of the test animals is homogeneous, so it can be used as an initial condition for SHE treatment.

High-fat diet can cause insulin resistance [40,44]. While the low-dose STZ injection induces β-cell destruction, resulting in hyperglycemia [40]. The occurrence of insulin resistance accompanied by pancreatic β-cell dysfunction, resulting in these cells failing to control blood glucose levels due to impaired insulin release. Abnormalities in β-cell function led to the development of T2DM [45]. Mice treated with a high-fat diet and STZ injection are an ideal model of T2DM, because they have similar features and patterns that are typical of the characteristics of T2DM, namely insulin resistance and pancreatic β-cell dysfunction. Therefore, a high-fat diet followed by low-dose STZ injection in rats is widely used to mimic the metabolic characteristics of human T2DM [40].

There are various pathways that explain how excess nutrients and fat can trigger insulin resistance until T2DM occurs through regulating the IRS/PI3K/Akt signaling pathway. Excess nutrients inhibit IRS tyrosine (py) phosphorylation through the hexosamine pathway, IKK/protein kinase C (PKC) pathway and ceramides signaling. Free fatty acid metabolites such as ceramides, acyl-CoA, diacylglycerol (DAG)) activate serine/threonine kinases such as PKC, nuclear factor kB (NFkB), inhibitory kB kinase β (IKKβ), which inhibit IRS and PKB/Akt, thereby inhibiting insulin signaling and preventing GLUT-4 translocation. Thus, increased levels of free fatty acids will inhibit glucose utilization by inhibiting insulin signaling pathways that stimulate muscles for glycogen synthesis which also occurs in T2DM [45].

Excess nutrients and the availability of cytokine can promote insulin resistance by activating rapamycin (mTOR) pathway [46]. Through this pathway, mTOR causes serine phosphorylation (pS) of IRS-I by activating S6 Kinase1 (S6K1), thus inhibiting IRS-I from activating phosphatidylinositol 3-kinase (PI3K) and Akt proteins, target proteins of the insulin metabolic pathway [45]. Activation of PI3K and Akt is necessary for the translocation of GLUT-4 from intracellular compartments to the plasma membrane [6]. Hence, if the activation of PI3K and Akt is

inhibited, it will inhibit the translocation of GLUT-4 to transport glucose from outside the cell into the cell [45].

In addition to the above pathways, obesity not only produces insulin resistance in skeletal muscle, but also leads to the development of T2DM through the process of obesity-induced inflammation by regulating the IRS/PI3K/Akt signaling pathway [45]. In the presence of excess nutrients, adipocytes or macrophages in adipose tissue or skeletal muscle release various types of proinflammatory cytokines through transcriptional activation of inflammatory genes such as iNOS and various types of proinflammatory cytokines (TNF-α, IL-1 and IL-6) activating c-jun N-terminal kinase (JNK) and inhibitor of kappa B kinase (IKK). Inhibition of pS of IRS-I by IKK and JNK through transcriptional activation of inflammatory genes such as iNOS. When there is phosphorylation of IRS-I on serine residues, IRS-I is inhibited so that there is no activation of PI3K and Akt proteins, resulting in inhibition of the translocation of GLUT-4 to the cell surface to transport glucose from into the cell. The enzyme iNOS is a major inflammatory mediator in obesity and causes insulin resistance in skeletal muscle, inhibiting adiponectin secretion from adipocytes. The accumulation of macrophages in adipose tissue in obese patients results in the expression of multiple genes that cause adipose tissue inflammation in obesity.

Induction of Diabetes Mellitus (DM) experimentally in experimental animals is by using chemicals that can selectively destroy pancreatic β-cells and one of the diabetogenic chemicals is streptozotocin (STZ). Streptozotocin can cause pancreatic β-cell death through DNA alkylation, because STZ has a methyl nitrosourea group that can bind to the nitrogenous base guanine at position O6. STZ can also damaging the DNA that will decrease NAD in β-cells and resulting in β-cells necrosis, causing less insulin to be release [47]. The alkylation of STZ from nitrosourea component ignited poly (ADP ribose) polymerase-1 (PARP-1) which causes NAD^+ depletion, thus leading to cell death [48]. In addition, nitrosourea groups can release nitric oxide (NO) which is one of the free radicals, thus increasing the production of oxidative stress [49].

Type 2 diabetes mellitus is characterized by hyperglycemia due to a insulin resistance and pancreatic β-cell dysfunction [50]. This is in accordance with the observation conditions in the first week after STZ injection, the glucose levels of rats in the C+, LD, MD and HD groups were more than 200 mg/dL, while the glucose levels of the C– group that was not induced by STZ were still within the normal range (< 150 mg/dL). Increased glucose levels are associated with reduced insulin production by pancreatic ß cells, so that the role of insulin as a glucose transporter into cells is also reduced. The reduced role of insulin results in high glucose levels in the circulation. Based on the above explanation, it can be confirmed that all rats injected with STZ have experienced hyperglycemia, with blood glucose levels > 200 mg/dL. Referring to [38], rats that have experienced hyperglycemia can be used as diabetic model rats

Homeostatic model assessment for insulin resistance (HOMA-IR) index in diabetic rats after SHE treatment

Insulin level in diabetic rats after SHE treatment

The mean plasma insulin levels of diabetic rats after being treated with SHE (**Table 4**) with the Kolmogorov-Smirnov test showed a normally distributed data distribution (p-value > *0.05*). The results of the homogeneity test (Levene's test) obtained p-value > *0.05*, which indicates that the variance obtained is homogeneous so that the data is tested using the ANOVA test. Based on the ANOVA test, there were no differences in plasma insulin levels in all treatment groups (p-value > *0.05*) (**Figure 6**). This shows that there is no difference in plasma insulin levels in all groups.

Patients with type 2 diabetes may have insulin levels that look normal or raised; nonetheless, failure to regulate blood glucose reveals a relative deficiency in glucose-stimulated insulin production [51]. The decrease in blood insulin is mainly due to the action of STZ, which damages pancreatic β cells. This can be seen in C+, which is a diabetic rat that is not treated with SHE; the insulin level is lower. Insulin levels that are not significantly different from healthy rats (C–) may indicate that it is suspected that endogenous

insulin in this animal model is not sufficiently altered to change insulin sensitivity and SHE administration can repair damage to β-cells.

Table 4 Mean plasma insulin levels of diabetic rats in SHE treatment for 4 weeks (28 days).

Group	Insulin level (ng/mL)
C–	2.51 ± 0.35
C+	1.96 ± 0.08
LD	2.4 ± 0.41
MD	2.01 ± 0.23
HD	2.17 ± 0.27

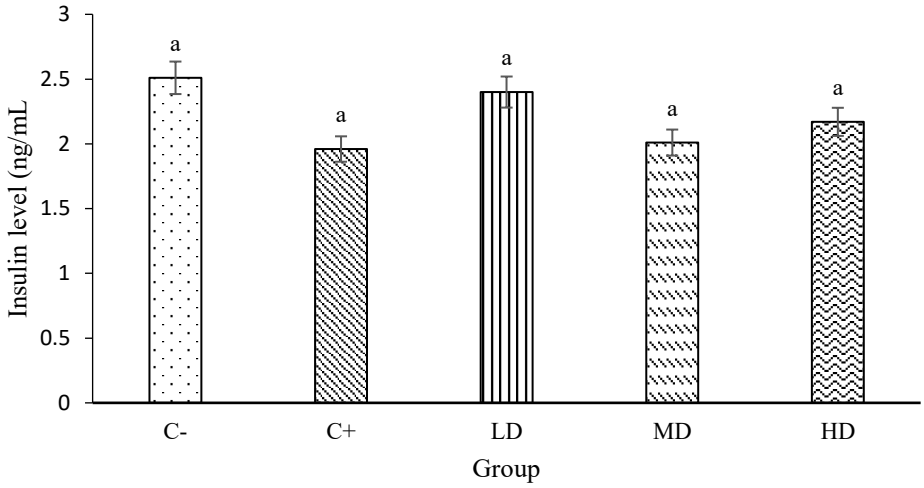

Figure 6 Diagram of plasma insulin levels of diabetic rats treated with various doses of SHE for 4 weeks (28 days). The same letter notation above the diagram indicates no difference between treatments in ANOVA test (*p*-value < *0.05*).

HOMA-IR index of diabetic rats after SHE treatment

To measure the level of insulin resistance, a homeostatic model assessment of insulin resistance called HOMA-IR (homeostasis model assessment) is used. This method calculates the insulin resistance formula based on serum glucose and insulin levels and is an extensively used method in clinical trials, epidemiological studies and research [12]. The calculation of the insulin resistance indicator (HOMA-IR) is done using the formula HOMA-IR = fasting blood glucose (mg/dL) × fasting insulin (ng/mL)/405. The formula was taken and elaborated from the HOMA-IR formula written by [28], HOMA-IR = fasting blood glucose (mmol/L)×fasting insulin (μU/mL)/ 22.5 (fasting blood glucose conversion unit: 1 mmol/L = 18.018018 mg/dL; insulin unit 1 μU/mL = 0.04 ng/mL).

To determine the effect of SHE on HOMA-IR index values, statistical tests were conducted. It is expected that by giving SHE, it will affect the decrease in oxidative stress, which will reduce the occurrence of further hyperglycemia and reduce insulin resistance, which will ultimately reduce the value of the HOMA-IR index.

The mean HOMA-IR index for each treatment (**Table 5**) was tested using the Kolmogorov-Smirnov test, which showed that the distribution of data obtained was normally distributed (*p*-value > *0.05*). Furthermore, the homogeneity test (Levene's test) was carried out, which obtained a homogeneous variance (*p*-value > *0.05*), so the data was tested using the ANOVA test. Based on the ANOVA test, there was a

difference in the value of the HOMA-IR Index in all treatment groups (p-value < *0.05*) and then Duncan's test was conducted to determine if there was a significant difference between each treatment.

Based on Duncan's test, the HOMA-IR Index values of rats given SHE in the MD (1.36 ± 0.25) and HD (1.37 ± 0.42) groups were significantly different from the diabetic rat group without treatment (C+) (1.95 ± 0.16), but the HOMA-IR Index values of these three groups were significantly higher (p-value < *0.05*) when compared to the healthy diabetic rat group C– (0.716 ± 0.11). The grouping of HOMA-IR index values between SHE treatments can be seen in **Figure 7**.

Table 5 Mean HOMA-IR index of diabetic rats in SHE treatment for 4 weeks (28 days).

Group	HOMA-IR Index
C–	0.716 ± 0.11
C+	1.95 ± 0.16
LD	1.63 ± 0.47
MD	1.36 ± 0.25
HD	1.37 ± 0.42

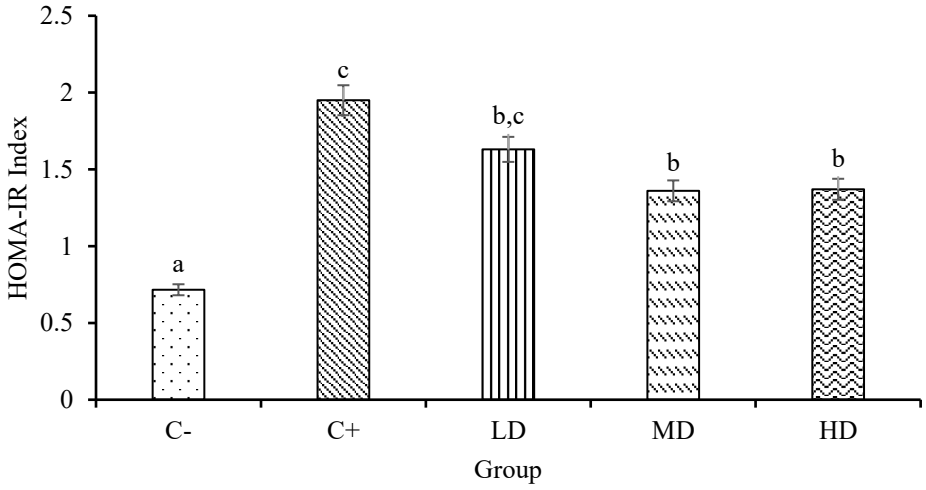

Figure 7 Diagram of HOMA-IR index of diabetic rats treated with various doses of SHE for 4 weeks (28 days). Different letter notations above the diagram indicate significant differences between treatments (p-value < *0.05*).

These findings indicate that the SHE MD and HD treatments significantly reduced the HOMA-IR value, although it did not reach the same value or smaller than the HOMA Index of the healthy group (C–). However, the HOMA-IR values of the SHE-treated rats and the healthy group were significantly lower than those of the diabetic rats. Based on the results of treatment on fasting blood glucose levels, SHE also significantly affects the decrease in fasting blood glucose levels in the MD and HD rat groups, so it can be concluded that the decrease in HOMA-IR levels in the MD and HD groups indicates that SHE is effective in reducing insulin resistance.

Homeostasis model assessment (HOMA) index is a robust tool with wide range of applications that had been recognized as a prognostic marker for impending metabolic disturbance and can provide insight for insulin sensitivity [52]. This method employs a singular blood glucose measurement alongside the corresponding insulin level to ascertain the insulin resistance (IR) level [11]. A HOMA-IR index value > 2.7 is declared insulin resistance [53]. Other

researchers stated that the HOMA-IR cutoff value for detecting insulin resistance was > 3.22 for pubertals and > 2.91 for postpubertal adolescents [54]. Although there are various interpretations of the HOMA-IR index value as mentioned above, the results of this study were compared with the HOMA-IR index in diabetic rats (C+). The results showed that increased sensitivity to insulin was evidenced by a decrease in the HOMA-IR index.

Hyperglycemia can be triggered by insulin resistance [55]. This can occur because sustained hyperglycemia can interfere with Akt/PKB activation, thus inhibiting the translocation of intracellular glucose transporter (GLUT-4) to the cell membrane [7]. Factors that support the occurrence of insulin resistance in patients with type 2 DM are not only hyperglycemia conditions but also due to deregulation of fatty acid metabolism. Elevated FFAs, as commonly found in obesity, are closely connected to insulin resistance and play a crucial role in the development of T2DM [56]. FFA can inhibit the activation PKB/Akt, thereby inhibiting insulin signaling pathway [57]. The elevated FFAs increase the ser^{307} phosphorylation of IRS-1 [56], which limits GLUT-4 transport to the cell surface and produces hyperglycemia [58].

Various studies report that there is a strong relationship between oxidative stress and insulin resistance [8,59,60]. One of the ROS formations is through the Ceramide Pathway. Reactive Oxygen Species (ROS) result from the accumulation of intramyocellular fatty acid metabolites and increased deposition of intramyocellular lipid metabolites (e.g., fatty acyl-CoAs, DAG) results in increased cytoplasmic acyl-CoA levels. These molecules stimulate numerous serine/threonine (Ser/Thr) kinases, such as PKC, IKK and cytokines, such as NFkB, TNF-α, and IL-6. Proteins from the insulin signaling pathway that are phosphorylated by Ser/Thr kinases will inhibit IRS1/IRS2 and PKB/Akt. In addition, activation of the iNOS enzyme, which is a major inflammatory mediator in obesity, will increase the production of high nitric oxide (NO), which ultimately produces peroxynitrite (ONOO-) free radicals. These free radicals cause s-nitrosylation or nitration of IRS-I, PI3K and/or Akt, which inhibits the action of all three [45].

Increased production of ROS that exceeds the antioxidant capacity of cells leads to increased oxidative stress accompanied by dysfunction and damage to pancreatic β-cell tissue [8,61], resulting in decreased insulin secretion [62]. Stated that ROS have been induce the β-cells dysfunction that leads to impaired insulin secretion and insulin resistance. The antioxidant properties of SHE has an effect on lowering insulin resistance. This is supported by the decrease in blood glucose levels and the decrease in oxidative stress and ROS formation based on the increase in MDA levels in SHE-treated rats. A well-accepted mechanism is the loss of its effect on insulin signaling disorders caused by ROS that induce serine/threonine phosphorylation of IRS, which will ultimately disrupt insulin signaling [40].

Blood glucose level in diabetic rats after SHE treatment

Measurement of the results of various doses of SHE treatment in diabetic rats was carried out on day 28, after 4 weeks of treatment. On day 28, the rats were fasted for 12 h before being sacrificed the next day for intracardiac blood collection. The plasma obtained, in addition to analyzing MDA and GSH levels as done above, also measured fasting blood glucose and insulin levels to obtain the calculation of the HOMA-IR Index. After SHE treatment, it is expected that the fasting blood glucose level will decrease.

To determine the effect of SHE treatment on fasting blood glucose levels of diabetic rats, statistical tests were conducted to compare each treatment. Data distribution of fasting blood glucose levels of all groups (**Table 6**) tested using Kolmogorov-Smirnov showed that the distribution of data obtained was normally distributed (p-value > *0.05*) but showed non-homogeneous variance (p-value < *0.05*) on Levene's test, requiring the Brown-Forsythe test. Significant differences were found among treatment groups (p-value < *0.05*). Independent t-tests revealed healthy rats (C–) had significantly lower glucose levels (124.8 ± 20.85 mg/dL) compared to MD (295 ± 76.82 mg/dL) and HD (256.8 ± 75.24 mg/dL), while all three groups had significantly lower levels than diabetic rats (C+) at 402.8 ± 34.8 mg/dL.).

Based on **Figure 8**, SHE treatment in MD and HD can significantly reduce blood glucose levels,

although the decrease is not the same as in the healthy rat group (C–). The results of this statistical test show that SHE reduces blood glucose levels in diabetic rats (DM2), although it has not reached the limit of normal blood sugar levels, which is less than 200 mg/dL. However, it can still be concluded that SHE has antidiabetic potential if given to diabetic rats

Table 6 Mean fasting blood glucose levels of diabetic rats on SHE treatment for 4 weeks (28 days).

Group	Fasting blood glucose level (mg/dL)
C–	124.8 ± 20.85
C+	402.8 ± 34.8
LD	250.4 ± 89.16
MD	295 ± 76.82
HD	256.8 ± 75.24

Description: C– (Negative Control): Normal/healthy mice. C+ (Positive control): Diabetic rats without treatment. LD (lower dose): Diabetic rats with SHE treatment 2mL/day. MD (middle dose): Diabetic rats treated with SHE 3mL/day. HD (upper dose): Diabetic rats treated with SHE 4mL/day.

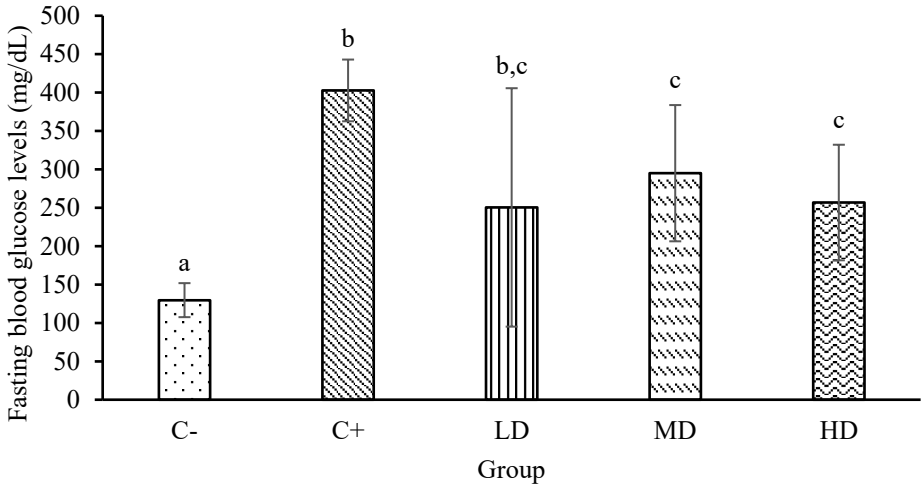

Figure 8 Diagram of fasting blood glucose levels of diabetic rats treated with various doses of SHE for 4 weeks (28 days). Different letter notations above the diagram indicate significant differences between treatments in independent t-test (*p*-value < *0.05*).

The lower fasting blood glucose levels in MD and HD in SHE treatments indicate that SHE can reduce blood glucose levels in diabetic rats. The reduction in fasting blood glucose also happened in mean blood glucose level. Based on **Figure 9**, there is a tendency to decrease blood glucose levels in diabetic rats treated with LD, MD and HD SHE until week 4 when the treatment is stopped. While the blood glucose levels of diabetic rats without SHE treatment (C+) showed an upward trend in blood glucose levels until the 4th week of the end of treatment.

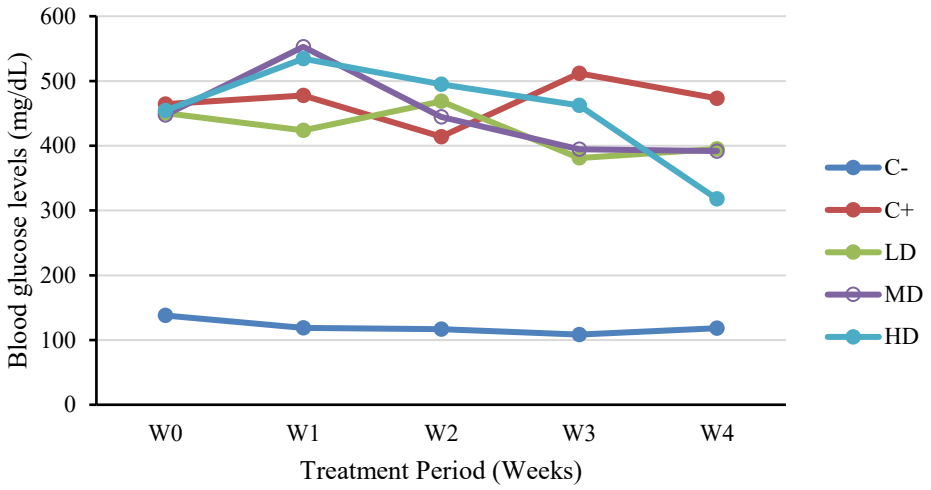

Figure 9 Graph of mean blood glucose levels of rats per week in SHE treatment for 4 weeks (28 days).

The Kolmogorov-Smirnov test showed the glucose data was normally distributed (p-value > *0.05*), but the Levene's test indicated non-homogeneous variance (p-value < *0.05*). Thus, the Brown-Forsythe test was used, revealing significant differences in fasting glucose levels across groups (p-value < *0.05*). Independent t-tests were then conducted. The t-tests showed SHE-treated diabetic rats (LD: 394.80 ± 48.61 mg/dL, MD: 391.80 ± 53.70 mg/dL, HD: 317.75 ± 105.84 mg/dL) had lower glucose levels (p-value < *0.05*) than untreated diabetic rats (C^+: 473.40 ± 27.20 mg/dL). However, LD and MD levels were higher than healthy rats (118.25 ± 10.31 mg/dL), while HD levels were similar to healthy rats. However, as shown in **Table 7** and **Figure 10**, the average blood glucose levels of SHE-treated rats LD, MD and HD at the end of the treatment still did not reach the normal limit (> 200 mg/dL), indicating that the rats were still in a hyperglycemic condition. Based on **Figure 9**, there is a trend of decreasing blood glucose levels in all SHE treatments as the treatment time increases, so there is a possibility that a decrease in blood glucose levels can be possible as the SHE treatment time increases. It can be concluded that the lack of treatment time allows the condition of hyperglycemia at the end of this study to still occur. This is reinforced by the results of statistical tests, which show that SHE significantly reduces blood glucose levels in diabetic rats even though it has not reached normal limits. Based on the results of statistical tests, it can be concluded that SHE has antidiabetic potential in diabetic rats. Decreased hyperglycemia is the most important indicator for successful diabetes treatment, because prolonged exposure to supraphysiological blood glucose levels can worsen tissue conditions [40].

High ROS generation can result in oxidative stress, which can promote the development of various diseases, including diabetes [50]. Suppression of oxidative stress with antioxidants is a useful therapy to prevent these diseases. Antioxidant can reduce oxidative stress by direct ROS scavenging. Antioxidants prevent the development of OS and reduce the buildup of DHAP and GA3P. However, by raising glutathione levels and lowering GPx activity, antioxidant actions will be triggered, reducing the development of OS in diabetic complications [63].

The antidiabetic properties of SHE may be related to its antioxidant activity. Albumin in SHE has important antioxidant activity. The protein contained in SHE is albumin as the main fraction. If we look at the content of amino acid residues in albumin, there are amino acids that are the main side that may be responsible for antioxidant activity, namely lysine, methionine [64], cysteine [65], as well as arginine, which also plays a role in GSH synthesis [66]. These molecules act through multiple binding sites and free radical scavenging properties directly, as well as indirectly by playing a role in GSH synthesis.

Table 7 Mean blood glucose levels of diabetic rats in SHE treatment for 4 weeks (28 days).

Group	Blood Glucose Levels (mg/dL)
C–	118.25 ± 10.31
C+	473.40 ± 27.20
LD	394.80 ± 48.61
MD	391.80 ± 53.70
HD	317.75 ± 105.84

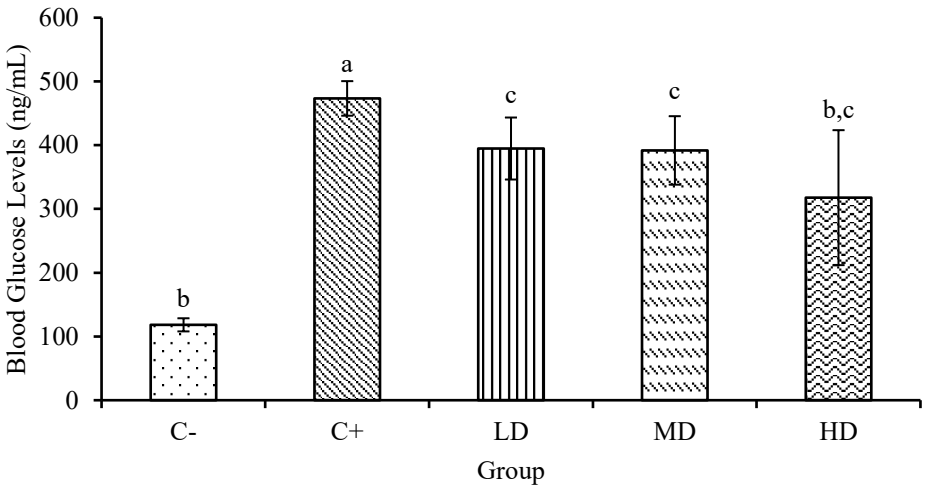

Figure 10 Diagram of blood glucose levels of diabetic rats treated with various doses of SHE for 4 weeks (28 days). Different letter notations above the diagram indicate significant differences between treatments in the Brown Forsythe test (p-value < *0.05*).

Preliminary research by [18] has proven that snakehead fish extract has the potential to be antidiabetic. Research on the potential of SHE in reducing oxidative stress, which is the effect of free radicals, has also been carried out by [33]. The results of this study concluded that SHE therapy has the potential to restore damaged pancreatic tissue in hyperglycemic rats due to ROS, thus achieving normal conditions. This study proves that SHE has antioxidant and antidiabetic potential in diabetic rats, which is indicated by a decrease in plasma MDA levels, a decrease in blood glucose levels and a decrease in insulin resistance by measuring the level of HOMA-IR insulin resistance and GLUT-4 density in striated muscle.

Malondialdehyde (MDA) level in diabetic rats after SHE treatment

Referring to [50], hyperglycemic conditions could trigger an imbalance between ROS production and cellular antioxidant system, causing oxidative stress. One of the biomarkers to assess oxidative stress is an increase in MDA [10,67,68], giving SHE to diabetic rats is expected to reduce ROS so that it can reduce MDA in plasma.

Table 8 Mean plasma MDA levels of diabetic rats in SHE treatment for 4 weeks (28 days).

Group	MDA Level (ng/mL)
C–	54.61 ± 16.29
C+	82.32 ± 17.98

Group	MDA Level (ng/mL)
LD	53.54 ± 6.18
MD	53.91 ± 8.89
HD	52.44 ± 9.72

The Kolmogorov-Smirnov test showed normal data distribution (*p*-value > *0.05*), allowing the one-way ANOVA (*p*-value = 0.018, *p*-value < *0.05*), which confirmed the effect of SHE on plasma MDA levels. Duncan test results showed no significant differences (*p*-value > *0.05*) in MDA levels between SHE treatments: LD (53.54 ± 6.18 ng/mL), MD (53.91 ± 8.89 ng/mL) and HD (52.44 ± 9.72 ng/mL). However, when compared to the MDA levels of diabetic rats without SHE treatment (C+) (82.32 ± 17.98 ng/mL), the value is lower and there is a significant difference (*p*-value < *0.05*). Meanwhile, when compared with healthy rats (C–), the three SHE treatment groups showed values that were not significantly different (*p*-value > *0.05*). Higher MDA levels in diabetic rats (C+) are indicated by the occurrence of oxidative stress in diabetic rats [33].

Based on the above analysis as well as **Table 8** and **Figure 11**, MDA levels are significantly reduced after SHE treatment. The administration of SHE in the diabetic rat group was able to restore MDA levels to be the same as the C– group, namely healthy rats. These results indicate that the administration of SHE has the potential as an antioxidant against diabetic rats (DM2), in accordance with the expectation that the administration of SHE in diabetic rats is expected to reduce ROS so as to reduce MDA in plasma

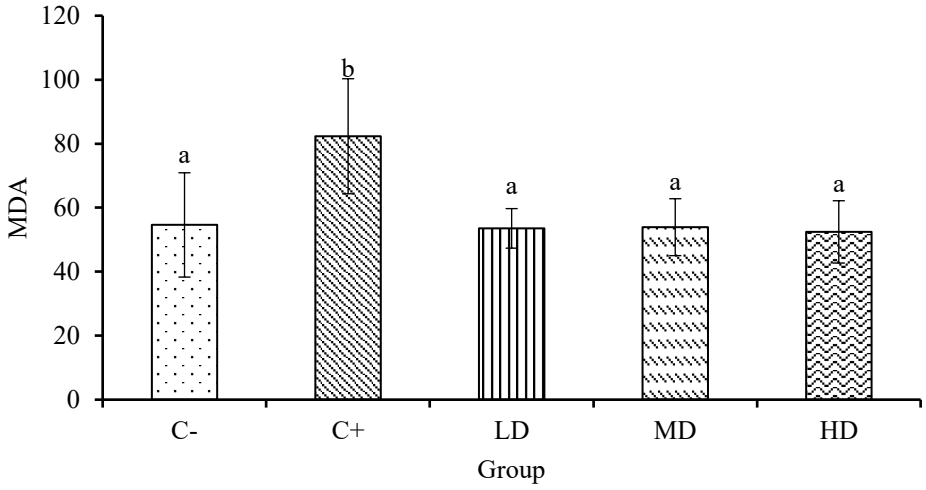

Figure 11 Diagram of plasma MDA levels of diabetic rats treated with various doses of SHE for 4 weeks (28 days). Different letter notations above the diagram indicate significant differences between treatments in ANOVA test (*p*-value < *0.05*).

Chronic hyperglycemia conditions in diabetes increased the production of ROS, further aggravate the oxidative stress [8]. Patients with diabetes are reported to have decreased antioxidant capacity accompanied by increased oxidative stress [69]. The destruction of cell by ROS occurs when the double bonds of polyunsaturated fatty acids in membranes bind with oxygen free radicals to become lipid peroxides [70]. Lipid peroxidation is a chain phenomenon that results in the formation of various active compounds that cause cell damage [71]. This increased oxidative stress will lead to increased levels of damage in cellular structure and possibly destroying the tissues [72]. Referring to previous research, it is known that diabetic rats show structural damage to the pancreas [73], liver [74] and testes [75]. According to [68], oxidative stress

in the body can also be seen through the examination of MDA levels, because MDA accumulation associated with ROS production and increase in lipid peroxidation. This is supported by the statement of [10], testing the concentration of MDA in various biological samples such as blood, exhaled breath condensate (EBC) and urine is a way to determine antioxidant activity. This was observed in diabetic rats prior to SHE treatment.

Higher MDA levels in diabetic rats are indicated by oxidative stress in diabetic rats [33]. Research conducted by [76] showed the occurrence of hyperglycemia accompanied by increased lipid peroxidation, as evidenced by a significant increase in MDA concentration in the serum of untreated diabetic rats. Treatment of various concentrations of SHE in this study can reduce MDA levels in diabetic rats. This treatment proved to be influential because the prevention of lipid peroxidation formation is beneficial and able to restricted the detrimental effect in T2DM caused by oxidative stress [77]. SHE treatment also prevents further hyperglycemia, which results in the polyol pathway. ROS generated by hyperglycemic conditions triggering the polyol pathway [78], thus increasing the formation of AGE. This route causes an abnormal accumulation of intracellular ROS in different organs of diabetes patients, such as the heart, vasculature, neurons, eyes and kidneys. [79]. This increase in serum MDA concentration in untreated diabetic rats was attributed to the destruction of erythrocyte membranes. This erythrocyte membrane suffered from oxidative damaged, increasing the MDA concentration [80]. The increased level of oxidative stress in diabetic animals is due to glucose autoxidation, protein glycation [8], lipid peroxidation [81] and low activity of antioxidant enzymes [82]. Oxidative stress has been linked to the pathogenesis of diabetes [8]. Therefore, suppression of oxidative stress is a useful new therapy to prevent the disease. Antioxidants can terminate the ROS damaging action [83], preventing the cellular damage [84].

The decrease in MDA levels in rats treated with SHE, to the same level as MDA in normal rats, indicates a decrease in oxidative stress in diabetic rats after being treated with SHE. Snakehead fish extract (SHE) could act as an antioxidant due to the content of albumin and amino acids in accordance with the previous discussion. In general, fish is a source of essential antioxidants and snakehead fish is one of the freshwater fish that is high in antioxidant content. Antioxidant activity of snakehead fish is provided by amino acids and fatty acids contained in its meat and muscle [85]. Fish produces more lipophilic antioxidants that effectively act as antioxidants. Amino acids are known to have significant antioxidant properties as synergists or primary antioxidants and are believed to be important metal chelators with significant potential in linoleic acid and linoleic acid methyl ester systems. *In vitro* antioxidant activity of *Channa* sp. is higher than that of other fish, such as Labeo fish [86].

The antidiabetic properties of SHE is not only related to antioxidant activity in scavenge the free radicals by donate an electron to neutralize [87] and break the chain of radical reactions to prevent damage from OS [88], but also to the mechanism of insulin signaling disruption caused by ROS that induce serine/threonine phosphorylation of IRS, thus disrupting the cellular redistribution of insulin signaling components [40]. The significant recovery of decreased MDA and GSH activity suggests that SHE has antioxidant potential, protective against oxidative stress induced by HFD/STZ-induced diabetes, through decreasing lipid peroxidation and increasing free radical scavenging activity. However, this study use blood-based biomarkers to assess MDA, which may not fully reflect tissue-specific pathophysiological changes associated with diabetes. Subsequent research should analyze MDA-specific organs to elucidate the effect of SHE as a therapeutic agent for managing diabetes and oxidative stress.

Glutathione (GSH) level in diabetic rats after SHE treatment

The occurrence of oxidative stress can trigger the activation of antioxidant responses [89], allowing it to reduce the production of ROS, decrease oxidative stress, transform ROS into harmless substances and increased the ROS enzymes activity [90]. As an endogenous antioxidant, GSH is a n essential antioxidant that provides protection against free radical [91]. The presence of exogenous antioxidants (SHE) is expected to maintain the content of endogenous antioxidants (GSH). In addition, the content of

glutamine, glycine and cysteine in SHE as potential precursors of GSH formation is expected to increase the content of GSH in the blood.

Data on the mean blood plasma GSH levels of rats in all tested treatments are contained in **Table 9**. The Kolmogorov-Smirnov test confirmed normal data distribution (p-value > *0.05*) and the variance was homogeneous, allowing the use of ANOVA. The ANOVA test showed significant differences in plasma GSH levels among treatment groups (p-value < *0.05*), prompting Duncan's post hoc test for further analysis. Based on Duncan's test (**Figure 12**), there is a significant difference (p-value < *0.05*) in the mean GSH levels of the diabetic rat group not treated with SHE C+ (8.08 ± 1.55 µmol/L) with the diabetic rat group treated with SHE MD (16.96 ± 5.05 µmol/L), but there is no significant difference (p-value > *0.05*) with SHE HD treatment (7.58 ± 5.14 µmol/L). There was no significant difference between the healthy rat group (C–) and all SHE LD, MD and HD treatment groups. It was concluded that the highest GSH levels were in the MD treatment group, higher and different from the diabetic C+ group and the same as the healthy C– group. While the LD and HD groups had the same GSH levels as diabetic rats.

Table 9 Mean plasma GSH levels of diabetic rats in SHE treatment for 4 weeks (28 days).

Group	GSH Level (µmol/L)
C–	10.24 ± 2.70
C+	8.08 ± 1.55
LD	12.42 ± 3.88
MD	16.96 ± 5.05
HD	7.58 ± 5.14

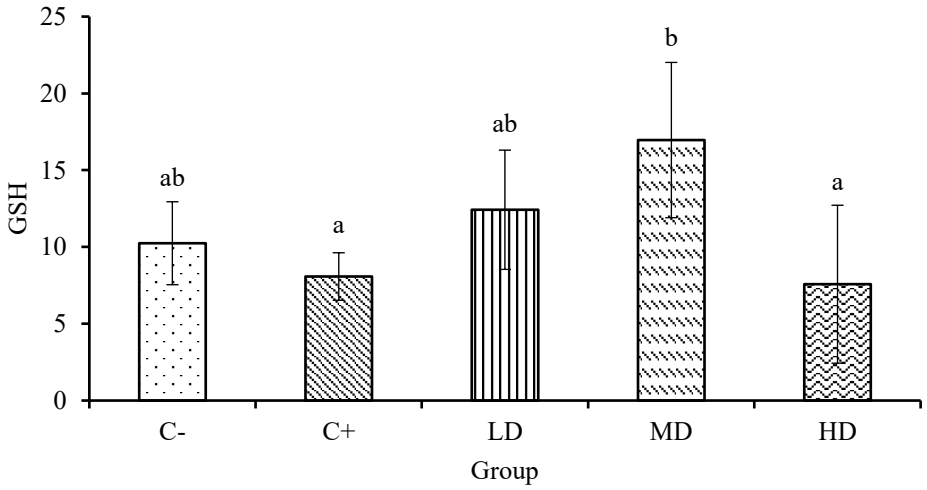

Figure 12 Diagram of plasma GSH levels of diabetic rats treated with various doses of SHE for 4 weeks (28 days). Different letter notations above the diagram indicate significant differences between treatments in ANOVA test (p-value < *0.05*).

Glutathione is a major intracellular antioxidant [92] and acts as a defense molecule against oxidative stress present [93]. Similar data were reported that the activity of the rate-limiting enzyme of GSH synthesis, glutamylcysteine synthase, increased with improved glycemic control [94]. There are three amino acids involved in GSH synthesis, namely cysteine, glutamic acid and glycine. The biosynthesis of antioxidant GSH formation involves two main enzymes, namely γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase [95]. The γ-glutamylcysteine synthetase enzyme converts the substrates glutamic acid and

cysteine into γ-glutamylcysteine [96]. Glutathione synthetase enzyme activity can convert γ-glutamylcysteine and glycine residues to become glutathione antioxidants (GSH) [97]. Furthermore, when insulin resistance and hyperglycemic occur, it will elevate the ROS level [98]. Hence, activation of endogenous or exogenous antioxidant responses is very important to neutralize the effect of ROS [50]. A known mechanism of protection against oxidative stress is by stimulating glutathione (GSH) synthesis [99]. Hyperglycemic conditions stimulate GSH to drown out free radicals by self-converting to form GSSG [100]. This explanation supports the low levels of GSH in the SHE HD treatment, which shows that the treatment of increased SHE levels is not followed by an increase in GSH levels and a decrease in MDA levels is not always accompanied by an increase in plasma GSH levels.

GSH levels in healthy rats (C–) were higher than in diabetic rats (C+) and SHE LD and MD treated rats. Intracellular GSH content and GSH/GSSG ratio are regulated in different amounts to maintain cellular homeostasis. Reduced glutathione (GSH) is having crucial role in managing oxidative stress [101] and its comparison with oxidized glutathione (GSSG) can be used as a marker of oxidative stress [97]. Patient with T2DM condition have low levels of GSH and high levels in its oxidized form (GSSG) [102]. Type 2 diabetic patients with uncontrolled blood sugar levels have suppressed GSH levels in their red blood cells and after treatment, red blood cell GSH levels increase almost twofold, as found in nondiabetic controls [103]. Under normal conditions, the GSH: GSSG ratio is ranging from 100:1 to 20:1. Meanwhile under oxidative stress, this ratio decreases to 5:1 and even 1:1 [96]. The known mechanism of protection against oxidative stress is by stimulating the synthesis of GSH by involving glutathione reductase and neutralization of free radicals resulting in scavenger products (GSSG) by involving glutathione peroxidase. Due to the absence of GSGG measurement, it is necessary to conduct further research to ensure that the condition of high GSH levels in healthy and SHE-treated rats in this study is accompanied by low GSH levels in diabetic rats (C+). Though, similar to the MDA assay, the GSH assay used a blood sample to assess the GSH. Future studies should examine organ-specific markers to clarify SHE's therapeutic effects on GSH-specific organs.

GLUT-4 density in striated muscle cell membrane in diabetic rats after SHE treatment

The administration of SHE is anticipated to attenuate reactive oxygen species (ROS) levels through its albumin content, thereby mitigating oxidative stress and preserving the integrity of insulin signaling pathways. Enhancement of the insulin signaling pathway is thought to increase GLUT-4 density on the surface of skeletal muscle cell membranes since GLUT-4 is an responsive transporter and will translocate to the plasma membrane after insulin stimulation [104].

GLUT-4 density in striated muscle cell membrane (**Table 10**) observed in this study was taken from rat tongue muscle. The calculation of GLUT-4 density is based on the histological preparation of transverse incisions of rat tongue muscle cells with immunohistochemical staining using antibodies for GLUT-4, whose images are presented in **Figure 14**. **Figure 14** shows that the density of GLUT-4 in the striated muscle cell membrane of diabetic rats (B) is lower than that of healthy rats (A) and SHE-treated rats (C), based on GLUT-4, which is stained with immunohistochemical staining in the form of brown grains.

Table 10 Mean density of striated muscle cell GLUT-4 from diabetic rats in SHE treatment for four weeks (28 days).

Group	GLUT-4 density (grains/100 µm)
C–	96.72 ± 3.18
C+	75.64 ± 8.41
LD	95.00 ± 11.10
MD	98.40 ± 5.68

Group	GLUT-4 density (grains/100 µm)
HD	94.42 ± 9.66

The mean data of striated muscle GLUT-4 density in all treatments were tested by ANOVA test. Kolmogorov-Smirnov test results showed that the distribution of data obtained was normally distributed (*p*-value > *0.05*). Based on the results of the homogeneity test, it shows that the variance obtained is homogeneous so that it is tested using the ANOVA test. The ANOVA test results showed a difference in striated muscle GLUT-4 density in all treatment groups (*p-value* < *0.05*) (**Figure 13**). Duncan's post hoc test showed a significant difference (*p*-value < *0.05*) in GLUT-4 density in striated muscle cell membranes between groups of diabetic rats that were not treated with SHE (C+) with all treatment groups (LD, MD and HD), as well as healthy rats without treatment (K). GLUT-4 density in striated muscle cell membranes in diabetic rats without SHE treatment (C+) (75.64 ± 8.41 grains/100 µm) was lower than GLUT-4 density in striated muscle cell membranes in the LD (95.00 ± 11.10 grains/100 µm), MD (98.40 ± 5.68 grains/100 µm), and HD (94.42 ± 9.66 grains/100 µm) groups as well as healthy rats without treatment C– (96.72 ± 3.18 grains/100 µm). The results of statistical analysis showed that the administration of SHE could significantly increase the GLUT-4 density of striated muscle cells in the diabetic rat group and reach the same condition as the GLUT-4 density in healthy rats.

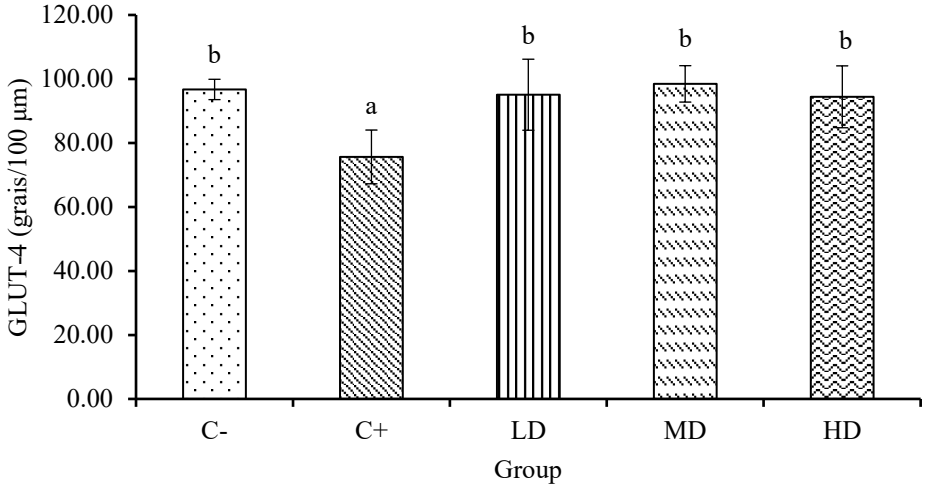

Figure 13 Diagram of GLUT-4 density in striated muscle cell membranes of rats treated with various doses of SHE for 4 weeks (28 days). Different letter notations above the diagram indicate significant differences between treatments in ANOVA test (*p*-value < 0.05).

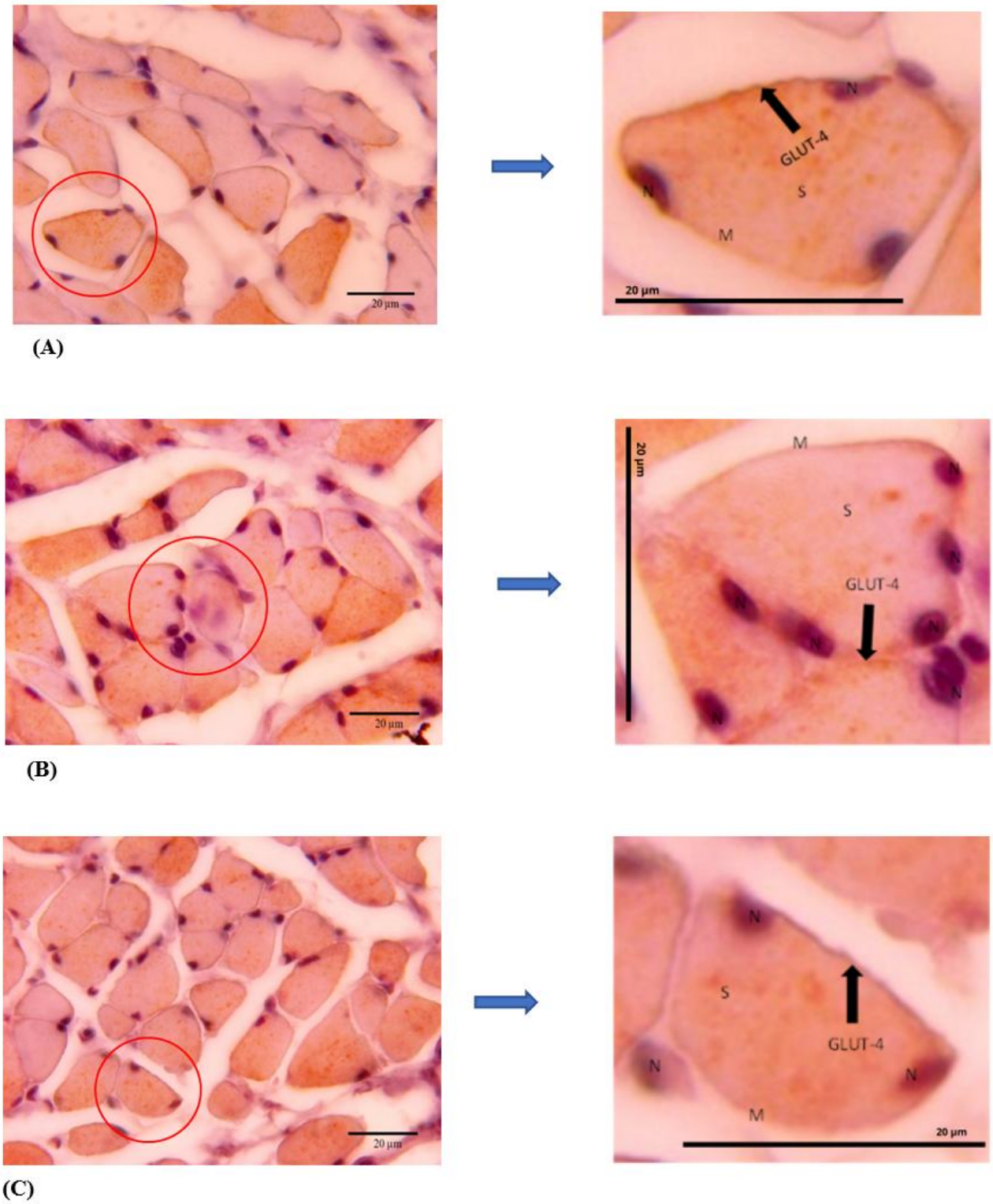

Figure 14 Histology of transverse section of striated muscle cells with immunohistochemical staining of GLUT-4 in 100×10 magnification, Chromogen DAB (diaminobenzidine) showing brown color, counter stain Hematoxylin. Description: A). Striated muscle of healthy rat (C–); B). Striated muscle of diabetic rat (C+); C). Striated muscle of diabetic rat with SHE treatment; M). Plasma membrane; N). Nucleus; S). Cytoplasm.

Based on the results presented in **Table 10**, diabetic rats (C+) exhibited the lowest density of GLUT-4 in muscle cell membranes compared to healthy and SHE-treated rats. This reduction is likely due to insufficient insulin stimulation, which is critical for GLUT-4 translocation. In diabetic conditions,

elevated oxidative stress impairs the IRS-2/PI3K/Akt signaling pathway—a key mediator of insulin action—thereby preventing GLUT-4 mobilization to the plasma membrane [40,105]. Sustained hyperglycemia further exacerbates insulin resistance by disrupting Akt/PKB activation, impairing intracellular GLUT-4 trafficking [40]. This mechanism is reflected in the observed lower insulin levels (**Table 4**) and elevated HOMA-IR values (**Table 5**) in diabetic rats, which correspond with reduced GLUT-4 membrane density.

GLUT-4 is an insulin-responsive glucose transporter typically stored in intracellular vesicles and its translocation to the cell surface is triggered by insulin signaling [104,106]. Under hyperglycemic conditions, this process is hindered by oxidative stress, which not only suppresses GLUT-4 expression but also disrupts insulin-mediated glucose uptake [7]. Chronic oxidative stress, exacerbated by persistent hyperglycemia, impairs insulin secretion and signaling by reducing GLUT transporter availability and activating stress kinases that block insulin pathways [8,50,107,108].

GLUT-4 density of SHE-treated rats (LD, MD, HD) have a similar result compared to the healthy rats (C–). This result stems from the amino acids in SHE-derived albumin, which play an important role in reducing the oxidative stress. Cysteine as one of the amino acids in albumin, reduce the number of hydroxyl radicals through reducing the Cys34 [30]. Another amino acid like lysine, methionine and arginine also has an antioxidant activity due to the presence of extra thiol, amine group, or thioether [32]. Albumin exerts significant antioxidant effects by neutralizing various reactive oxygen species (ROS). It directly scavenges peroxyl and superoxide radicals, thereby preventing lipid peroxidation and cellular damage. Its ability to bind hydrophobic molecules, such as fatty acids and pharmacological agents, further limits their participation in oxidative processes, including singlet oxygen–mediated damage. Moreover, albumin indirectly reduces ROS production by inhibiting NADPH oxidase activity through suppression of the cytosolic subunit p47phox recruitment to the membrane [109]. This decreased ROS will improve the IRS-2/PI3K/Akt-22 signaling pathway, thus resulting in improved GLUT-4 density in SHE-treated rats.

Based on the above explanation, the administration of SHE in diabetic rats reduces oxidative stress as indicated by the decrease in MDA levels in the plasma of SHE-treated rats and results in no interference with insulin signaling so that Akt/PKB activation occurs, which results in the translocation of glucose transporters (GLUT-4) intracellularly to the cell membrane. In **Figure 14** GLUT-4 density can be seen in the cell membrane, which is stained with immunohistochemical staining in the form of brown-colored grains.

Conclusions

This study highlights the antioxidant and antidiabetic properties of snakehead fish extract (SHE) in diabetic rats, supported by a series of experimental findings. SHE demonstrated significant antioxidant activity, with a concentration of 125 µg/mL effectively reducing ABTS radicals by 50% and this inhibitory effect increased at higher concentrations. Administration of SHE led to a marked decrease in plasma malondialdehyde (MDA) levels, suggesting its potential role in alleviating oxidative stress, although it did not significantly alter glutathione (GSH) levels. Additionally, SHE enhanced GLUT-4 density in the membranes of striated muscle cells, reduced insulin resistance—evidenced by a lower HOMA-IR index—and effectively lowered blood glucose levels. The antidiabetic mechanism of SHE in diabetic rats involved direct effects such as improved insulin sensitivity, increased cell membrane GLUT-4 density and reduction of oxidative stress, as well as indirect effects such as lowering the MDA levels. These findings indicate that SHE could be a promising natural therapeutic agent for managing diabetes and oxidative stress. However, this study has limitations. The use of blood-based biomarkers for MDA and GSH may not fully capture tissue-specific oxidative changes. Moreover, the total protein analysis did not quantify albumin levels or characterize the amino acid profile of SHE, which are critical to understanding its bioactivity. Future studies should include organ-specific analyses and detailed profiling of SHE's protein and amino acid constituents to better elucidate its mechanisms and support its development as a natural therapeutic agent for diabetes management.

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Declaration of Generative AI in Scientific Writing

The authors acknowledge the use of QuillBot and Copilot by Microsoft as generative AI tools during the manuscript preparation process, specifically for correcting grammar. These tools were not employed for generating content or interpreting data. The authors assume full responsibility for the manuscript's content and conclusions.

CRediT Author Statement

Nurlita Abdulgani: Conceptualization, Formal analysis, Methodology, Resources, Writing – Review & Editing.

Dwi Winarni: Methodology, Supervision, Writing – Review & Editing, and Validation.

Win Darmanto: Methodology, Supervision, Writing – Review & Editing, and Validation.

Dewi Hidayati: Formal analysis, Validation, and Visualization.

Muhammad Zamharir Rojafi: Project Administration, Writing & Editing.

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